Characteristics of the Calcium-Triggered Mitochondrial Permeability Transition in Nonsynaptic Brain Mitochondria: Effect of Cyclosporin A and Ubiquinone 0

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Abstract: The objective of the present study was to assess the capacity of nonsynaptic brain mitochondria to accumulate Ca2+ when subjected to repeated Ca2+ loads, and to explore under what conditions a mitochondrial permeability transition (MPT) pore is assembled. The effects of cyclosporin A (CsA) on Ca²⁺ accumulation and MPT pore assembly were compared with those obtained with ubiquinone 0 (Ub_{0}), a quinone that is a stronger MPT blocker than CsA, when tested on muscle and liver mitochondria. When suspended in a solution containing phosphate (2 mM) and Mg^{2+} (1 mM), but no ATP or ADP, the brain mitochondria had a limited capacity to accumulate Ca²⁺ (210 nmol/mg of mitochondrial protein). Furthermore, when repeated Ca2+ pulses (40 nmol/mg of protein each) saturated the uptake system, the mitochondria failed to release the Ca^{2+} accumulated. However, in each instance, the first Ca^{2+} pulse was accompanied by a moderate release of Ca^{2+} , a release that was not observed during the subsequent pulses. The initial release was accompanied by a relatively marked depolarization, and by swelling, as assessed by light-scattering measurements. However, as the swelling was <50% of that observed following addition of alamethicin, it is concluded that the first Ca²⁺ pulse gives rise to an MPT in a subfraction of the mitochondrial population. CsA, an avid blocker of the MPT pore, only marginally increased the Ca2+-sequestrating capacity of the mitochondria. However, CsA eliminated the Ca2+ release accompanying the first Ca²⁺ pulse. The effects of CsA were shared by Ub₀, but when the concentration of Ub₀ exceeded 20 μ M, it proved toxic. The results thus suggest that brain mitochondria are different from those derived from a variety of other sources. The major difference is that a fraction of the brain mitochondria, studied presently, depolarized and showed signs of an MPT. This fraction, but not the remaining ones, contributed to the chemically and electron microscopically verified mitochondrial swelling. Key Words: Calcium—Mitochondria—Swelling—Brain—Rat— Cyclosporin A.

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Mitochondria are considered key players in necrotic and apoptotic cell death (Bernardi et al., 1998, 1999; Lemasters et al., 1998). There is extensive evidence that damage to mitochondria, encompassing a decreased capacity for oxidative phosphorylation, is a major cause of brain cell death due to ischemia and reperfusion (Rehncrona et al., 1979; Fiskum, 1983; Hillered et al., 1984; Sims, 1990; Siesjö, 1992; Allen et al., 1995; Almeida et al., 1995; Nicholls and Budd, 1998; Fiskum et al., 1999). This impairment of mitochondrial function is not necessarily present in the early recirculation period, but may appear after a delay, the duration of which varies with the length of the ischemic period (e.g., Sims and Pulsinelli, 1987; Almeida et al., 1995; Kuroda and Siesjö, 1997). Our understanding of the mechanisms of this mitochondrial damage is incomplete. However, breakdown of the lipid backbone of the mitochondrial membranes by phospholipase A₂ activation or by lipid peroxidation has been incriminated (Nakahara et al., 1991; Sun and Gilboe, 1994), as has inactivation of respiratory complexes (Wagner et al., 1990; Allen et al., 1995; Almeida et al., 1995). Damage may be mediated by reactive oxygen species and reactive nitrogen species, such as nitric oxide, hydroxyl radicals, and peroxynitrite (Richter et al., 1995; Heales et al., 1999).

In addition, under adverse conditions, such as gross mitochondrial Ca^{2+} accumulation or oxidation of thiol groups, a mitochondrial permeability transition (MPT) pore may be formed that is permeable to molecules with a mass of <1,500 Da. As a result, the electrochemical H⁺ gradient (which is essential for oxidative phosphorylation) is dissipated, and mitochondrial swelling occurs due to entry of water from the cytosol (Szabo and Zoratti, 1991; Bernardi et al., 1994, 1999; Gunter and Gunter,

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Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CsA, cyclosporin A; MPT, mitochondrial permeability transition; Rh, rhodamine; RR, ruthenium red; Ub₀, ubiquinone 0.

1994; Bernardi and Petronilli, 1996). Evidence has been obtained that an MPT occurs during reperfusion of cardiac muscle and liver cells, and that this MPT is a major factor causing reperfusion damage in these tissues (Crompton and Costi, 1990; Griffiths and Halestrap, 1995; Nieminen et al., 1995).

Identification of pore assembly in many in vitro systems is aided by the fact that the MPT pore is blocked (relatively specifically) by cyclosporin A (CsA). It is now known that CsA, when allowed to pass the bloodbrain barrier, markedly ameliorates brain damage caused by forebrain and focal ischemia (Uchino et al., 1995, 1998; Li et al., 1996; Butcher et al., 1997; Matsumoto et al., 1999; Yoshimoto and Siesjö 1999), as well as in hypoglycemic coma (Friberg et al., 1998).

Most of the in vitro data on the assembly of an MPT pore have been obtained by using mitochondria isolated from tissues such as heart, liver, and skeletal muscle, and it has even been questioned if an MPT can be elicited in brain mitochondria (Kristal and Dubinsky, 1997; Berman et al., 1998). However, recent studies give support to the hypothesis that, at least under some circumstances, an MPT pore is also assembled in brain mitochondria. Kristal and Dubinsky (1997) found that isolated nonsynaptic brain mitochondria underwent swelling when exposed to Ca^{2+} or P_i, and similar results were obtained when cultured astrocytes were exposed to Ca²⁺ loads. However, in both situations, CsA was only partially protective. In another study (Andreyev et al., 1998), a mixed population of brain mitochondria (both synaptic and nonsynaptic) were found to undergo an MPT, but only in the absence of ATP and Mg^{2+} , i.e., under conditions in which only small amounts of Ca^{2+} can be accumulated by the mitochondria.

To elucidate further the mechanisms of MPT formation in brain tissue, we examined the ability of isolated nonsynaptic brain mitochondria to accumulate repeated Ca^{2+} loads, and the ability of these Ca^{2+} loads to elicit mitochondrial swelling by measuring Calcium Green-5N fluorescence and light scattering, respectively. The results were compared with those obtained on muscle mitochondria. In addition, to support our spectrophotometric studies, we examined mitochondrial swelling by electron microscopy. Furthermore, we studied the effects of not only CsA, but also ubiquinone 0 (Ub₀) on Ca^{2+} uptake by brain nonsynaptosomal mitochondria and on MPT pore opening. The latter was done because Ub_0 has been shown to increase markedly the capacity of mitochondria from other sources to accumulate Ca2+ and to block the Ca^{2+} -triggered MPT (Fontaine et al., 1998*a*,*b*).

MATERIALS AND METHODS

Isolation of nonsynaptic brain mitochondria

We isolated nonsynaptic brain mitochondria from 300-g male Wistar rats (Simonsen Laboratories Inc., Gilroy, CA, U.S.A.) using a modification of the method of Sims (1990). Following decapitation, brains were rapidly removed and placed in ice-cold isolation buffer containing 320 mM sucrose,

1 mM K₂EDTA, and 10 mM Tris (pH 7.4). All subsequent homogenization and centrifugation steps were carried out at 4°C. The cerebellum and underlying structures were removed, and the remaining brain tissue was used. Each hemisphere was minced separately with scissors in 3 ml of isolation buffer and homogenized by hand in a Potter-Elvehjem homogenizer. The resulting homogenate was centrifuged at 1,330 g_{av} for 3 min. The supernatant was decanted, and the pellet was resuspended in half of the original volume and recentrifuged as above. The pooled supernatant was centrifuged at 21,200 g_{av} for 10 min. The supernatant from this centrifugation step was decanted, and the pellet obtained was resuspended in 15% Percoll (3 ml) and layered into centrifuge tubes containing a preformed two-step discontinuous density gradient consisting of 3.5 ml of 23% Percoll on top of 2.5 ml of 40% Percoll. The gradients were centrifuged at 30,700 g_{av} for 10 min. The mitochondrial fraction, located at the interface between the bottom two layers, was removed and diluted 1:4 in isolation buffer and recentrifuged at 16,700 g_{av} for 10 min. The supernatant was decanted and the pellet resuspended in buffer containing 320 mM sucrose, 0.1 mM K₂EDTA, and 10 mM Tris-HCl (pH 7.4). Aliquots were removed for protein determinations, then bovine serum albumin (10 mg/ml) was added to the mitochondria, and the suspension was centrifuged at 6,900 g_{av} . The supernatant was removed, and the mitochondrial pellet was then gently resuspended in an equal volume of buffer used for mitochondrial Ca²⁺ uptake experiments (see below) and stored on ice.

Isolation of heart mitochondria

After decapitation, hearts were rapidly removed and placed in ice-cold isolation buffer containing 210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 50 mM Tris (pH 7.4). The tissue was finely minced with scissors and then homogenized in 10 ml of isolation buffer using a Tissuemizer motorized homogenizer (Tekmar Co., Cincinnati, OH, U.S.A.). The homogenate was centrifuged at 1,300 $g_{\rm av}$ for 3 min. The supernatant was decanted, and the pellet was resuspended in 5 ml of isolation buffer and centrifuged as above. The pooled supernatant was centrifuged at 10,000 g_{av} for 10 min. The resulting supernatant was removed together with the top layer of the pellet (which contains "light" mitochondria and/or damaged mitochondria), and the dark-brown "dense" mitochondrial pellet was resuspended in isolation buffer with a lower EDTA concentration (0.1 mM EDTA). After aliquots were removed for protein measurements, the mitochondria were centrifuged at 6,800 g_{av} for 10 min. The mitochondrial pellet obtained was then gently resuspended in buffer and stored on ice prior to the Ca²⁺ uptake experiments.

Mitochondrial protein measurements

Mitochondrial protein concentrations were measured using a Lowry DC kit (Bio-Rad) with bovine serum albumin used as a concentration standard.

Mitochondrial oxygen consumption

The measurement of oxygen consumption rates was performed using a custom-made incubation chamber with a water jacket and a micro-Clark electrode (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.) fitted into the top of the chamber. The incubation medium was stirred constantly using an electromagnetic stirrer and magnetic stirring bar. The oxygen consumption studies were conducted at 37°C in respiration medium consisting of 100 mM KCl, 75 mM mannitol, 25 mM sucrose, 5 mM phosphate-Tris, 10 mM Tris-HCl, and 50 μ M EDTA (pH 7.4). Approximately 0.2 mg of mitochondrial protein was added into an incubation chamber in a total volume of 400 μ l of respiration medium. The concentrations of substrates used were 5 m*M* glutamate and 5 m*M* malate. State 3 respiration was induced by the addition of ADP, and the respiratory control ratio was calculated from the ratio of the state 3/state 4 oxygen consumption rates, with and without ADP, respectively (Chance and Williams, 1956).

Measurement of mitochondrial Ca²⁺ uptake

Extramitochondrial free Ca²⁺ was measured according to the methods described by Murphy et al. (1996). Isolated mitochondria (0.5 mg of protein) were resuspended in 2 ml of buffer containing 125 m*M* KCl, 2 m*M* K₂HPO₄, 1 m*M* MgCl₂, 1 μ *M* EGTA, 20 m*M* Tris (pH 7.2 at 37°C), 5 m*M* glutamate, and 5 m*M* malate. Free Ca²⁺ was monitored by use of a hexapotassium salt of Calcium Green-5N (0.1 μ *M*). Fluorescence was recorded continuously in a water-jacketed cuvette holder at 37°C using a Perkin–Elmer LS-50B fluorescence spectrometer with excitation and emission wavelengths of 506 and 532 nm, respectively. At the end of each experiment, calibrations were performed to establish (a) a zero (by adding 20 μ l of 0.1 *M* EGTA) and (b) a saturated Ca²⁺ level (by adding 20 μ l of 1 *M* CaCl₂). Free Ca²⁺ concentration was calculated from the following equation:

$$[\mathrm{Ca}^{2+}]_{\mathrm{free}} = K_{\mathrm{D}} \frac{[F - F_{\mathrm{min}}]}{[F_{\mathrm{max}} - F]}$$

where *F* is the fluorescence of the indicator at experimental Ca^{2+} levels, F_{min} is the fluorescence in the absence of Ca^{2+} (after adding EGTA), and F_{max} is the fluorescence of the Ca^{2+} -saturated probe (1 *M* CaCl₂ added). A Calcium Green-5N K_D for Ca^{2+} of 4.3 was used (Rajdev and Reynolds, 1993). To calculate the total amount of Ca^{2+} taken up by mitochondria, a calibration pulse was recorded by addition of a known quantity of CaCl₂ to the medium with mitochondria where the Ca^{2+} uptake was prevented by 5 μ *M* rotenone or 0.5 μ *M* antimycin. As the buffer contains EGTA and phosphate, which can bind Ca^{2+} , the ordinate axes on the figures are labeled as added nanomoles of Ca^{2+} per milligram of protein, which correspond to the fluorescence level associated with a known quantity of Ca^{2+} added to the medium, normalized to 1 mg of mitochondrial proteins.

Monitoring of the mitochondrial membrane potential

Changes in mitochondrial membrane potential were measured in the presence of 0.2 μ M rhodamine (Rh) 123 as described by Emaus et al. (1986). The excitation and emission wavelengths for Rh 123 were 503 and 525 nm, respectively.

Measurement of mitochondrial swelling

Mitochondrial swelling was estimated from the changes of light scattering (at 90° to the incident light beam) at 540 nm (for both excitation and emission wavelengths) measured in mitochondrial suspensions (0.5 mg of protein in 2 ml) using the same buffer as for the mitochondrial Ca²⁺ experiments above. The experiments were performed in a water-jacketed cuvette holder at 37°C using a Perkin–Elmer LS-50B fluorescence spectrometer. Each experiment was terminated by the addition of alamethicin (40 μ g/mg of protein) to induce a maximal swelling of the whole mitochondrial population. This was confirmed to be the case with the electron micrographs obtained (see below). In the figures, light intensity after alamethicin treatment represents 0, and the light intensity before Ca²⁺ addition is marked as 100 on the ordinate.

Fixation of mitochondria for electron microscopy

For electron microscopy, mitochondria were incubated under the specific conditions and centrifuged at 13,000 g for 5 min. The pellet obtained was fixed overnight with 4% glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.4) at 4°C, postfixed with 1% osmic acid in 0.1 *M* cacodylate buffer, dehydrated, and embedded in LX-112 (Ladd) epoxy resin. Thin sections were observed with a Zeiss 10/A transmission electron microscope.

RESULTS

We will describe changes in the capacity of brain mitochondria to sequester Ca²⁺, as well as the accompanying alterations in membrane potential and in mitochondrial volume. The changes observed will be correlated to the electron micrograph appearance of Ca²⁺loaded mitochondria. After that, we will consider the effects of CsA and Ub₀. However, it seems reasonable that we begin by obtaining control data by illustrating how heart mitochondria respond to Ca²⁺ loading under conditions similar to those used in the experiments on brain mitochondria. The mitochondria used in this study were well coupled. The respiratory control ratios of the nonsynaptic brain mitochondria and heart mitochondria used were always ≥ 5 and ≥ 8 , respectively. The experiments where qualitative changes are reported were repeated three or four times to confirm reproducibility.

Ca²⁺ loading of heart mitochondria

Figure 1 shows that heart mitochondria buffered two consecutive Ca²⁺ loads (40 nmol of Ca²⁺/mg of mitochondrial protein) before they had been saturated and released the Ca2+ accumulated, probably because an MPT pore was opened. Measurement of light scattering did not suggest that an MPT pore opened after a single Ca^{2+} addition (curve a). However, when the Ca^{2+} dose was increased to 100 nmol of Ca2+/mg of protein, progressive swelling occurred (curve b). By increasing the added Ca²⁺ to 400 nmol of Ca²⁺/mg of protein, the swelling rate increased further (curve c). The results thus suggest that both the swelling and the release of Ca^{2+} reflect the opening of an MPT pore. This contention was also supported by electron micrographs (Fig. 2), which show that following the addition of 400 nmol of Ca^{2+}/mg of protein, the majority of the heart mitochondria showed high amplitude swelling with damaged cristae.

Ca²⁺ loading of brain mitochondria

As Fig. 3 (upper panel) shows, brain mitochondria had a seemingly better capacity to sequester repeated Ca²⁺ loads than heart muscle mitochondria. There were two additional features that the brain mitochondrial preparations displayed. First, the initial Ca²⁺ uptake was followed by a small release; this is illustrated more clearly in the middle panel of Fig. 3. Second, when the mitochondria were saturated, they did not release the Ca²⁺ accumulated. However, when carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; 3 μM) was added to the buffer, a release of Ca²⁺ was observed, indicating that mitochondria are still loaded with the Ca²⁺.



FIG. 1. Measurement of Ca²⁺ uptake and swelling of isolated heart mitochondria. Upper panel: The Ca2+ uptake by mitochondria (0.5 mg of protein in 2 ml) in the presence of 5 mM glutamate and 5 mM malate was measured by using Calcium Green-5N (0.1 μM) as an indicator of the free Ca²⁺ concentration. The mitochondria were resuspended in buffer containing 125 m/ KCl, 1 m/ MgCl₂, 2 m/ K₂HPO₄, 20 m/ Tris (pH 7.2). Equivalent pulses of CaCl₂ (40 nmol of Ca²⁺/mg of protein = 10 μM) were added to mitochondria every 3 min until a spontaneous release of Ca²⁺ occurred. The small horizontal bars represent the time points when the Ca2+ pulses were added to the buffer. Mit, addition of mitochondria to the buffer. See Materials and Methods for description of the label on the ordinate. Lower panel: Swelling of heart mitochondria (0.5 mg of protein in 2 ml of buffer of the same composition as above without the Calcium Green-5N) was assessed from measurement of light scattering at 540 nm. Curve a represents the changes in light scattering following addition of the standard 40 nmol of Ca²⁺/mg of protein pulse (no swelling was observed). An addition of 100 nmol of Ca2+/mg of protein to the mitochondrial suspension was followed by progressive swelling (curve b). The rate of swelling was increased further when 400 nmol of Ca2+/mg of protein was added to the isolated heart mitochondria (curve c). EM represents the time points when the mitochondria were removed and processed for electron microscopy. A.U., arbitrary units.

Mitochondrial Ca^{2+} accumulation was blocked by ruthenium red (RR) (Fig. 3, lower panel). Thus, when RR was present in the buffer, Ca^{2+} added to the incubation medium greatly increased the initial Ca^{2+} concentration in the buffer, as no uptake was observed. As RR is a relative specific inhibitor of mitochondrial Ca^{2+} uptake, these data suggest that the added Ca^{2+} was accumulated by the mitochondria. Changes in mitochondrial membrane potential, as measured by Rh 123 fluorescence, are shown in Fig. 4. The upper part of the figure illustrates an experiment in which Ca^{2+} was added at 3-min intervals. Addition of Ca^{2+} caused a rapid, but small, depolarization, which was followed by a gradual loss of membrane potential. Additional Ca^{2+} pulses resulted in progressive depolarization until the mitochondrial membrane potential reached the residual Donnan potential. This was confirmed at the end of experiments by the induction of complete depolarization with 3 μM CCCP.

The lower panel of Fig. 4 illustrates an experiment in which 10 min was allowed to pass before a second addition of Ca^{2+} was made. The results demonstrate that



FIG. 2. Photomicrographs of heart mitochondria processed following incubation in standard reaction buffer for 5 min (**A**) and after additions of 400 nmol of Ca²⁺/mg of protein (**B**). Following Ca²⁺ treatment, almost the whole population of the heart mitochondria show massive swelling with disrupted cristae. Scale bar = 0.7 μ m.



FIG. 3. Uptake of Ca²⁺ by nonsynaptosomal brain mitochondria. **Upper panel:** Uptake of Ca²⁺ by brain mitochondria (0.5 mg of protein in 2 ml) when the standard 40 nmol of Ca²⁺/mg of protein pulses were added to the medium every 3 min until there was no evidence of further uptake. At the end of the experiment, CCCP (3 μ *M*) was added. The experimental conditions are identical to those described in the upper panel of Fig. 1. Mit, addition of mitochondria to the buffer. **Middle panel:** Uptake of Ca²⁺ following two Ca²⁺ pulses that were separated by a 10-min period, to show the different response of brain mitochondria as compared with the heart mitochondria. **Lower panel:** Inhibition of Ca²⁺ uptake by RR. The incubation buffer contained 0.5 μ *M* RR. Labels on the ordinate axes correspond to the fluorescence intensity associated with a known quantity of added Ca²⁺, normalized to 1 mg of mitochondrial protein.

the first addition caused a substantial loss of membrane potential (cf. effect of CCCP).

The results illustrated in Fig. 4 should be compared with those of Fig. 5, which shows the effect of a single and of repeated additions of Ca^{2+} on the light scattering measured at 540 nm. A decrease in light scattering is believed to reflect mitochondrial swelling caused by an MPT. The results demonstrate that most of the swelling observed after repeated Ca^{2+} additions was the effect of the first addition, i.e., the one that caused some Ca^{2+} release and that substantially depolarized the membranes.

As alamethic in caused at least twice as much change in light scattering as Ca^{2+} additions, the combined results suggest that the first Ca^{2+} wave is associated with an MPT in a subpopulation of the mitochondria that leads to limited mitochondrial depolarization and to partial swelling.

To shed further light on this issue, mitochondrial preparations were examined in the electron microscope before and after Ca^{2+} addition. As shown in Fig. 6, Ca^{2+} caused swelling of part of the mitochondrial population. This was in contrast to alamethicin, which triggered swelling of the whole mitochondrial population. As the swelling was similar whether one or several Ca^{2+} additions were made, the results support our conclusion that the first Ca^{2+} pulse triggers an MPT. Additional support is provided by the effect of CsA (see below).



FIG. 4. Changes in mitochondrial membrane potential as assessed by Rh 123. Fluorescence excited at 503 nm and emitted at 527 nm was measured in standard buffer with 0.2 μ M Rh 123. Addition of brain mitochondria (Mit) caused a decrease in light intensity due to fluorescence quenching of Rh 123. After a stable reading was obtained, Ca²⁺ pulses (40 nmol of Ca²⁺/mg of protein) were added every 3 min (arrows, **upper panel**) until no change in mitochondrial membrane potential was observed. At the end of the experiment, CCCP (3 μ M) was added to the incubation buffer to ensure that the mitochondria are fully depoded during identical conditions, with the second one applied after a 10-min delay; at the end of the experiment, CCCP (3 μ M) was added.



FIG. 5. Swelling of brain mitochondria as assessed by changes in light scattering at 540 nm. Brain mitochondria (0.5 mg of protein) were incubated in the same buffer as described in Fig. 1 without Calcium Green-5N. To induce swelling, a single Ca2 pulse (40 nmol of Ca²⁺/mg of protein) was added into incubation buffer (upper panel). Ten minutes after the Ca2+ addition, alamethicin (Alm; 40 μ g/mg of protein) was added to the buffer to induce a complete swelling of all mitochondria in suspension. Lower panel: Changes in light scattering when mitochondria were exposed to equivalent Ca2+ pulses every 3 min. The small horizontal bars represent the time points of Ca2+ additions. EM corresponds to conditions when the mitochondria were processed for electron microscopy. Light intensity in the lower panel is expressed in arbitrary units (A.U.), where 100 corresponds to the light recorded before Ca2+ addition and the zero value represents the light intensity following alamethicin treatment.

Effect of CsA and of Ub₀

The immunosuppressant drug CsA is reported to have two effects on isolated mitochondria from tissue other than the brain: it is a virtually specific blocker of the MPT, and it increases the capacity of the mitochondria to sequester Ca²⁺ (for reviews, see Bernardi et al., 1992; Duchen et al., 1993; Murphy et al., 1996). Recently, Ub₀ has been shown to have a similar effect on mitochondria from liver and muscle tissue (Fontaine et al., 1998*a*,*b*).

Figure 7 shows that CsA had little effect on Ca²⁺ accumulation by the mitochondria; in fact, CsA increased the capacity of the mitochondria to sequester Ca²⁺ by only 10% (from ~ 210 to ~ 230 nmol of Ca²⁺/mg of protein). However, CsA prevented the moderate release of Ca²⁺ following the first Ca²⁺ pulse.

J. Neurochem., Vol. 74, No. 5, 2000

When added at a dose of 5 μM , Ub₀ had very similar effects. When the concentration was increased to 20 and 40 μM , however, the Ca²⁺ sequestration capacity was progressively decreased, and when the uptake system was saturated, Ca²⁺ was released from the mitochondria (see Fig. 8).



FIG. 6. A: Electron photomicrograph of brain mitochondria incubated in the standard buffer for 5 min with no treatment. **B:** Brain mitochondria exposed to one standard Ca²⁺ pulse (40 nmol of Ca²⁺/mg of protein) for 5 min. One can identify swollen mitochondria with damaged matrix (asterisks) and rather unaffected compact mitochondria. **C:** Mitochondria swollen due to alamethicin treatment (40 μ g/mg of protein). Scale bar = 0.5 μ m.

Both CsA and Ub₀ stabilized the membrane potential following repetitive additions of Ca²⁺ (Fig. 9). Thus, in the presence of CsA, the first three Ca²⁺ pulses induced a small decrease in membrane potential, which quickly recovered. Further Ca²⁺ additions then caused progressive depolarization until finally the mitochondria were fully depolarized, as confirmed by the addition of CCCP.

As Fig. 10 shows, both CsA and Ub₀ reduced Ca²⁺induced swelling in a dose-dependent manner and, at optimal concentrations, the swelling was prevented completely. These concentrations were ~ 1 μM for CsA and 20–40 μM for Ub₀. When swelling had been prevented (or minimized), it appeared (or was augmented) when additional Ca²⁺ pulses were delivered (data not shown).

As it was suggested that Ub₀ affects the electron flow via complex I, thereby modulating the Ca²⁺ retention capacity and sensitivity of mitochondria to Ca²⁺-induced MPT (Fontaine et al., 1998*a*,*b*), we also measured the Ca²⁺ uptake by nonsynaptic brain mitochondria that were energized with the complex II-linked substrate suc-



FIG. 7. Effect of CsA on Ca²⁺ uptake by brain mitochondria. Mitochondria incubated in the standard buffer (see Fig. 1) in the presence of 0.1 and 1 μ M CsA were exposed to repeated Ca²⁺ additions (40 nmol of Ca²⁺/mg of protein) every 3 min. CsA inhibited the transient Ca²⁺ increase following the first Ca²⁺ pulse. Small horizontal bars represent the time points when the Ca²⁺ was added to the buffer.



FIG. 8. Effect of Ub₀ on Ca²⁺ uptake by brain mitochondria. Mitochondria incubated in standard buffer with different concentration of Ub₀ were exposed to repeated Ca²⁺ pulses. At high Ub₀ concentration, the brain mitochondria seemingly responded to Ca²⁺ pulses as heart mitochondria, with massive release of accumulated Ca²⁺ after they were overloaded.

cinate. With succinate as substrate, the mitochondrial Ca^{2+} accumulation following Ca^{2+} additions was more rapid than with malate plus glutamate (see Fig. 11). However, after four Ca^{2+} pulses, there was a marked transient increase in extramitochondrial Ca^{2+} concentration, and further Ca^{2+} additions led to a gradual saturation of the uptake system (Fig. 11, upper panel). In the presence of Ub₀, the retention capacity of mitochondria using succinate as substrate was also increased (see Fig. 11). Thus, Ub₀ was effective when either complex I-linked or complex II-linked substrates were used for mitochondrial respiration.

In summary, CsA at a concentration of 1 μ M had only a small effect on the Ca²⁺ sequestration capacity of brain mitochondria, but prevented the partial release of Ca²⁺ accompanying the first Ca²⁺ pulse, reversed the small depolarization of mitochondrial membranes following the first two or three Ca²⁺ pulses, and prevented the mitochondrial swelling that was elicited by a single Ca²⁺ pulse. Ub₀ had, in general, similar effects, and we could not identify any qualitative or quantitative differences between CsA and Ub₀. It seems clear, however; that Ub₀



FIG. 9. Changes in mitochondrial membrane potential due to Ca^{2+} additions as monitored by Rh 123 in the presence of CsA (**upper panel**) and Ub₀ (**lower panel**). When exposed to Ca^{2+} pulses in the presence of CsA (1 μ M), mitochondria depolarized only transiently with full recovery of the membrane potential after three repeated Ca²⁺ additions. Further Ca²⁺ additions then progressively depolarized the brain mitochondria (upper panel). The lower panel shows changes in membrane potential due to Ca²⁺ additions when Ub₀ (40 μ M) was present in the incubation buffer. Similarly, as with CsA, the Ca²⁺ pulses induced small transient depolarization in membrane potential. Following four Ca²⁺ additions, the mitochondria depolarized fully. Mit, addition of mitochondria to the buffer.

at high concentrations was toxic because it reduced the Ca^{2+} sequestration capacity of the mitochondria and caused massive release of Ca^{2+} when the mitochondria were saturated with Ca^{2+} . We recognize that when this occurred, brain mitochondria behaved as those isolated from heart tissue.

DISCUSSION

Mechanisms of Ca^{2+} uptake by isolated mitochondria, or by mitochondria in isolated cells, have attracted considerable interest mostly because such uptake is considered a trigger of membrane depolarization and of the assembly of an MPT. The majority of such studies have been carried out on mitochondria from liver, heart, and skeletal muscle (Fontaine et al., 1998*a*,*b*); however, comparable studies have been performed on the neuronal cell line GT1-7 (Murphy et al., 1996). In all of these studies, the mitochondria could absorb a limited number of Ca^{2+} pulses before they were saturated with Ca^{2+} . At that point, they released the Ca^{2+} accumulated, probably because an MPT pore was opened in the Ca^{2+} -loaded mitochondria. Much interest has been devoted to the pore-blocking drug CsA and the similarly acting Ub₀. Both of these drugs increase the capacity of the mitochondria to sequester Ca^{2+} , probably because they reduce the mitochondrial depolarization caused by Ca^{2+} accumulation (see Results and Fontaine et al., 1998*b*). Similar results on CsA were reported by Murphy et al. (1996) on GT1-7 cells. These authors also studied cells overexpressing Bcl-2 and found that Bcl-2 expression



FIG. 10. Effect of CsA and Ub₀ on Ca²⁺-induced swelling of brain mitochondria. **Upper panel:** Effect of CsA on swelling of brain mitochondria induced by addition of 40 nmol of Ca²⁺/mg of protein. Mitochondria (0.5 mg of protein) were incubated in standard buffer (see Fig. 1) except that different concentrations of CsA were present. The curves show Ca²⁺-induced swelling with (a) no CsA present, (b) 0.1 μ M CsA, (c) 0.5 μ M CsA, (d) 1 μ M CsA, (e) 5 μ M CsA, and (f) 10 μ M CsA. **Lower panel:** Swelling of brain mitochondria after Ca²⁺ additions where the incubation buffer contained different concentration of Ub₀. The curves represent changes in light scattering due to Ca²⁺ addition when the buffer contained (a) no Ub₀, (b) 5 μ M Ub₀, (c) 10 μ M Ub₀, (d) 20 μ M Ub₀, (e) 40 μ M Ub₀, and (f) 80 μ M Ub₀. The recorded light intensity is expressed in arbitrary units (A.U; see Figs. 1 and 5).



FIG. 11. Effect of Ub₀ on Ca²⁺ uptake by brain mitochondria energized with succinate. Brain mitochondria (0.5 mg) were resuspended in standard incubation buffer except that the buffer contained succinate (10 mM), but no malate and glutamate. The changes in buffer Ca²⁺ concentration were recorded with Calcium Green-5N fluorophore. To energize mitochondria, succinate and 5 μ M rotenone were added into the buffer. Ca²⁺ pulses (40 nmol of Ca²⁺/mg of protein) were added repeatedly every 3 min until there was no further Ca²⁺ uptake observed (**upper panel**). **Lower panel:** In the presence of 20 μ M Ub₀, the mitochondria rapidly took up the Ca²⁺ from the buffer until they were overloaded and released the accumulated Ca²⁺.

potentiated the capacity of mitochondria to sequester Ca^{2+} .

As stated in the introductory section, several groups have found that brain mitochondria are less prone to assemble an MPT, and it has been questioned if an MPT acts as an important modulator of ischemic cell death in the brain (Kristal and Dubinsky, 1997; Andreyev et al., 1998; Berman et al., 1998). However, it has remained a challenge that CsA, when allowed to pass the blood– brain barrier, is markedly neuroprotective. This is what inspired us to study in some detail how a nonsynaptosomal population of brain mitochondria responds to repeated Ca²⁺ pulses.

The primary objective of this study was to explore how nonsynaptosomal brain mitochondria differ from those derived from other tissues, such as liver and heart, when subjected in vitro to Ca^{2+} loads that normally would be expected to lead to an MPT, and to cause release of Ca^{2+} from Ca^{2+} -loaded mitochondria. An equally important objective was to assess how CsA, an established antiischemic drug, affects Ca^{2+} accumulation of isolated mitochondria and the assembly of an MPT pore. A secondary objective was to establish if Ub₀ mimics the effect of CsA, or if it acts by different mechanisms. Another, secondary objective was to explore whether energization by a complex II substrate (succinate) would give results similar to those obtained with glutamate plus malate.

Our results proved that brain mitochondria behave in a radically different way from liver, heart, and skeletal muscle mitochondria, studied by others, and heart mitochondria, as examined in the present study. Thus, under the experimental conditions used in our present studies, following saturation of the Ca²⁺ uptake system, brain mitochondria did not respond with a large release of their sequestrated Ca²⁺. As stated, however, there was a small transient release of mitochondrial Ca²⁺ after the first Ca²⁺ pulse, suggesting that some part of the mitochondrial population underwent an MPT and that Ca²⁺ released from this subpopulation of mitochondria was then accumulated by other mitochondria within the incubation medium. It is of note, however, that this second population of mitochondria did not release the Ca²⁺ accumulated.

The effect of CsA on the ability of nonsynaptic brain mitochondria to accumulate Ca^{2+} was only moderate compared with the effects of CsA on liver or muscle mitochondria (Fontaine et al., 1998*a*,*b*). Similar effects of CsA were also reported by Murphy et al. (1996) on their GT1-7 cell line. However, our studies unequivocally showed that CsA prevented the partial efflux of Ca^{2+} that accompanied the first Ca^{2+} pulse, which depending on the CsA dose entirely prevented the Ca^{2+} -induced swelling of the mitochondria, and which reversed the depolarizations during the first two or three Ca^{2+} pulses.

The changes in Rh 123 fluorescence cannot be readily interpreted in our study as depolarization or recovery of mitochondrial membrane potential. This is because of the heterogeneity of the nonsynaptic brain mitochondrial population. Following addition of 40 nmol of Ca^{2+}/mg of protein, some of the mitochondria depolarized due to an MPT, but the rest of the population could still have maintained their membrane potential, which is reflected in their ability to accumulate additional Ca²⁺. Thus, a moderate increase in Rh 123 fluorescence intensity may reflect complete depolarization of a subfraction of the mitochondria, without significant changes in membrane potential in the rest of the mitochondrial population. Data on mitochondrial membrane potential suggest that the saturation of the Ca^{2+} uptake after several Ca^{2+} pulses is due to complete depolarization of mitochondria to the resting Donnan potential. The fact that no Ca²⁺ release was observed suggests that membrane integrity was preserved.

Our data, showing only incomplete swelling of the nonsynaptic mitochondrial population, together with the fact that the mitochondrial accumulation of Ca²⁺ after the first Ca²⁺ pulse was always preceded by a transient increase in Ca^{2+} in the incubation medium, suggest that there is a subpopulation of nonsynaptic mitochondria that undergo the MPT at lower Ca²⁺ concentrations than other nonsynaptic mitochondria. Nonsynaptic mitochondria are derived from a variety of cell types representing particularly neurons and glia. Thus, such a mitochondrial population may give rise to a heterogeneous response to Ca^{2+} exposure. This hypothesis is also supported by the fact that within the rat brain there are different subpopulations of nonsynaptic and synaptic mitochondria that have different oxygen consumption rates with NADlinked and FAD-linked substrates, as well as different activities of primary dehydrogenase enzymes, such as pyruvate dehydrogenase and glutamate dehydrogenase (Lai and Clark, 1979). In addition, it is known that synaptic and nonsynaptic mitochondria have different activities of the respiratory chain complexes (Bates et al., 1994; Almeida et al., 1995), and that mitochondrial membrane potentials in a single cell can be heterogeneous (Smiley et al., 1991). Thus, these different mitochondrial populations may have different resting membrane potentials and depolarize to different extents when exposed to the same amount of Ca²⁺. This could explain why some mitochondria may be able to take up only a small amount of Ca²⁺ before they are overloaded, whereas other mitochondria have a much larger capacity for Ca²⁺ uptake.

The recent results reported by Fontaine et al. (1998*a*) raise the possibility that electron flow through mitochondrial complex I is a major modulator of MPT pore opening, and that this is the site where Ub₀ acts. One could argue that if Ub₀ only affects electron flow via complex I, it should have no effect on mitochondria energized with a complex II substrate. However, in our experiments, mitochondria incubated in the presence of succinate and Ub₀ were relatively resistant to Ca²⁺ challenges and, at least at a concentration of 20 μ M Ub₀, the partial release of Ca²⁺ after repeated pulses was blocked. This suggests that Ub₀ suppresses the MPT induced by Ca²⁺ in succinate-respiring mitochondria.

These data thus suggest that nonsynaptic brain mitochondria behave differently from mitochondria from liver or muscle, particularly with respect to MPT pore opening, and also support the concept of mitochondrial heterogeneity with respect to Ca^{2+} accumulation and MPT pore opening. The data also show that although CsA can increase the capacity of brain mitochondria to accumulate Ca^{2+} and to resist Ca^{2+} -induced mitochondrial swelling, the concentration of CsA that the mitochondria are exposed to is critical. The data obtained using succinate and Ub₀ suggest that electron flow via respiratory complexes has a controlling/modulatory role in MPT formation in nonsynaptic brain mitochondria, as well as in liver and skeletal muscle mitochondria (Fontaine et al., 1998*a*,*b*).

The studies of Andreyev et al. (1998) were carried out on a mixture of synaptic and nonsynaptic mitochondria. They reported the following results. First, when mitochondria took up Ca^{2+} , they released up to 40% of their cytochrome c content in a cyclosporin-insensitive manner. When ATP and Mg^{2+} were present, this process was not accompanied by mitochondrial swelling, as assessed by absorbance measurements. The conclusions drawn were that cytochrome c released by brain mitochondria does not require an MPT; however, in the absence of ATP and Mg²⁺, Ca²⁺ triggers a CsA-sensitive highamplitude swelling and release of matrix solutes. The results reported again raise the question whether an MPT occurs in vivo. It seems particularly justified to examine mitochondrial behavior in the presence of Mg²⁺ and phosphate, without and with ATP or ADP, because these are the conditions prevailing in ischemic and postischemic tissues, respectively.

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