

Heterogeneity of the calcium-induced permeability transition in isolated non-synaptic brain mitochondria

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Abstract

Calcium overload of neural cell mitochondria plays a key role in excitotoxic and ischemic brain injury. This study tested the hypothesis that brain mitochondria consist of subpopulations with differential sensitivity to calcium-induced inner membrane permeability transition, and that this sensitivity is greatly reduced by physiological levels of adenine nucleotides. Isolated non-synaptosomal rat brain mitochondria were incubated in a potassium-based medium in the absence or presence of ATP or ADP. Measurements were made of medium and intramitochondrial free calcium, light scattering, mitochondrial ultrastructure, and the elemental composition of electron-opaque deposits within mitochondria treated with calcium. In the absence of adenine nucleotides, calcium induced a partial decrease in light scattering, accompanied by three distinct ultrastructural morphologies, including large-amplitude swelling, matrix vacuolization and a normal appearance. In the

presence of ATP or ADP the mitochondrial calcium uptake capacity was greatly enhanced and calcium induced an increase rather than a decrease in mitochondrial light scattering. Approximately 10% of the mitochondria appeared damaged and the rest contained electron-dense precipitates that contained calcium, as determined by electron-energy loss spectroscopy. These results indicate that brain mitochondria are heterogeneous in their response to calcium. In the absence of adenine nucleotides, approximately 20% of the mitochondrial population exhibit morphological alterations consistent with activation of the permeability transition, but less than 10% exhibit evidence of osmotic swelling and membrane disruption in the presence of ATP or ADP.

Keywords: brain, calcium, mitochondria, permeability transition, rat.

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Mitochondrial dysfunction plays an important role in neuronal cell death caused by ischemia, hypoglycemic coma and trauma (for review see Siesjö *et al.* 1999; Fiskum 2000). In particular, the mitochondrial membrane permeability transition (MPT) may contribute to neuronal damage following ischemia, as suggested by strong neuroprotection observed in animals administered cyclosporin A (CsA), an inhibitor of the MPT (Uchino *et al.* 1995; Friberg *et al.* 1998; Sullivan *et al.* 1999). This contention is also supported by neuroprotective effect of minocycline, which inhibits the calcium-induced MPT in brain mitochondria (Zhu *et al.* 2002). The MPT can be induced under adverse conditions when mitochondria are overloaded with calcium and/or subjected to oxidative stress. The MPT is also modulated by several other physiological variables such as pH, magnesium, phosphate, mitochondrial redox state, free radical levels,

adenine nucleotide levels and cytosolic calcium (for review see Gunter and Pfeiffer 1990; Bernardi 1999; Dubinsky *et al.* 1999).

The MPT causes a dramatic increase in the permeability of the mitochondrial inner membrane accompanied by uncoupling of oxidative phosphorylation, osmotic swelling and

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Abbreviations used: AdNT, ATP/ADP translocator; CsA, cyclosporin A; ESI, electron spectroscopic imaging; EELS, electron-energy loss spectroscopy; MPT, mitochondrial permeability transition.

release of solutes of molecular weight up to 1500 Da from the mitochondria to the cytosol (for review see Zoratti and Szabo 1995; Bernardi 1999). The MPT has also been proposed to mediate the release of cytochrome *c* and other mitochondrial proteins into the cytosol where they activate the cascade of caspase protease activities that mediate apoptosis (Zamzami and Kroemer 2001). The MPT and its regulation have been studied and characterized primarily under *in vitro* conditions in sucrose-based media using mitochondria isolated from liver and heart tissues. Recently, the MPT and the effects of CsA were also described in brain mitochondria under conditions that more closely resemble those *in vivo*, i.e. using potassium-based media containing physiological levels of magnesium and phosphate (Andreyev *et al.* 1998; Andreyev and Fiskum 1999; Kristian *et al.* 2000; Brustovetsky *et al.* 2001; Kristian *et al.* 2001).

Although the MPT has been characterized extensively *in vitro* using isolated mitochondria, there is no compelling evidence that it is induced *in vivo* within the brain and that the MPT is a primary cause of mitochondrial dysfunction that mediates neuronal cell death. In contrast to mitochondria from other tissues, e.g. liver, brain mitochondria appear relatively resistant to calcium-induced MPT (Andreyev and Fiskum 1999; Berman *et al.* 2000). There is also an apparent heterogeneity in the response of brain mitochondria to calcium-induced MPT (Kristian *et al.* 2000). Several studies have shown that physiological concentrations of adenine nucleotides are potent inhibitors of the MPT (Haworth and Hunter 1979; Le Quoc and Le Quoc 1988; Rottenberg and Marbach 1989; Novgorodov *et al.* 1992). Thus, the question arises of whether CsA can exert a significant effect on the MPT *in vivo* if it is already inhibited by normal levels of adenine nucleotides. The finding that CsA can affect the phosphorylation state of proteins, for example BAD, which can affect mitochondrial retention of pro-apoptotic proteins independent of the MPT (Uchino *et al.* 2002), further questions the role of the MPT *in vivo*. The present studies were undertaken to examine the interaction between CsA and adenine nucleotides in regard to their effect on the calcium-induced MPT in brain mitochondria under conditions closely resembling those *in vivo*. To this end we studied calcium accumulation by isolated non-synaptosomal rat brain mitochondria in the presence of ATP, ADP and/or CsA. Furthermore, we examined calcium-induced mitochondrial ultrastructural alterations and volume changes.

Materials and methods

Isolation of non-synaptic brain mitochondria

Non-synaptic rat brain mitochondria were isolated as described previously (Kristian *et al.* 2000), using minor modifications of the method of Sims (1990). Male 300-g Wistar rats were used for this study. Animals were allowed water and food *ad libitum* for the 24 h

before death. All animal procedures were carried out according to National Institutes of Health and the University of Maryland, Baltimore animal care and use committee guidelines. Rats were killed by decapitation, forebrains were rapidly removed, chopped and homogenized in ice-cold isolation buffer (225 mM mannitol, 25 mM sucrose, 10 mM Hepes, 1 mM K₂EDTA, pH 7.4, at 4°C). The homogenate was centrifuged at 1330 *g* for 3 min, and the pellet obtained was re-suspended and re-centrifuged at 1330 *g* for 3 min. The pooled supernatants were centrifuged at 21 200 *g* for 10 min. The pellet was re-suspended in 15% Percoll (Sigma, St Louis, MO, USA) and layered on pre-formed Percoll gradients (40 and 23%). The Percoll gradients were then centrifuged at 31 700 *g* for 10 min. The mitochondrial fraction located at the interface of the lower two layers was removed, diluted with isolation buffer and centrifuged at 16 700 *g* for 10 min. The purified mitochondrial pellet was re-suspended in isolation buffer. Aliquots were removed for protein determination and bovine serum albumin was added to the mitochondria (10 mg/mL), to bind any free fatty acids, which can uncouple brain mitochondria. After the final centrifugation at 6800 *g* for 10 min, the mitochondria were re-suspended in 50 μ L isolation medium without EDTA. The isolated mitochondria used in these studies exhibited respiratory control ratios of ≥ 5.0 , defined as oxygen consumption in the presence of ADP (state 3 respiration) divided by respiration in the absence of ADP (state 4 respiration) in the presence of the respiratory substrates malate (5 mM) and glutamate (5 mM).

Calcium uptake by brain mitochondria

The hexapotassium salt of the fluorescent dye Calcium Green-5N (Molecular Probes, Portland, OR, USA) was used to record the changes in extramitochondrial free calcium concentration in incubations containing a suspension of isolated brain mitochondria, as described previously (Kristian *et al.* 2000; Murphy *et al.* 1996). Isolated mitochondria (0.5 mg) were re-suspended in 2 mL buffer containing 125 mM KCl, 2 mM K₂HPO₄, 1 mM MgCl₂, 1 μ M EGTA, 20 mM Tris-HCl, pH 7.4, 37°C). To minimize the calcium contamination of the experimental medium we used supra-pure potassium chloride (Merck, Darmstadt, Germany). However, it was still necessary to add 1 μ M EGTA to the medium in order to keep the calcium concentration below 1 μ M. Glutamate (5 mM) and malate (5 mM), or 10 mM succinate (with 2 μ M rotenone) were used as Complex-I linked and Complex II-linked substrates respectively. In some experiments, ATP (2.5 mM) or ADP (0.2 mM) and oligomycin (2.5 μ g per mg protein) were present in the incubation media. Calcium Green-5N fluorescence was measured with a Hitachi 2500 (Tokyo, Japan) fluorescence spectrometer, with an excitation wavelength of 506 nm and an emission wavelength of 532 nm. All experiments were performed at 37°C.

Measurement of mitochondrial swelling

Mitochondrial swelling was quantified by measuring light scattering at 540 nm, as described previously (Kristian *et al.* 2000) using a Hitachi 2500 fluorescence spectrometer. Mitochondria were re-suspended in the incubation buffer described above (0.5 mg protein in 2 mL). At the end of each experiment, the non-selective pore-forming molecule alamethicin (40 μ g per mg protein) was added as a calibration standard to cause maximal swelling of the entire mitochondrial population (Andreyev and Fiskum 1999; Kristian *et al.* 2000). All experiments were performed at 37°C.

Electron microscopy

After incubation of mitochondria under the specific conditions described above, the mitochondrial suspension was removed and centrifuged at 10 000 *g* for 4 min. The mitochondrial pellet was fixed with 4% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, at 4°C, and post-fixed with 1% osmium. The samples were dehydrated through increasing concentrations of ethanol, and embedded in LX112 epoxy resin. Ultrathin sections were obtained with a Reichert Ultracut E microtome (Reichert-Jung, Austria), and stained with uranyl acetate or viewed unstained with a Zeiss 10/a transmission high-voltage electron microscope (Zeiss, Gottingen, Germany). In samples obtained following the addition of calcium, the number of mitochondria with damaged cristae compared with mitochondria with normal condensed morphology was calculated by counting 400 mitochondria on sections from four independent samples. The values are expressed as mean \pm SD.

Electron spectroscopic imaging (ESI) and electron-energy loss spectroscopy (EELS)

ESI and EELS were used to determine the elemental composition of the deposits within the mitochondrial matrix. Unstained ultrathin sections were analyzed with a LEO 912 energy-filtering transmission electron microscope with an integrated Omega electron-energy spectrometer (Leo Electron Microscopy Ltd, Cambridge, UK). Primary beam electrons (accelerating voltage 100 kV) interact with electrons of the specimen, resulting in inelastically scattered electrons which have suffered an element-specific energy loss. With an energy-filtering transmission electron microscope these electrons are selected with a spectrometer and can be used either for imaging (ESI) or analyzed as intensity versus energy loss (EELS) (Kortje *et al.* 1991).

Intramitochondrial free calcium concentration measurement

Mitochondria were loaded with fura-4f/AM and the intramitochondrial free calcium concentration was estimated from the fluorescence of fura-4f-loaded mitochondria as described by Li *et al.* (1995) for fura-2-loaded brain mitochondria. Mitochondrial protein (5 mg) was incubated in 0.5 mL medium that contained 225 mM mannitol, 75 mM sucrose, 10 mM Hepes, pH 7.4, 1% bovine serum albumin, 5 mM NaCl, 1 mM ADP, 0.5 mM MgCl₂, 0.1 mM EGTA and 5 μ M Fura-4f/AM, at 25°C for 10 min. The mitochondrial suspension was then centrifuged at 6600 *g* for 6 min. The mitochondrial pellet was re-suspended in 1.5 mL ice-cold medium containing 225 mM mannitol, 25 mM sucrose, 0.05 mM EGTA and 10 mM Hepes, pH 7.4, and re-centrifuged at 6600 *g* for 6 min. The resulting pellet was re-suspended in approximately 50 μ L of the same mannitol–sucrose medium. The equivalents of 0.5 mg of mitochondrial protein were used for experiments. The experimental medium was the same as that used for calcium uptake and mitochondrial swelling measurements. Fura-4f fluorescence was measured with a Hitachi 2500 fluorescence spectrometer at 510 nm emission, with the excitation wavelengths at 340 and 380 nm, in a stirred, temperature-controlled cuvette. The fluorescence ratio at 340 nm/380 nm was used to calculate the matrix Ca²⁺ concentration using a *K*_d of 770 nM for fura-4f (Montero *et al.* 2001). At the end of each experiment, a calibration was performed in order to obtain a minimum and maximum ratio by adding 10 μ L 0.1 M EGTA followed by 10 μ L 1 M CaCl₂ in the presence of 1 μ M

ionomycin, a calcium-selective ionophore. For calculation of intramitochondrial free calcium concentration from the fluorescence signal, we used ratiometric methods according to Grynkiewicz *et al.* (1985). To test whether the fura-4f-loaded mitochondria were unaffected functionally, we measured the changes in light scattering after calcium additions and compared them with the results obtained in mitochondria that were not subjected to the loading procedure. As the fluorescence signal is affected by background light scattering, before calculation of the intramitochondrial free calcium concentration, the experiments were repeated with non-fura-4f-loaded mitochondria under identical conditions and the signal obtained was subtracted from the data obtained with fura-4f-loaded mitochondria. All experiments were performed at 37°C. Experiments in which quantitative changes are reported were repeated three times to confirm reproducibility.

Results

Calcium uptake and calcium-induced swelling of brain mitochondria

Figure 1 shows accumulation of calcium by brain mitochondria energized by either Complex I-linked or Complex II-linked substrates. The rate of Ca²⁺ sequestration was more rapid in medium containing the Complex II-linked substrate, succinate (Fig. 1a, right panel), compared with mitochondria energized with the Complex I-linked substrates malate and glutamate (Fig. 1a, left panel). This difference in respiratory substrate-dependent Ca²⁺ influx has been attributed to a relatively low MPT activity in the presence of succinate (and rotenone) (Kristian *et al.* 2000; Kristian *et al.* 2001) for skeletal muscle mitochondria (Fontaine *et al.* 1998a; Fontaine *et al.* 1998b). In agreement with this explanation, the rate of Ca²⁺-induced swelling (decrease in light scattering) was slower with succinate than with malate plus glutamate (Fig. 1b).

Increasing levels of added calcium led to a maximal extent of swelling that was only 40–50% of that obtained by the subsequent addition of alamethicin, a non-selective pore-forming molecule (Fig. 1b). Thus, calcium uptake appeared to cause swelling of only a subpopulation of the isolated non-synaptic brain mitochondria.

As a further test of whether calcium-induced changes in light scattering reflect volume alterations in some mitochondria only or reflect incomplete swelling of the whole mitochondrial population, we processed the mitochondrial samples for electron microscopic examination. Figure 2 shows electron micrographs of mitochondria incubated in the experimental medium without calcium addition (Fig. 2a), mitochondria exposed to 40 or 100 nmol Ca²⁺ per mg protein (Figs 2b and c), and mitochondria treated with alamethicin (Fig. 2d). Uptake of 100 nmol Ca²⁺ per mg protein caused massive swelling of 20 \pm 5% of mitochondria (*); however, a fraction of the mitochondrial population appeared normal (n)

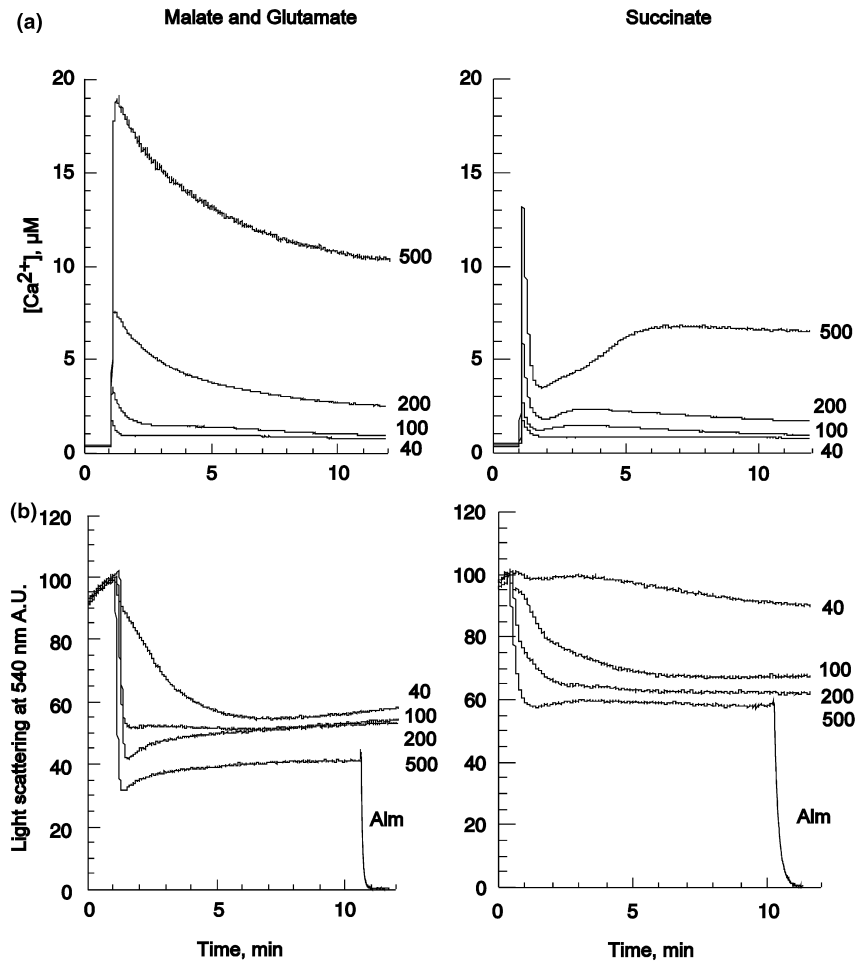


Fig. 1 (a) Accumulation of calcium by brain mitochondria energized by either Complex I-linked or Complex II-linked substrates. Brain non-synaptic mitochondria (0.25 mg/ml) were suspended in 125 mM KCl, 20 mM Tris-HCl, 2 mM K_2HPO_4 , 1 mM $MgCl_2$, 1 μ M EGTA, 5 mM malate, 5 mM glutamate (left panel) or 10 mM succinate and 2 μ M rotenone (right panel). In order to measure the changes in extramitochondrial calcium concentration, 0.1 μ M Calcium Green-5N was added, and the fluorescence was followed. (b) Calcium-induced swelling of brain mitochondria

as assessed by light scattering technique. The experimental conditions were the same as described above, except that no Calcium Green-5N was added. Numbers at each trace indicate the amount of added Ca^{2+} (nmoles per milligram mitochondrial protein). Each experiment was terminated by addition of alamethicin (Alm; 40 μ g per mg protein) as illustrated in the trace for 500 nmol Ca^{2+} per mg protein. The level of light intensity following alamethicin addition was defined as zero (maximal swelling of mitochondria).

whereas other mitochondria exhibited an electron-dense matrix with extensive vacuolation ($6.4 \pm 1.6\%$; \rightarrow) (Fig. 2c). The electron-dense matrix morphology was observed only in mitochondrial samples treated with 100 nmol Ca^{2+} per mg protein. Treatment of mitochondria with 40 nmol Ca^{2+} per mg protein resulted in swelling of an even smaller subpopulation of brain mitochondria ($< 10\%$) (Fig. 2b). As expected, alamethicin treatment caused massive swelling of the entire mitochondrial population (Fig. 2d).

Calcium uptake and changes in light scattering in the presence of adenine nucleotides

In order to examine whether the heterogeneity of brain mitochondria in response to calcium challenges is also

apparent under conditions more closely resembling those that exist within cells, we incubated brain mitochondria in high-potassium medium containing magnesium and 2.5 mM ATP, known physiological inhibitors of an MPT. In the presence of ATP, the capacity of brain mitochondria to accumulate and retain calcium was markedly increased (Fig. 3). Following addition of 2 μ mol Ca^{2+} per mg protein mitochondrial calcium uptake was followed by a delayed calcium release. Interestingly, under these conditions mitochondria energized with Complex-I and Complex-II showed similar rates of calcium uptake. However, in the presence of malate and glutamate, the calcium uptake was followed by a progressive release of calcium, which was not observed with succinate as substrate.

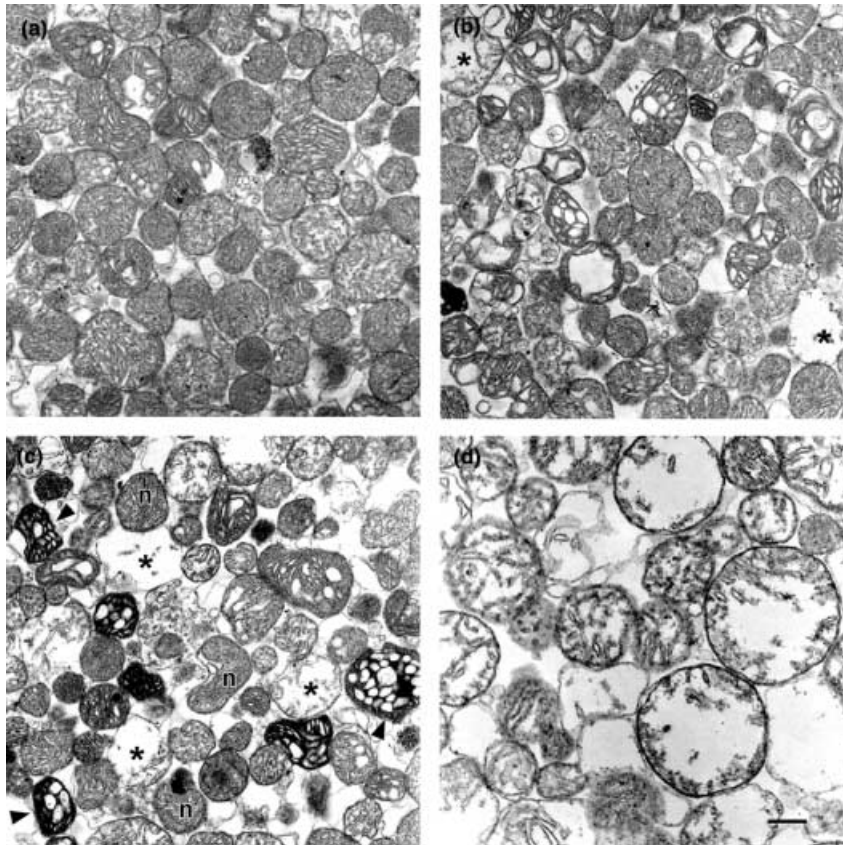


Fig. 2 Electron photomicrograph of brain mitochondria incubated in the standard buffer (a) and when exposed to 40 nmol Ca^{2+} per mg protein (b) or 100 nmol Ca^{2+} per mg protein (c) for 5 min, and after alamethicin treatment (d). One can identify swollen mitochondria with

damaged cristae (*), electron-dense mitochondria expressing vacuolization (arrowhead) and also mitochondria with normal morphology (n). Scale bar, 1 μm .

As the observed delayed release of calcium may be the result of MPT pore opening, we examined light scattering of the mitochondrial suspensions under these conditions. In contrast to the decrease in light scattering observed in the absence of ATP, mitochondrial calcium uptake induced an increase in light scattering in the presence of ATP. This response to calcium was observed with both Complex I-linked and Complex II-linked substrates. These observations are in agreement with the results reported by Andreyev *et al.* (1998), who suggested that the increase in optical density of brain mitochondria during calcium accumulation in the presence of ATP is due to formation of intramitochondrial calcium precipitates, as reported earlier for isolated liver mitochondria (Weinbach and Von Brand 1965; Weinbach and Von Brand 1967).

Electron microscopic examination of brain mitochondria exposed to calcium in the presence of ATP revealed that electron-dense granules were formed within the mitochondrial matrix (Fig. 4). Furthermore, as Fig. 4 shows, part of the mitochondrial population displayed damaged cristae with low electron density and disrupted mitochondrial membranes

($10.8 \pm 4\%$; $n = 4$). A control sample that was not treated with calcium contained fewer damaged mitochondria ($3.5 \pm 1.2\%$; $n = 4$).

As the electron-dense granules were formed during calcium accumulation we analyzed the topographical distribution of elements throughout the mitochondrial preparations by ESI and EELS. Typical electron-energy loss spectra obtained from control and calcium-treated samples are shown in Fig. 5(a), which shows a clear calcium peak in the spectrum obtained from the calcium-treated sample. ESI at an energy loss of 250 eV (below the carbon edge; in order to reduce the contribution of carbon signal) produced dark-field images displaying a structure-sensitive contrast (Fig. 5b). Figure 5(c) is a photographic negative of this image, giving a transition electron microscopic-like image of the sample. After subtraction of ESI beyond (360 eV) and below (330 eV) the $\text{Ca-L}_{2,3}$ edge, the calcium distribution within the sample could be observed (Fig. 5e). Figure 5 also shows an artificially colored electron spectroscopic image (Fig. 5d), the topographical calcium distribution (Fig. 5e) and overlap of these images from the

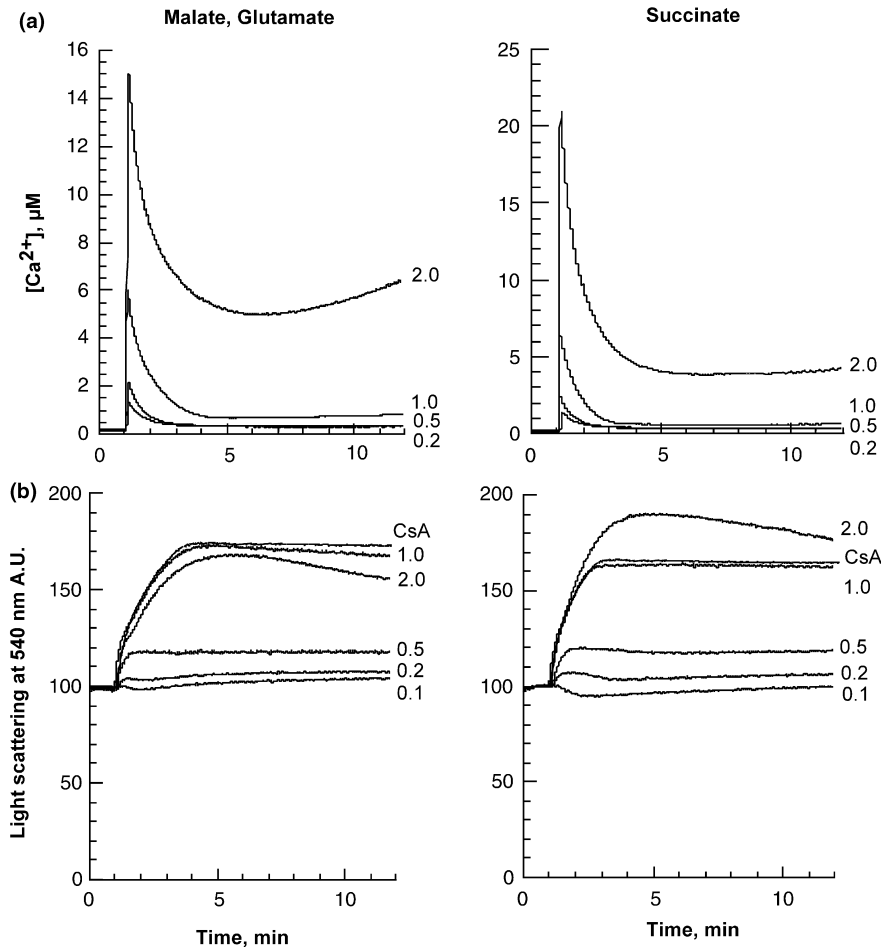


Fig. 3 Calcium uptake by brain mitochondria (a, upper panels) and changes in light scattering (b, lower panels) induced by calcium in the presence of ATP. Mitochondria were re-suspended in the buffer described in Fig. 1. The medium also contained ATP (2.5 mM). Calcium additions were followed by a rapid transient increase in extramitoch-

ondrial calcium concentration and the light scattering showed an increase proportional to the amount of added calcium. Numbers at each trace indicate the amount of added Ca^{2+} ($\mu moles$ per milligram mitochondrial protein).

calcium-treated sample (Fig. 5f). These data show that the electron-dense granules found in the matrix contain high levels of calcium.

In addition to ATP, ADP also enhances calcium uptake by brain mitochondria and inhibits MPT pore opening (Rottenberg and Marbach 1989). In the presence of 0.2 mM ADP, calcium at levels of 0.2 or 0.5 $\mu mol/mg$ induced an increase in light scattering of the mitochondrial suspensions (Fig. 6). Addition of 1.0 $\mu mol Ca^{2+}$ per mg protein usually resulted in a more complex response in the presence of ADP than it did with ATP. Uptake of calcium was accompanied by an initial increase in light scattering, followed by transient decrease, then a secondary increase (Fig. 6b). As this level of Ca^{2+} is very close to the maximal mitochondrial uptake capacity, the temporal pattern of swelling varied somewhat among different mitochondrial preparations (compare with Fig. 6d, for example). However,

as the Ca^{2+} concentration was increased to 2.0 μmol per mg protein, a level that was consistently greater than the uptake capacity (Fig. 6a), the extent of light scattering was substantially greater than that observed at 1.0 $\mu mol/mg$ and similar to that observed in the absence of adenine nucleotides (see Fig. 1). The MPT inhibitor CsA prevented the decrease in light scattering induced by 1.0 $\mu mol Ca^{2+}$ per mg protein and also accelerated net calcium uptake (Figs 6c and d). These data thus suggest that non-synaptic brain mitochondria can undergo an MPT in the presence of ADP and that the inhibitory effects of CsA and ADP are additive. As the mechanism of protection of mitochondria by both ADP and ATP is considered to be via their effects on the ATP/ADP translocator (AdNT) (Klingenberg 1985), one might expect CsA to have similar effects on calcium-induced changes in the presence of ATP and ADP. However, addition of CsA to the medium containing ATP

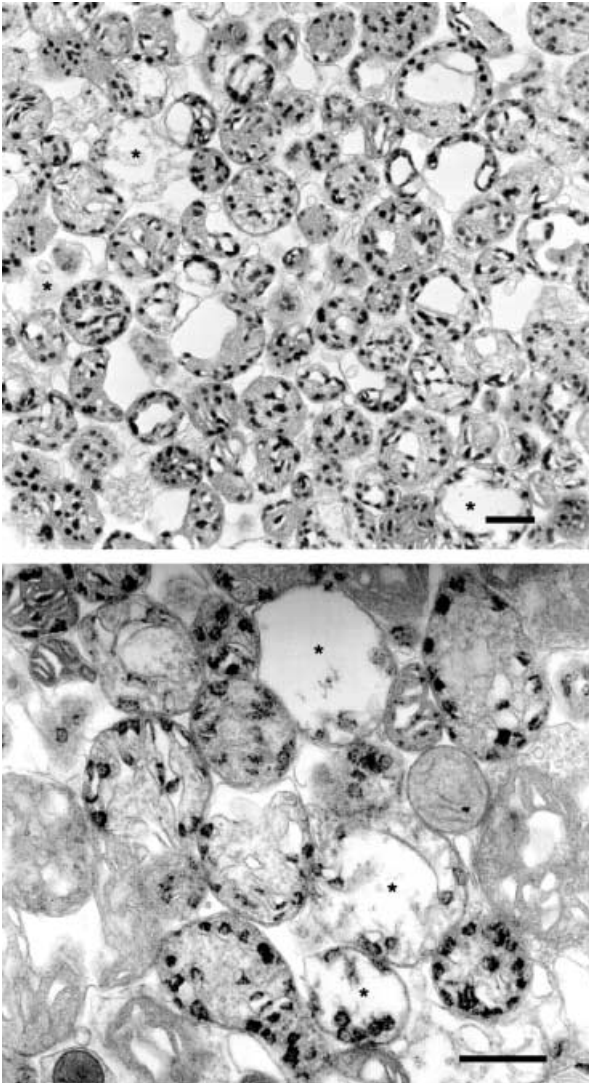


Fig. 4 Photomicrographs of brain mitochondria re-suspended in the presence of 2.5 mM ATP and exposed to 1 $\mu\text{mol Ca}^{2+}$ per mg protein. Most mitochondria contain electron-dense granules within the matrix. Although most of the mitochondria do not show any damage there is a subpopulation expressing disrupted cristae and with low electron density (*). Scale bar, 1 μm .

did not significantly affect the calcium-induced changes in light scattering (Fig. 3).

The addition of ADP exhibited less protection than ATP against the calcium-induced MPT, although ADP is reportedly a more potent blocker of the MPT than ATP (Rottenberg and Marbach 1989). As the MPT is triggered by intramitochondrial calcium accumulation, we compared the changes in mitochondrial matrix calcium concentration following calcium additions in the presence of both ADP and ATP. The extramitochondrial calcium concentration increased to higher levels in the presence of ADP (Fig. 7a) than the same

calcium additions in the presence of ATP (Fig. 7b). This suggests that the calcium uptake was faster with ATP in the medium. However, the intramitochondrial calcium transients were shorter when ATP was added to the medium (Fig. 7b), suggesting that the calcium binding was more efficient under these conditions.

Discussion

The results of experiments measuring mitochondrial calcium uptake support our previous findings that brain mitochondria retain larger amounts of calcium when energized with the Complex II-linked substrate succinate than mitochondria respiring in the presence of Complex I-linked substrates. Furthermore, the data strongly support the hypothesis that there is a clear heterogeneous sensitivity among non-synaptosomal brain mitochondria with respect to calcium-induced MPT. This heterogeneity was reflected by incomplete swelling (decreased light scattering) following large calcium additions in the absence of adenine nucleotides. Moreover, electron microscopic examination of brain mitochondria revealed that calcium treatment caused heterogeneous morphological changes. The levels of calcium that led to maximal swelling as measured by light scattering did not result in an apparent size increase among the entire mitochondrial population. Multiphase temporal changes in light scattering (Fig. 6) may also be explained by differential responses of separate mitochondrial populations. The explanation for this heterogeneous response of non-synaptosomal brain mitochondria to calcium exposure is unknown, although a differential capacity for phosphate uptake (which accompanies calcium accumulation) by different subpopulations of mitochondria may be responsible (see Kristian *et al.* 2001).

Several laboratories have reported that adenine nucleotides, specifically ADP and ATP, are strong inhibitors of the MPT (Haworth and Hunter 1979; Le Quoc and Le Quoc 1988; Rottenberg and Marbach 1989; Beutner *et al.* 1998). It has been suggested that binding of adenine nucleotides to the AdNT transforms it to the M-state and prevents its participation in MPT pore formation (Le Quoc and Le Quoc 1988). The ADP concentration required for binding to the AdNT and inhibition of the MPT is approximately 10 μM (Rottenberg and Marbach 1989); (Beutner *et al.* 1998). The total tissue concentration of ADP is approximately 0.2 mM and, similarly, tissue ATP levels under normal physiological conditions are near 3 mM (Folbergrova *et al.* 1995). Our results with non-synaptic brain mitochondria, as well as those reported by Andreyev *et al.* (1998) for synaptic plus non-synaptic mitochondria, indicate that when brain mitochondria are exposed to calcium in the presence of millimolar concentrations of ATP, their calcium uptake capacity is markedly increased. Uptake of calcium under

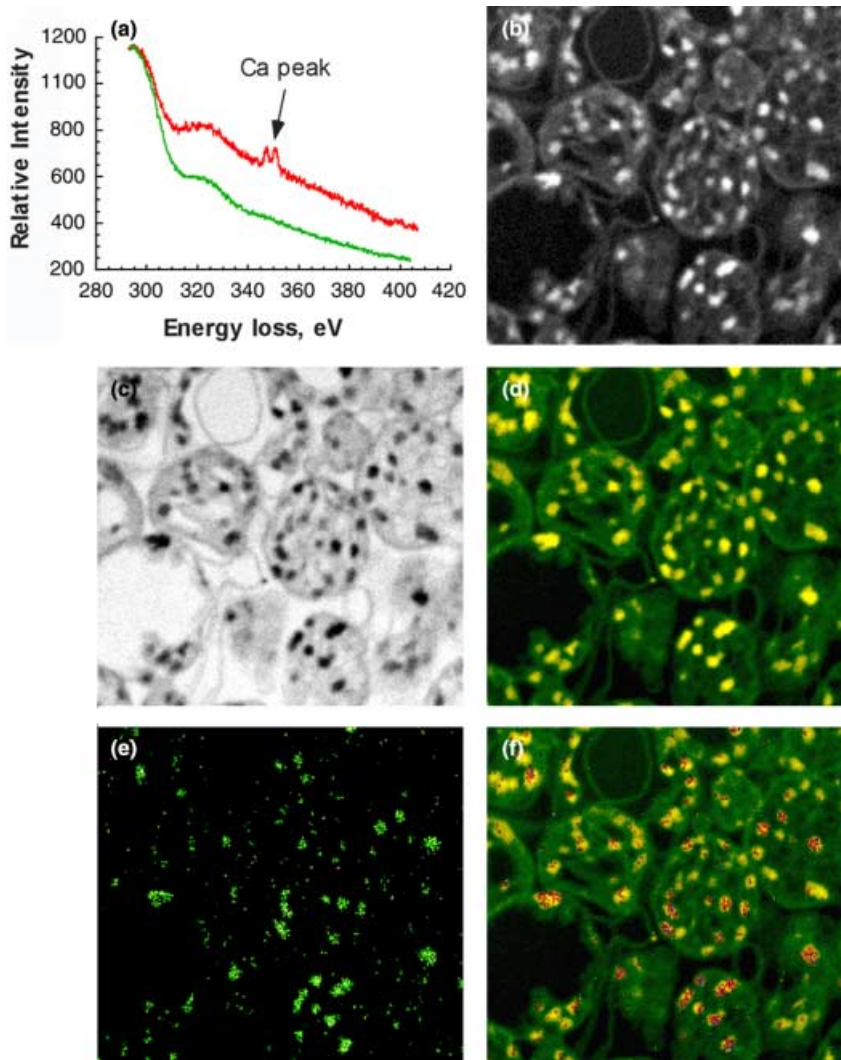


Fig. 5 Electron-energy loss spectrum obtained from mitochondria, and topographic distribution of calcium within the mitochondria after calcium uptake in the presence of ATP. (a) Spectrum from control sample (no calcium addition to mitochondrial suspension) and spectrum obtained from mitochondria incubated in medium containing ATP (2.5 mM) and 1 $\mu\text{mol Ca}^{2+}$ per mg protein. (b) Structure-sensitive contrast image from mitochondria exposed to calcium (1 $\mu\text{mol Ca}^{2+}$ per mg protein) in the presence of ATP. This image was produced by ESI at 250 eV energy loss. (c) Photographic negative of image (b), showing the localization of precipitates within the mitochondrial matrix. (d) Artificially colored image obtained by ESI at 250 eV energy loss. (e) Calcium distribution obtained by employing an element-specific energy window for calcium. After subtraction of ESI below 330 eV and beyond 360 eV, the calcium distribution image was obtained. (f) Overlap of images (c) and (e) shows that the electron-dense precipitates contain high levels of calcium.

these conditions results in an increase in light scattering, in contrast to the decreased light scattering observed in the absence of adenine nucleotides. In the absence or presence of adenine nucleotides, the mitochondrial calcium uptake capacity was greater in the presence of succinate than in the presence of Complex I respiratory substrates, indicating that the potentiation of calcium uptake and retention by succinate and adenine nucleotides is mediated by different mechanisms.

Ultrastructural analysis of mitochondria challenged with calcium in the presence of ATP revealed that the increase in light scattering accompanying calcium uptake is due to the formation of electron-dense precipitates within the mitochondrial matrix. As expected, elemental analysis of mitochondrial samples by EELS demonstrated that the matrix granules contain high levels of calcium. The composition of these precipitates isolated from calcium-loaded liver mitochondria was reported to be a mixture of hydroxyapatite and whitlockite (Weinbach and Von Brand 1967). Thus, in

addition to calcium, phosphate and carbonate are probably present within the granules that form in brain mitochondria following calcium uptake in the presence of adenine nucleotides.

In the presence of ATP, relatively large amounts of calcium are required to saturate the calcium uptake capacity and reverse the increase in light scattering. Thus, there is a possibility that very high calcium concentrations eliminate the effects of CsA (Novgorodov *et al.* 1992); (Brustovetsky and Dubinsky 2000). Another explanation for the lack of CsA effect on changes in light scattering in the presence of ATP is that inhibition of a decrease in light scattering caused by the small fraction of mitochondria that undergo osmotic swelling is obscured by the increase in light scattering caused by calcium precipitation within the vast majority of mitochondria that do not swell.

The higher calcium uptake capacity of brain mitochondria in the presence of ATP compared with ADP cannot be readily explained by differential inhibition of the MPT pore opening.

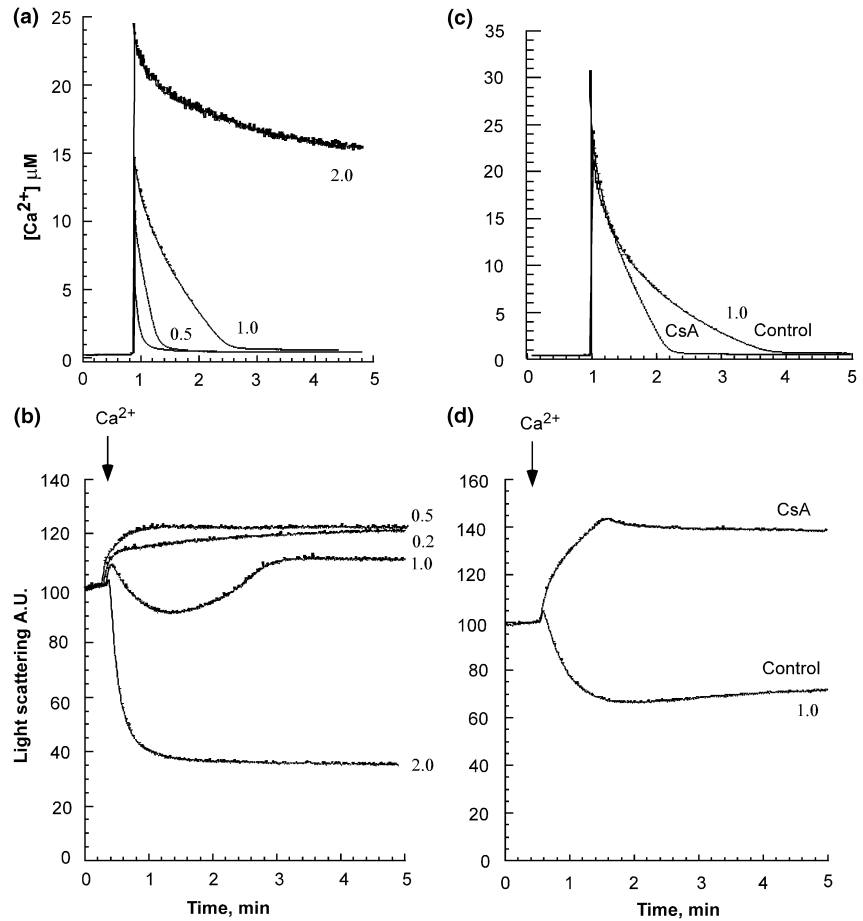


Fig. 6 Calcium-induced swelling (a) and calcium uptake (b) by brain mitochondria in the presence of ADP (0.2 mM). Mitochondria were re-suspended in the medium described in Fig. 1. The experiment started by addition of oligomycin (2.5 μg per mL) and ADP (0.2 mM). Following incubation of mitochondria in the medium for 2 min calcium was added. Numbers at each trace indicate the quantity of calcium (micromoles per milligram mitochondrial protein). Effect of CsA on calcium-induced swelling (c) and calcium uptake (d) by brain mitochondria in the presence of ADP. Experiments were carried out as described in (a) and (b) except that CsA (1 μM) was present in the incubation medium where indicated.

The concentration of either ATP or ADP necessary for maximal inhibition of the MPT is approximately 10 μM whereas millimolar levels of either adenine nucleotide were used in our experiments. In the presence of ATP, the duration of the rise in the intramitochondrial free calcium concentration was shorter than that in the presence of ADP (Fig. 7). As both ATP and ADP are implicated in promoting intramitochondrial Ca^{2+} and phosphate precipitation (Carafoli *et al.* 1965), this observation suggests that ATP is more effective. ATP is also more effective than ADP at protecting mitochondria against respiratory inhibition caused by large calcium loads. (Villalobo and Lehninger 1980) Finally, it is also possible that ATP hydrolysis contributes to the electrogenic H^+ ejection necessary to drive electrophoretic calcium influx under our experimental conditions. Although the high membrane potential generated by respiration-dependent H^+ efflux normally inhibits ATP hydrolysis, this potential collapses during the extremely rapid influx of calcium that occurs at high extramitochondrial calcium concentrations. The subsequent hydrolysis of ATP but not ADP can then sustain mitochondrial Ca^{2+} influx even if respiration is impaired.

Although the fraction of mitochondria that are sensitive to calcium-induced swelling is much less in the presence than in

the absence of adenine nucleotides, an apparent heterogeneous response to calcium occurs under both conditions. Considering the numerous neural cell types from which non-synaptosomal brain mitochondria are isolated, this heterogeneity might reflect differences in the sensitivity of mitochondria to the permeability transition both within and among different brain cells.

Heterogeneous mitochondrial morphological alterations have also been observed in animal models of cerebral ischemia. Recent ultrastructural studies of neuronal mitochondria after ischemic insult revealed several types of changes in mitochondrial morphology. Following 3–5 h of focal ischemia in rats, many neuronal mitochondria were moderately to severely swollen (Solenski *et al.* 2002). Dispersed around the swollen mitochondria were mitochondria with poorly defined cristae and increased matrix density. When ischemia was followed by reperfusion for 2 h, some mitochondria demonstrated dilated cristae within an electron-dense matrix. These morphological changes are similar to those we observed when isolated mitochondria were exposed to 100 nmol Ca^{2+} per mg protein in the absence of adenine nucleotides. At 24 h of reperfusion, many mitochondria exhibited an electron-lucent matrix, but some mitochondria

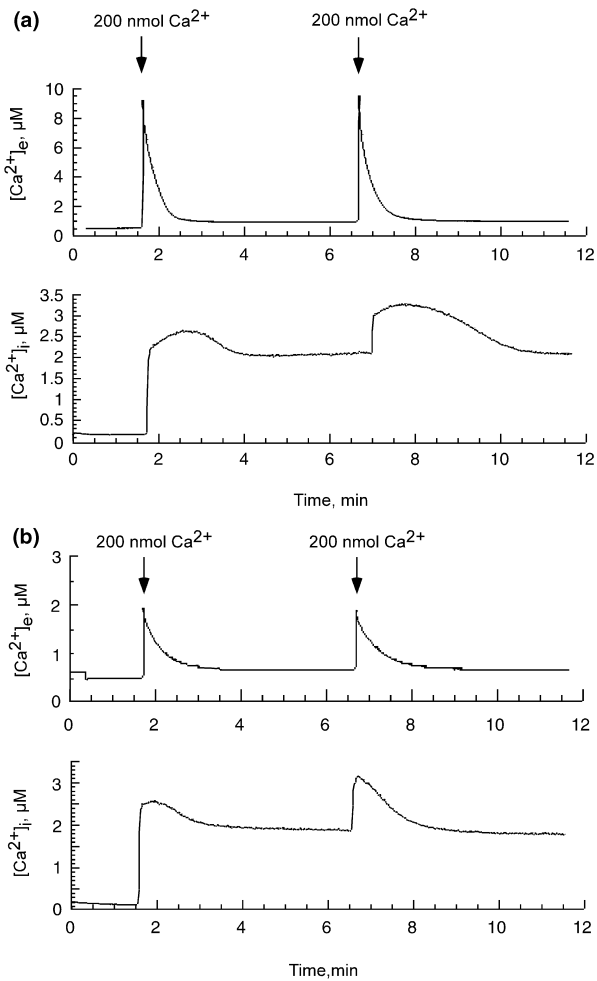


Fig. 7 Changes in extramitochondrial and intramitochondrial calcium concentrations following calcium addition to the suspension of brain mitochondria incubated in medium containing ATP or ADP. The changes in extramitochondrial calcium concentration were measured by use of Calcium Green-5N dye. Intramitochondrial calcium concentration was assessed by measurement of fluorescence of mitochondria loaded with fura-4f fluorophore. (a) Mitochondria were re-suspended in standard medium containing 0.2 mM ADP and oligomycin. Calcium transients were induced by addition of 200 nmol Ca^{2+} per mg protein. (b) Experiments were carried out in the presence of 2.5 mM ATP.

contained deposits of very electron-dense material within the matrix (Solenski *et al.* 2002). These mitochondria resemble the morphology of our isolated brain mitochondria challenged with calcium in the presence of ATP.

The study by Solenski *et al.* (2002) focused only on mitochondrial morphological changes within cortical neurons following focal ischemia and reperfusion. However, similar pleomorphic mitochondrial alterations have been observed in astrocytes and other non-neuronal cells in a monkey permanent focal ischemia model (Garcia *et al.* 1971), a

feline transient focal ischemia model (Garcia *et al.* 1977) and in rat models of transient global cerebral ischemia (Petito and Babiak 1982; Petito 1986). Astrocyte and oligodendrocyte histopathology in ischemic stroke has also been shown to occur following a period of ischemia (30 min) when neurons display only minor structural alterations (Pantoni *et al.* 1996). Therefore ischemic mitochondrial structural and functional abnormalities are heterogeneous both among and within different types of brain cells, and are dynamically linked to the duration of ischemia and reperfusion.

Because the isolated non-synaptosomal brain mitochondria used in our investigation represent mitochondria originating from both neurons and non-neuronal brain cells, the observed heterogeneity to calcium-induced MPT may reflect a differential sensitivity of mitochondria from neurons and glial cells to this phenomenon. A recent study in which primary cultures of cortical astrocytes were exposed to oxygen and glucose deprivation demonstrated a cyclosporin A-sensitive loss of mitochondrial membrane potential (Reichert *et al.* 2001). We have directly compared the effects of a calcium ionophore on the mitochondrial membrane potential of cortical astrocytes and cerebellar granule neurons, and found only the mitochondrial membrane potential of astrocytes to be protected by cyclosporin A (Fiskum *et al.* 2001). These *in vitro* observations, taken together with the heterogeneous responses of mitochondria within different cells to cerebral ischemia/reperfusion, support the hypothesis that the responses of brain mitochondrial subpopulations to supra-normal levels of calcium result from phenotypic variability among mitochondria relevant to the pathophysiology of stroke and other forms of brain injury.

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