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Distinct characteristics of Ca^{2+}-induced depolarization of isolated brain and liver mitochondria

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Abstract

Ca^{2+}-induced mitochondrial depolarization was studied in single isolated rat brain and liver mitochondria. Digital imaging techniques and rhodamine 123 were used for mitochondrial membrane potential measurements. Low Ca^{2+} concentrations (about 30–100 nM) initiated oscillations of the membrane potential followed by complete depolarization in brain mitochondria. In contrast, liver mitochondria were less sensitive to Ca^{2+}; 20 μM Ca^{2+} was required to depolarize liver mitochondria. Ca^{2+} did not initiate oscillatory depolarizations in liver mitochondria, where each individual mitochondrion depolarized abruptly and irreversibly. Adenine nucleotides dramatically reduced the oscillatory depolarization in brain mitochondria and delayed the onset of the depolarization in liver mitochondria. In both type of mitochondria, the stabilizing effect of adenine nucleotides completely abolished by an inhibition of adenine nucleotide translocator function with carboxyatractyloside, but was not sensitive to bongkrekic acid. Inhibitors of mitochondrial permeability transition cyclosporine A and bongkrekic acid also delayed Ca^{2+}-depolarization. We hypothesize that the oscillatory depolarization in brain mitochondria is associated with the transient conformational change of the adenine nucleotide translocator from a specific transporter to a non-specific pore, whereas the non-oscillatory depolarization in liver mitochondria is caused by the irreversible opening of the pore.

Keywords: Mitochondria; Calcium; ATP; Adenine nucleotide translocator; Permeability transition; Membrane potential

1. Introduction

Among other mitochondrial functions, energy production and Ca^{2+} uptake are crucial for maintaining neuronal viability. Both of these functions are critically dependent on mitochondrial membrane potential ($\Delta\Psi_m$). The respiratory chain, located in the inner mitochondrial membrane, creates the membrane potential of ~150 mV negative to the cytosol, which drives the phosphorylation of ADP to ATP [1]. ATP synthesized by mitochondria is a main source of energy in brain, where oxidative phosphorylation supplies 95% of total ATP [2]. Additionally, $\Delta\Psi_m$ provides the electrical gradient for mitochondrial Ca^{2+} uptake via the Ca^{2+}-uniporter. Mitochondrial Ca^{2+} sequestration buffers cytoplasmic Ca^{2+} elevations in neurons under physiological conditions [3–6]. However, excessive Ca^{2+} sequestration occurs in some pathological conditions and makes a critical contribution to neuronal injury [7–10].

Mitochondrial Ca^{2+} overload in combination with oxidative stress and ATP depletion induces opening of mitochondrial permeability transition pore (mPTP) [11,12]. The mPTP is a large conductance nonspecific pore formed in mitochondrial membranes by a conformational change of several proteins that putatively include the adenine nucleotide translocator (ANT), cyclophilin D and porin [12,13]. mPTP can operate in two modes, with both high conductance and low conductance forms of the pore [11]. Persistent opening of mPTP in high conductance mode is involved in many forms of cell injury, including ischemia–reperfusion, oxidative stress and apoptosis [12,14]. Tran-

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Abbreviations: $[Ca^{2+}]$, external Ca^{2+} concentration; mPTP, mitochondrial permeability transition; ANT, adenine nucleotide translocator; $\Delta\Psi_m$, mitochondrial membrane potential; HBS, HEPES-buffered solution; Rh123, rhodamine 123; CA, carboxyatractyloside; BA, bongkrekic acid; CsA, cyclosporine A

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sient mPTP opening in a lower conductance mode may contribute to physiological Ca^{2+} signaling [15,16].

The $\Delta\Psi_m$ is a dynamic parameter. Recent data show that $\Delta\Psi_m$ can change spontaneously in a number of different cell types. Spontaneous changes in $\Delta\Psi_m$ were reported in neuroblastoma cells [17,18], cardiomyocytes [19–22], astrocytes [21,22], neurons [23], smooth muscle cells [24–26] and pancreatic B-cells [26]. A range of mechanisms has been reported to be responsible for the transient changes in $\Delta\Psi_m$. Several laboratories have concluded that this phenomenon is caused by mitochondrial mPTP [16,18]. Others reported the involvement of the F_1F_oATPase [23], Ca^{2+}-influx through mitochondrial Ca^{2+}-uniporter [19], mitochondrial membrane anion channels [27] and free radicals released from the matrix side of mitochondria [20].

An important limitation in using intact cells to study of $\Delta\Psi_m$ dynamics is a lack of direct access for drug application to mitochondria and potentially also interference from the cellular environment. These can be avoided in isolated mitochondria preparations. In most studies on isolated mitochondria, $\Delta\Psi_m$ was measured as an average signal from mitochondrial suspension. This method does not capture $\Delta\Psi_m$ dynamics from individual organelles. The first report of transitory mitochondrial depolarization in individual mitochondria was published in 1997 by Ichas et al [16]. By imaging of mitochondria isolated from Ehrlich cells the authors showed a propagated depolarization and calcium wave in mitochondria immobilized in a gel which was dependent on transient opening of mPTP. mPTP was also involved in CsA-sensitive spontaneous short-lasting and prolonged depolarizations in isolated heart mitochondria [28,29]. However, other studies suggested that transient depolarizations in isolated heart mitochondria are result from transient opening of a pH sensitive and proton conductive channel [30,31].

Recently, we have applied fluorescence imaging techniques to monitor $\Delta\Psi_m$ in single isolated brain mitochondria. We have shown [32,33] that low concentration of Ca^{2+} (~30 nM) triggers fluctuations of $\Delta\Psi_m$ in isolated brain mitochondria. These fluctuations are inhibited by both ATP and ADP; carboxyatractyloside, a blocker of ANT, reversed the adenine nucleotide effect. We have also shown that free radical over-production and mitochondrial K(ATP) channels are not involved in this phenomenon. CsA was not able to stop the oscillation activity when applied after the onset of fluctuations. Also, we did not detect a leakage of calcein during the fluctuations, indicating that the fluctuations in $\Delta\Psi_m$ do not reflect opening of large conductance mPTP. However, the exact mechanisms of the $\Delta\Psi_m$ fluctuations remained unclear. In the present study, we compared Ca^{2+}-induced changes of $\Delta\Psi_m$ in liver and brain mitochondria in order to gain further insight into the mechanism of the depolarization. We show here that the dynamics of Ca^{2+}-induced depolarization in brain mitochondria is different from that in liver mitochondria. We suggest that Ca^{2+} induces transient conformational change of ANT to a nonspecific pore in brain mitochondria, whereas in liver mitochondria this transformation of ANT is non-reversible.

2. Materials and methods

2.1. Materials

All materials and reagents were purchased from Sigma (St. Louis, MO) unless otherwise specified. Carboxyatractyloside and bongkrekic acid were obtained from Calbiochem (La Jolla, CA), and rhodamine 123 (Rh123) was purchased from Molecular Probes (Eugene, OR).

2.2. Isolation of mitochondria

All procedures using animals were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee.

Rat brain mitochondria were isolated from the cortex of male Sprague–Dawley rats using a Percoll gradient method described by Sims [34] with minor modifications. The isolation buffer contained (in mM): mannitol 225, sucrose 75, EDTA 0.5, HEPES 5, 1 mg/ml fatty acid free BSA, pH adjusted to 7.3 with KOH. Brain tissue was homogenized using a glass/glass homogenizer in isolation buffer containing 12% Percoll and carefully layered on the top of a 12%/24%/42% discontinuous gradient of Percoll. After 11 min of centrifugation at 31,000×*g*, the mitochondrial fraction was collected from the top of the 42% Percoll layer of the gradient and then washed twice. For the final wash, we used isolation buffer where BSA was omitted and the concentration of EDTA was reduced to 0.1 mM.

For isolation of rat liver mitochondria, we used the same isolation buffer as was used for brain mitochondria. The liver tissue was homogenized using glass/glass homogenizer, centrifuged for 10 min at 1300×*g*, then the pellet was discarded, and the supernatant was centrifuged again for 10 min at 10,000×*g*. The resulting supernatant was removed together with the top layer of the pellet, which contains damaged mitochondria, and the dark-brown "dense" mitochondrial pellet was resuspended in isolation buffer and centrifuged again for 10 min at 10,000×*g*. For the final spin (11,000×*g* for 10 min), the pellet was resuspended in the isolation buffer where BSA was omitted and the concentration of EDTA was reduced to 0.1 mM. The mitochondrial pellet obtained was then gently resuspended in the isolation buffer and stored on ice prior the experiments.

All isolation procedures were carried out at 0–2 °C. During experimentation, mitochondria were stored on ice at a final concentration of 15–20 mg protein/ml in isolation medium until use. The experiments were performed up to

1–5 h after preparation. The protein concentration in each preparation was determined by the Biurett method using a plate reader.

2.3. Fluorescence measurements

All imaging experiments were performed at room temperature in KCl-based HEPES-buffered solution (HBS) containing (in mM): KCl 125, K_2HPO_4 2, HEPES 5, $MgCl_2$ 5, EGTA 0.2, malate 5, glutamate 5, pH 7.0. To attach the mitochondria to the coverslip, 31 mm glass coverslips were washed with 70% ethanol, then with H_20 and dried prior to use. The brain mitochondria were added to HBS at a final concentration of 0.75–1 mg protein/ml immediately before each experiment. A 20-μl drop of brain mitochondrial suspension was placed in the middle of the coverslip for 5–7 min. The liver mitochondrial suspension was placed on the coverslip at a concentration of 15–20 mg protein/ml for about 1 min. The coverslips with the mitochondrial suspension were placed into a 700-μl perfusion chamber that was then mounted onto a microscope fitted for fluorescence imaging as described below. The mitochondria were perfused with HBS buffer at 7 ml/min; after 1 min of perfusion, mitochondria which had not adhered to the coverslip were effectively washed out of the recording chamber. Typically, the density of attached mitochondria was 400,000–500,000 mitochondria per square millimeter. Thus, approximately 2,000 mitochondria were present in a field observed with a 100× objective.

For $\Delta\Psi_m$ measurements, we used the potentiometric probe rhodamine 123 (Rh123, Molecular Probes, Eugene, OR). For fluorescence recording, we used a BX50WI Olympus Optical (Tokyo, Japan) microscope fitted with an Olympus Optical LUM PlanFI 100× water immersion quartz objective. The fluorescence was monitored using excitation light provided by a 75 W xenon lamp-based monochromator (T.I.L.L. Photonics GmbH, Martinsried, Germany), and emitted light was detected using a CCD camera (Orca; Hamamatsu, Shizouka, Japan). Mitochondria were illuminated at 490 nm, and emitted fluorescence was passed through a 500-nm-long pass dichroic mirror and a 535/25 nm band pass filter (Omega Optical). Fluorescence data were acquired and analyzed using Simple PCI software (Compix Inc, Cranberry PA). Fluorescence was measured in 100–150 individual mitochondria for each coverslip. The objects smaller than 0.3 μm were not analyzed. Background fluorescence, determined from 3 to 4 mitochondrion-free regions of the coverslip, was subtracted from all signals. All experiments were repeated 4–6 times using mitochondria prepared from different animals.

$\Delta\Psi_m$ was monitored using an unquenched concentration of Rh123. Rh123 was present in the perfusion medium during the experiment; no preloading was necessary. Since $\Delta\Psi_m$ in brain and liver mitochondria was different in our preparation, an unquenched concentration of Rh123 was determined as 200 nM for brain and 100 nM for liver mitochondria. We also adjusted the exposure time for the imaging of Rh123 fluorescence singly for both types of mitochondria to obtain an optimal fluorescence level between 0 and 150 units. An intensity of Rh123 fluorescence is represented by an arbitrary value and is not directly comparable between the two types of mitochondria. Since Rh123 was used at concentrations below the quench limit, a decrease in fluorescence intensity indicated mitochondrial depolarization and the increase in intensity represented mitochondrial hyperpolarization.

2.4. Calculation of divalent ion concentration

Free Ca^{2+} and Sr^{2+} in the medium, containing EGTA, ATP and ADP, was estimated using the Max-Chelator program (Webmaxc Standard by C. Patton, http://www.stanford.edu/~cpatton/maxc.html). EGTA-free buffer was assumed as a nominal Ca^{2+}-free and contaminant [Ca^{2+}] was not taken into account.

2.5. Statistics

Statistical analysis was performed using Prism 3.0 (Graph Pad Software, San Diego CA). All the data are presented as mean ± S.E. Comparisons were made using Student's *t*-test, with *P* values less than 0.05 taken as significant.

3. Results

3.1. Ca^{2+} induces different events in brain and liver mitochondria

As we have shown previously, about 70–80% of single isolated brain mitochondria attached to the coverslip were initially polarized as detected by Rh123 fluorescence [32,33]. Non-fluorescent, presumably depolarized, mitochondria were excluded from analysis. In the presence of 200 μM, EGTA in the perfusion medium the Rh123 fluorescence was stable in all polarized mitochondria. An increase of the external free Ca^{2+} concentration ([Ca^{2+}]) by about 30 nM triggered $\Delta\Psi_m$ oscillations in brain mitochondria (Fig. 1A). Fig. 1B shows that an increase in [Ca^{2+}] by about 120 nM completely depolarized mitochondria within 5 min; in some mitochondria, $\Delta\Psi_m$ oscillated prior to complete depolarization. The number of mitochondria that underwent this oscillatory activity, as well as the amplitude and the duration of the oscillations diminished with increased [Ca^{2+}]. In all other experiments on brain mitochondria we increased [Ca^{2+}] by 30 nM Ca^{2+}, which completely depolarized about 90% of mitochondria within 10 min (see for example Fig. 2C).

As in brain mitochondria, Rh123 fluorescence was detected in about 80% of liver mitochondria attached to the coverslip. However, liver mitochondria were not

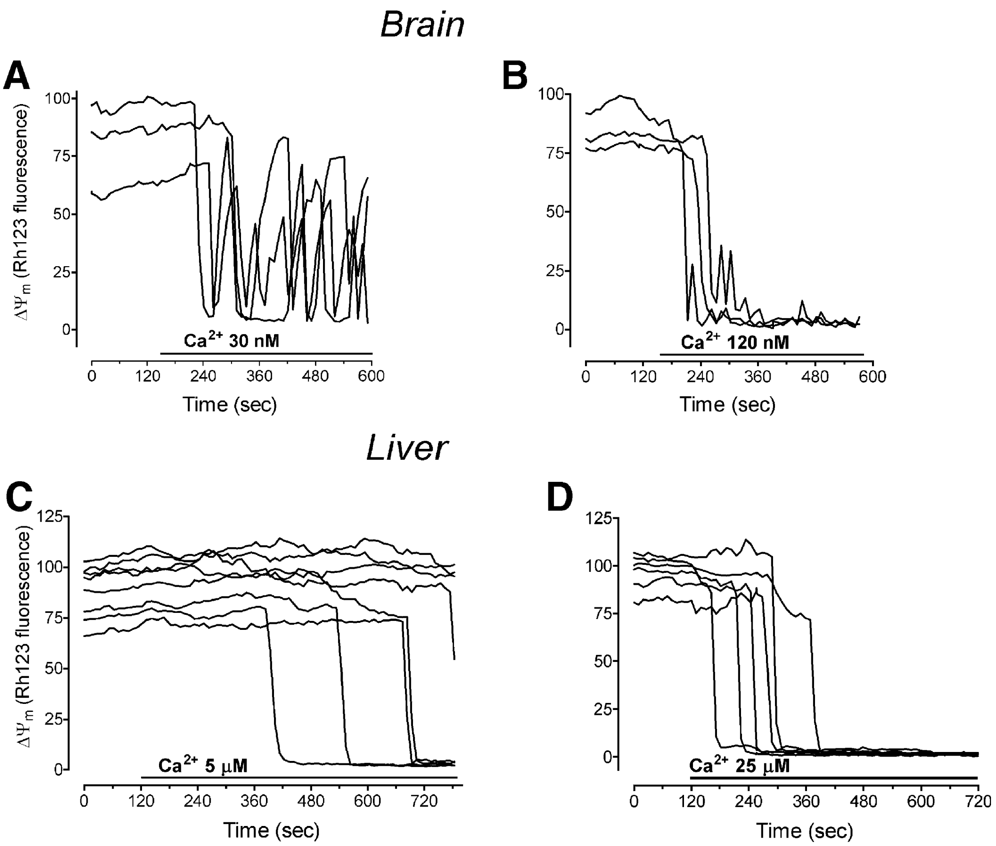

Fig. 1. Ca^{2+}-induced depolarization in brain and liver mitochondria. (A, B) Oscillatory depolarization in brain mitochondria in response to the increase of $[Ca^{2+}]$ by about 30 nM and 120 nM. (C, D) Non-oscillatory depolarization in liver mitochondria evoked by 5 µM and 25 µM Ca^{2+}. Each trace represents the changes in $\Delta\Psi_m$ (Rh123 fluorescence) in individual mitochondrion. Near zero fluorescence level indicates a complete $\Delta\Psi_m$ depolarization.

sensitive to very low $[Ca^{2+}]$. 5 µM Ca^{2+} was required to induce a visible mitochondrial response within 10 min of the Ca^{2+} application (Fig. 1C). An increase in $[Ca^{2+}]$ decreased the latency of $\Delta\Psi_m$ depolarization (Fig. 1D). However, none of liver mitochondria underwent an oscillatory depolarization and the Ca^{2+}-induced change in $\Delta\Psi_m$ was rapid and irreversible. The time between the beginning of Ca^{2+} application and the onset of mitochondrial depolarization varied within the mitochondrial population and depended on Ca^{2+} concentration. In subsequent experiments on liver mitochondria, we used 20 µM Ca^{2+} to depolarize the whole mitochondrial population within 7–10 min.

The mechanism of Ca^{2+}-induced collapse of $\Delta\Psi_m$ in liver mitochondria is believed to result from the activation of mPTP opening [11]. In contrary, the mechanism of Ca^{2+}-induced oscillations in brain mitochondria is not clear. However, in both cases Ca^{2+} induced an abrupt change in $\Delta\Psi_m$ at the level of the individual mitochondrion; the key difference is the reversibility of the loss of $\Delta\Psi_m$. A comparison of the Ca^{2+}-induced oscillatory depolarization in brain mitochondria with the non-oscillatory depolarization in liver mitochondria could thus potentially clarify the mechanism of Ca^{2+}-induced changes in brain $\Delta\Psi_m$.

3.2. Adenine nucleotides diminish the effect of Ca^{2+}

As we have reported previously [33], 100 µM ATP or ADP reversibly inhibited the $\Delta\Psi_m$ oscillations in brain mitochondria. Here, we treated both brain and liver mitochondria with 100 µM adenine nucleotides for 5 min before and during the Ca^{2+} application. To exclude the possible role of the ATP synthase/ATPase in the effects of adenine nucleotides, ATP and ADP were used in the presence of the ATP synthase blocker oligomycin (2 µM). Both ATP and ADP greatly reduced the Ca^{2+} effect in brain mitochondria (Figs. 2A–C) and profoundly delayed the onset of the depolarization in liver mitochondria (Figs. 2B–F). In brain mitochondria, both ATP and ADP reduced the number of oscillating mitochondria and the frequency of the $\Delta\Psi_m$ oscillations to the same extent and with similar potency. In liver mitochondria, ADP reduced the depolarizing action of Ca^{2+} more effectively than ATP (Fig. 2F). It should be noted that in about 50% of ATP or ADP-treated liver mitochondria, the depolarizations were biphasic: a slow phase followed by the second phase of abrupt and more complete depolarization (Fig. 2E). A similar biphasic depolarization was reported by Huser and Blatter in heart mitochondria [29]. The inhibitory effect of ADP on transient depolarizations in heart mitochondria was also shown by

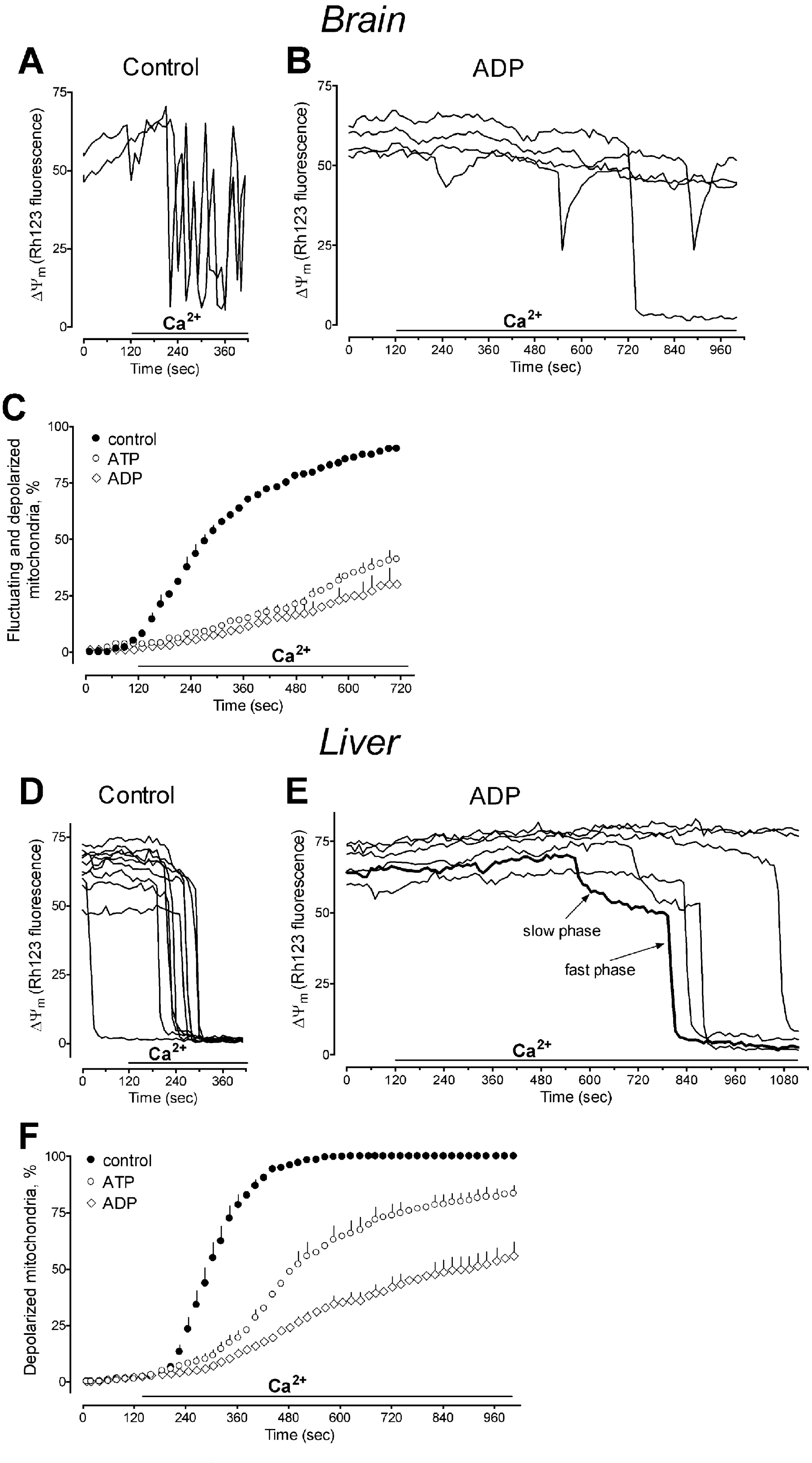

Fig. 2. Adenine nucleotides decrease the effect of Ca^{2+} in brain (A–C) and liver (D–F) mitochondria. Representative traces of individual mitochondria show Ca^{2+}-induced changes in Rh123 fluorescence in the control (A, D) and in the presence of ADP+oligomycin (B, E). Summarized data (C, F) show the percentage of oscillating and (or) depolarized mitochondria during Ca^{2+} application. ATP or ADP (100 μM) in the presence of oligomycin (2 μM) were present 5 min before and during Ca^{2+} application. $[Ca^{2+}]$ was elevated by 30 nM in brain and 20 μM in liver mitochondria. 100 individual mitochondria were analyzed in each experiment. Mean±S.E. is calculated from 4 experiments.

Hattori et al. [31], however, this effect was counteracted by oligomycin, and $\Delta\Psi_m$ oscillations were not sensitive to Ca^{2+}.

3.3. Effect of the modulators of adenine nucleotide translocase (ANT)

We have shown previously that carboxyatractyloside (CA), which inhibits ATP/ADP translocation by ANT, prevented the stabilizing action of ATP and ADP on Ca^{2+}-induced oscillations in brain mitochondria [33]. This raises the question of the involvement of the ANT in the effect of adenine nucleotides. Here, we compared the effect of ANT inhibition in both brain and liver mitochondria. For this purpose, we used CA and bongkrekic acid (BA), ANT ligands which bind to different states of the ANT. Fig. 3 shows the effect of CA and BA on the Ca^{2+}-induced depolarization in the brain and liver mitochondria and the combined effect of CA and BA with adenine nucleotides. BA significantly reduced both oscillatory depolarization in brain mitochondria and non-oscillatory depolarization in liver mitochondria. CA had an opposite effect compared to BA in the liver mitochondria, where it accelerated Ca^{2+}-depolarization, but had no effect alone in the brain

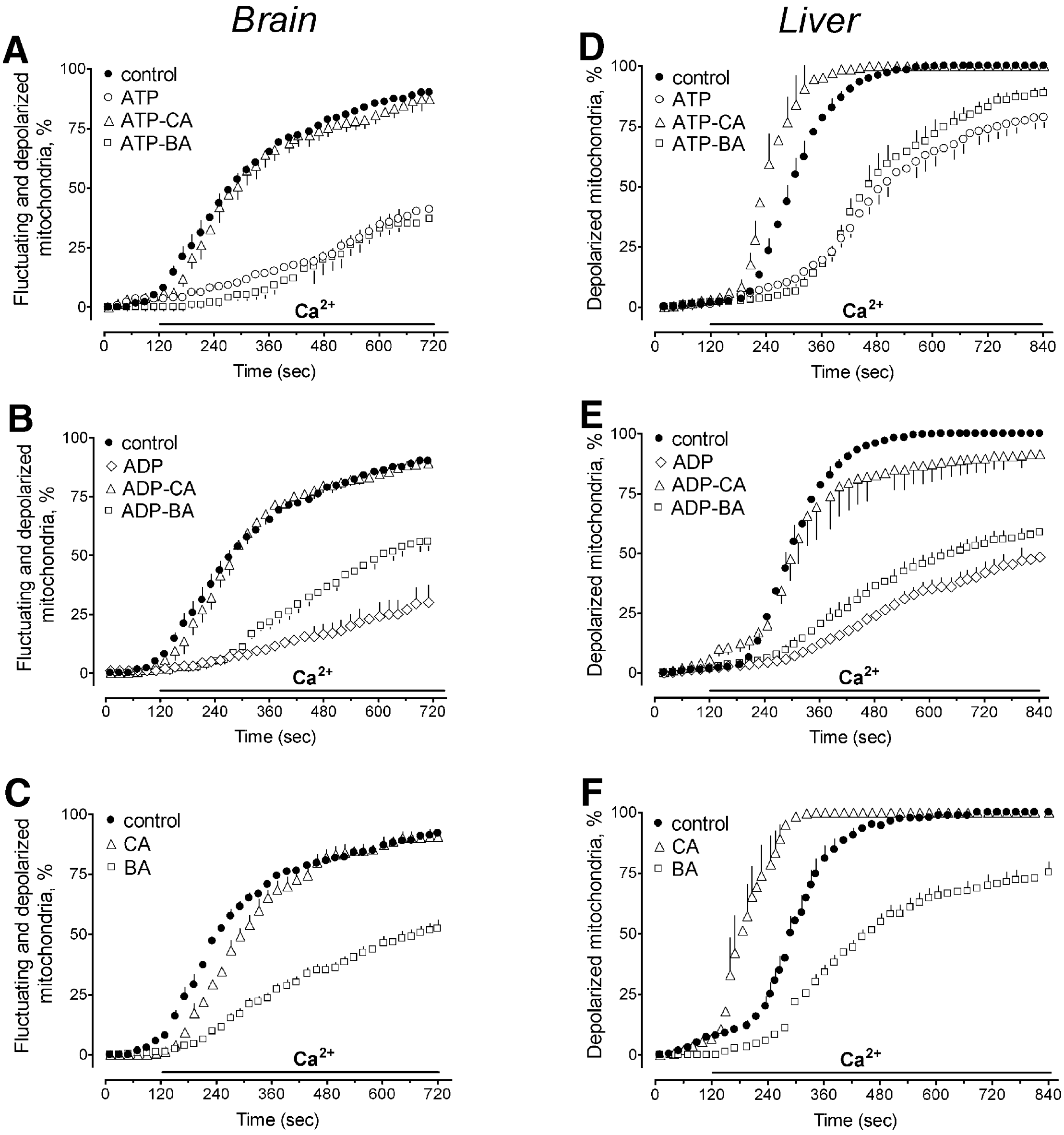

Fig. 3. Effect of CA and BA on the percentage of oscillating and (or) depolarized mitochondria during Ca^{2+} application in brain (A–C) and liver (D–F) mitochondria. (A, D) Controls (●) represent the response of untreated mitochondria to Ca^{2+}. In other experiments mitochondria were treated with 100 μM ATP (○) or with a combination of ATP and 2 μM CA (△) or ATP and 2 μM BA (□). (B, E) Effect of 100 μM ADP (◇), ADP+CA (△) and ADP+BA (□) on brain and liver mitochondria exposed to Ca^{2+}. (C, F) Effect of CA and BA on the percentage of mitochondria responded to Ca^{2+}. $[Ca^{2+}]$ was increased by 30 nM in brain and 20 μM in liver mitochondria. In all experiments adenine nucleotides were used in combination of 2 μM oligomycin. Each curve represents the mean ± S.E. calculated from 4 to 5 experiments with 100 individual mitochondria analyzed in each experiment.

mitochondria (Figs. 3C, F). Next, we checked the effect of ANT ligands on action of adenine nucleotides. As demonstrated in Figs. 3A–B and D–E, CA fully abolished the stabilizing effect of ATP and ADP in the both brain and liver mitochondria. In contrast, BA did not change significantly the ATP effect in either type of mitochondria or the effect of ADP in liver mitochondria. BA slightly reduced the effect of ADP in the brain mitochondria, but this change was much smaller than the complete reversal produced by CA.

The different effect of CA and BA cannot be explained simply by the depletion of matrix ATP and ADP, but other mechanisms must be involved. ANT ligands affect (CA facilitating and BA impeding) a Ca^{2+}-triggered conformational change of the ANT which converts the ANT from a specific transporter to a non-specific pore [12,35]. Adenine nucleotides decrease the probability of the conformational change of the ANT to the mPTP [12,14,35]. Thus, it is possible that the conformational change of the ANT is involved in the effects of CA and BA on $\Delta\Psi_m$. To test this hypothesis, we next used CsA, which influences the transformation of ANT to mPTP by a different mechanism—CsA prevents binding of cyclophilin-D to ANT, which inhibits mPTP opening.

3.4. *Effect of cyclosporin A*

Brain and liver mitochondria were treated with CsA (1 μM) for 5 min before and during the Ca^{2+} application. As illustrated in Figs. 4A–C, CsA produced a modest delay in the onset of the Ca^{2+} oscillations in the brain mitochondria. This effect was more modest than that produced by adenine nucleotides. In the liver mitochondria (Figs. 4D–F), CsA more substantially delayed the Ca^{2+}-induced depolarization. An example of the $\Delta\Psi_m$ changes in single liver mitochondria (Fig. 4E) shows that CsA made the initial slow phase of depolarization more apparent, although this slow phase was still followed by the abrupt dissipation of $\Delta\Psi_m$. This slow phase of $\Delta\Psi_m$ depolarization was never observed in brain mitochondria. In liver mitochondria, the effect of CsA was similar in magnitude to the effect of adenine nucleotides. This result supports the suggestion that adenine nucleotides, CA and BA all produce their effect by influencing of the transformation of ANT to the mPTP.

3.5. *Sr^{2+} depolarizes mitochondria in a different manner*

Ca^{2+} is required to bind the ANT to convert it from the carrier mode into a nonspecific pore [35]. However, other divalent metals such as Sr^{2+}, Mn^{2+}, Ba^{2+} and Mg^{2+} do not activate mPTP, even though several are transported by the calcium uniporter into the matrix. Instead, these metals inhibit mPTP by competing with Ca^{2+} for the trigger site [36]. To depolarize mitochondria by a mechanism presumably different from that induced by Ca^{2+}, we used the non-mPTP trigger Sr^{2+}. In contrast to Ca^{2+}, Sr^{2+} induced slow changes in the $\Delta\Psi_m$ in each individual brain mitochondrion with no sign of the oscillatory activity (Fig. 5A). This figure shows that the time-course of the decline in $\Delta\Psi_m$ was concentration-dependent; higher concentrations caused faster depolarization, but no oscillations appeared before complete depolarization. Sr^{2+}-induced depolarization of liver mitochondria was also different from the Ca^{2+}-induced depolarization. $\Delta\Psi_m$ decreased slowly in each mitochondrion without a delay. As in brain mitochondria, the time-course of the $\Delta\Psi_m$ in liver mitochondria depended on Sr^{2+} concentration (Fig. 5C). The brain mitochondria were more sensitive to Sr^{2+} compared to the liver; 60 μM Sr^{2+} depolarized brain mitochondria almost completely within 10 min, whereas liver mitochondria required 320 μM Sr^{2+} (Figs. 5B, D). A blocker of the mitochondrial Ca^{2+}-uniporter, ruthenium red, completely abolished the Sr^{2+}-induced depolarization (data not shown), indicating that Sr^{2+} enters to the matrix through the Ca^{2+}-uniporter. Sr^{2+}-induced depolarization was not sensitive to adenine nucleotides or CsA (Figs. 5B, D) suggesting that this depolarization is not associated with mPTP.

4. Discussion

In the present study, we have demonstrated that Ca^{2+}-induced depolarization of mitochondria is not the same in mitochondria from different tissues. Through measurements of $\Delta\Psi_m$ in single isolated mitochondria, we have shown that very low concentrations of Ca^{2+} trigger reversible depolarization (oscillations) in brain mitochondria, and that these reversible events precede complete mitochondrial de-energization. The duration of the oscillations and the percentage of mitochondria that oscillated before complete depolarization were dependent on [Ca^{2+}], diminishing with the increase of [Ca^{2+}]. In contrast, Ca^{2+} did not initiate transient depolarization in liver mitochondria. Instead, each individual mitochondrion depolarized rapidly and irreversibly. Transient mitochondrial depolarizations have been observed in different cell types [17–26] and in isolated mitochondria [16,28–33]. Although the exact role of this phenomenon is unknown, an involvement in cell signaling was suggested. The observation that Ca^{2+}-induced $\Delta\Psi_m$ oscillations are typical for brain, but not for liver mitochondria raises the possibility that this phenomenon plays a specific role in neuronal function.

Many investigations of spontaneous changes in $\Delta\Psi_m$ have concluded that the mPTP plays a critical role in this phenomenon [16,18,28,29]. We had previously suggested that oscillatory activity in $\Delta\Psi_m$ of brain mitochondria initiated by low doses of Ca^{2+} reflects an intermediate, unstable state of mitochondria possibly associated with neuronal injury. We also investigated the possible role of the mPTP in this event. The lack of calcein movement across the mitochondrial membrane during Ca^{2+}-oscillations and an inability of CsA to stop the oscillations [32] argued against the ‘classical’ mPTP, which has been largely defined

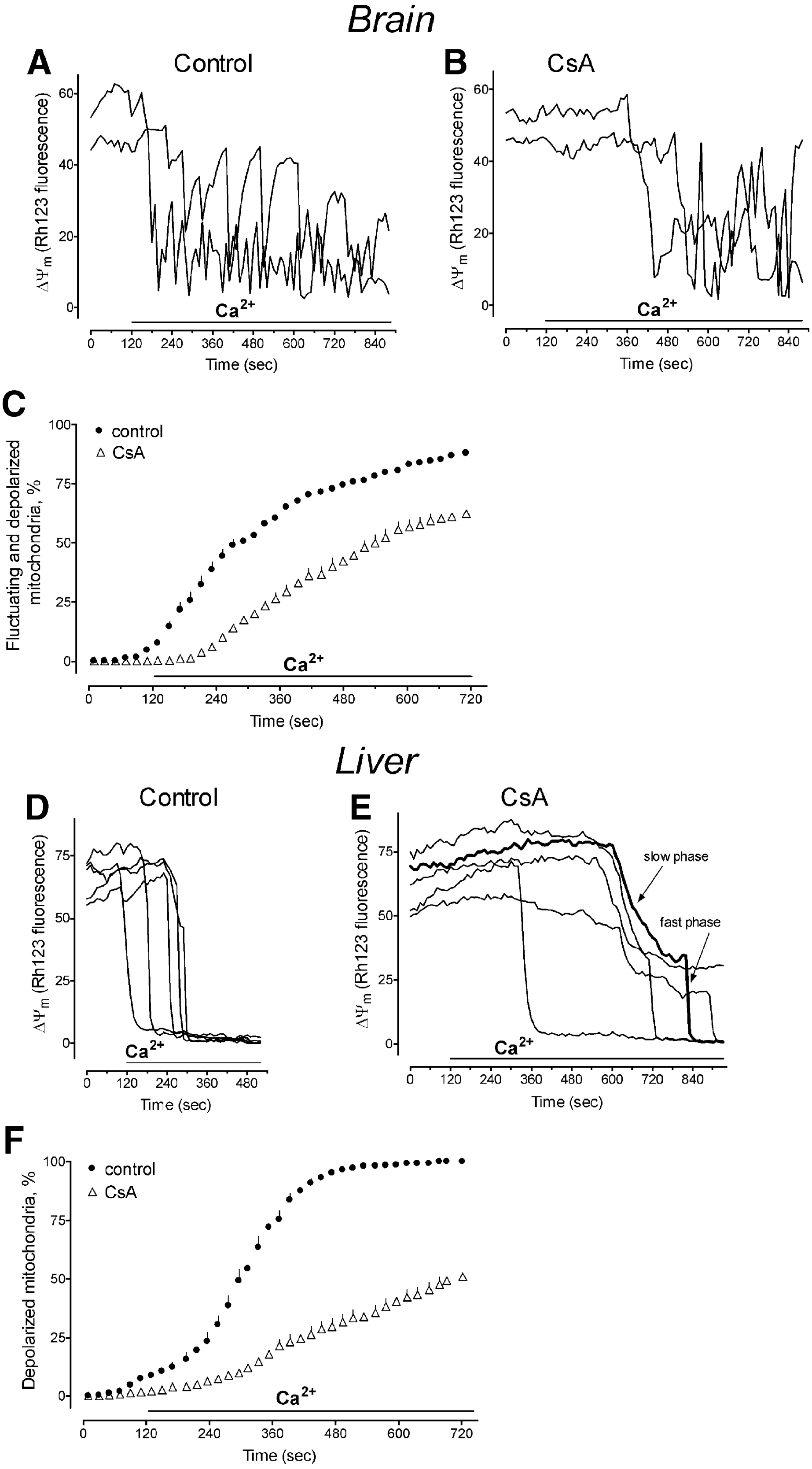

Fig. 4. CsA inhibits Ca^{2+}-induced depolarization in brain (A–C) and liver (B–F) mitochondria. (A and D) Representative traces show changes response of individual control mitochondria to Ca^{2+}. (B and E) Response of individual mitochondria treated with 1 μM CsA to Ca^{2+}. (C and F) Summary data show the percentage of oscillating and (or) depolarized mitochondria during the Ca^{2+} application. Each curve represents the mean (± SE) of 6 experiments with 100–150 individual mitochondria analyzed in each experiment. CsA was added for 5 min before recording and was present during the Ca^{2+} application. $[Ca^{2+}]$ was elevated by 30 nM in brain and 20 μM in liver mitochondria.

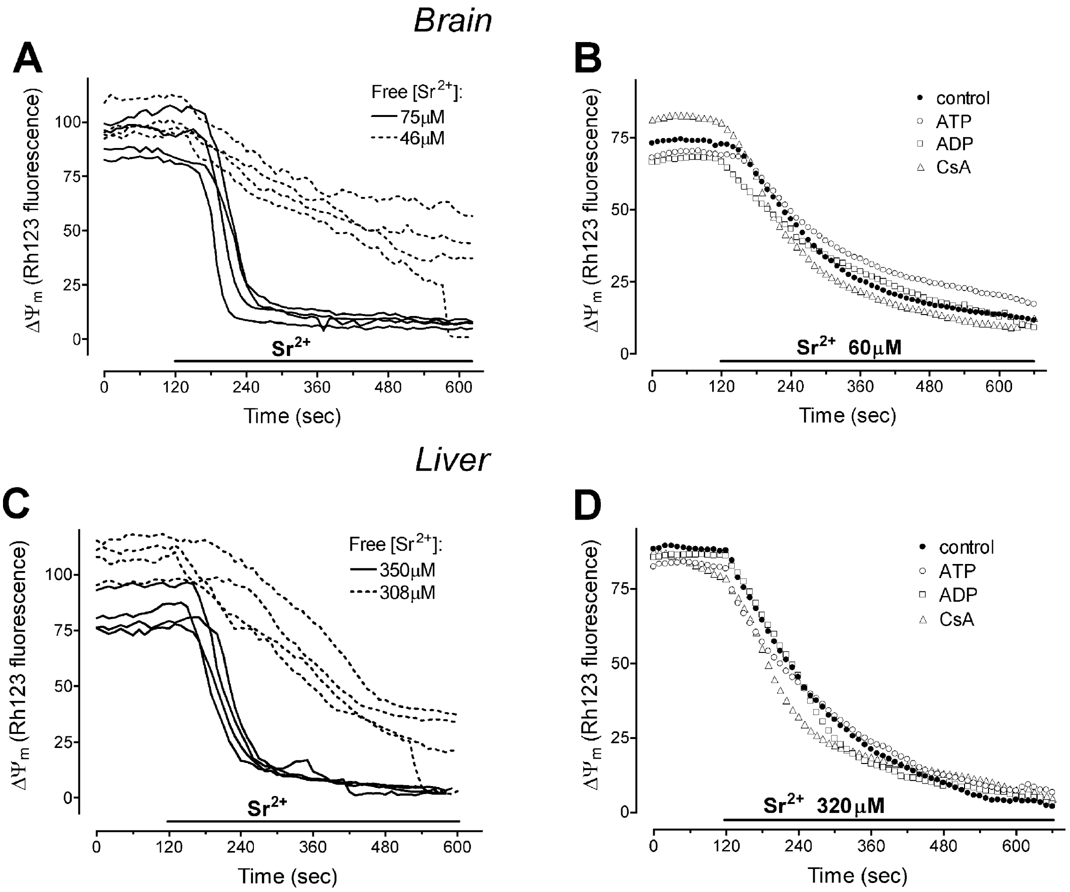

Fig. 5. Sr^{2+}-induced depolarization in brain (A, B) and liver (C, D) mitochondria. (A, C) Time-course of Sr^{2+}-induced depolarization in individual mitochondria exposed to low and high Sr^{2+} concentration. Each trace represents a single mitochondrion. (B, D) Mean traces of 4–5 experiments with 100–150 mitochondria analyzed in each experiment. All treatments (2 μM CsA, 100 μM ATP or ADP in the presence of 2 μM oligomycin) were 5 min before and throughout Sr^{2+} exposure.

by studies in liver mitochondria. In this work, we compared the properties of Ca^{2+}-depolarization in brain and liver mitochondria. As expected, CsA delayed Ca^{2+}-depolarization in liver mitochondria, consistent with the involvement of mPTP. The present studies used more sensitive approaches for detecting the effects of modulatory agents. With these more sensitive analytical methods we found that if CsA is applied a few minutes before the onset of oscillations, it can slightly delay the oscillatory activity. Furthermore, adenine nucleotides had basically similar inhibitory effects on both brain and liver depolarization. Additionally, BA and CA had essentially similar influences on adenine nucleotides effects in both types of mitochondria. These new observations renewed the question about a possible role of mPTP in the $\Delta\Psi_m$ oscillations in brain mitochondria.

It is widely considered that mPTP is a non-specific pore that opens in the mitochondrial membrane under conditions of elevated intramitochondrial Ca^{2+}, especially when this is accompanied by oxidative stress and depleted adenine nucleotides. Although it was reported by Kokoszka et al. [37] that ANT is not essential for the mPTP, the data from several other laboratories show that ANT is the pore-forming component of the mPTP [12,14,35]. Mitochondrial Ca^{2+} overload triggers the interaction between mitochondrial cyclophylin D (CyP-D) and the ANT, leading to the transformation of the ANT from a selective ADP-ATP antiporter to a non-selective pore [35]. Adenine nucleotides inhibit Ca^{2+} binding to the ANT, preventing pore opening [12,35]. CsA inhibits the mPTP by preventing CyP-D binding to the ANT. In our experiments on liver mitochondria both adenine nucleotides and CsA had similar effects on Ca^{2+}-induced depolarization, indicating that the delay in the depolarization was related to the inhibition of the mPTP. It is also interesting that only when mPTP was inhibited, Ca^{2+} induced slow changes in $\Delta\Psi_m$ (the first phase of mitochondrial depolarization) in liver mitochondria, which was followed by the typical collapse of $\Delta\Psi_m$. Presumably, the fast phase reflects the mPTP opening. Despite of the different sensitivity of Ca^{2+}-induced depolarization of brain mitochondria, the basic properties of adenine nucleotides and CsA modulation are qualitatively similar to those seen in liver mitochondria.

We also examined the effects of specific ANT ligands BA and CA on Ca^{2+}-induced depolarization in both types of mitochondria (Fig. 3). Both CA and BA inhibit the transport of adenine nucleotides across the inner mitochondria membrane. However, in our experiments, CA and BA had opposite effects: CA enhanced Ca^{2+}-depolarization and abolished the stabilizing action of adenine nucleotides, whereas BA reduced the depolarization and did not alter adenine nucleotides effects. Therefore, the effects of CA and BA on $\Delta\Psi_m$ cannot be explained by the depletion of matrix ADP and ATP caused by lack of ADP-ATP transport. It is known that ANT ligands inhibit function of ANT by changing its conformational state. CA binding to the ANT from the cytosolic surface decreases ADP binding affinity and activates mPTP opening; BA binding to the matrix surface of the ANT increases ADP binding affinity and inhibits mPTP [14]. Based on this, we hypothesize that the effects of adenine nucleotides, CA and BA on Ca^{2+}-induced depolarization in liver and brain mitochondria are due to the modulation of ANT transformation from a selective ADP-ATP antiporter to a non-selective pore. The difference in Ca^{2+} sensitivity could be the result of a difference in the extent of Ca^{2+} uptake by the mitochondria, or possibly even a difference in the Ca^{2+} sensitivity of the ANT isoforms that dominate in each tissue. Unfortunately, the methods used in this study cannot distinguish these possibilities.

The hypothesis that the actions of Ca^{2+} are due to modulation of mPTP is supported by the experiments where mitochondria were depolarized by Sr^{2+}. Sr^{2+} does not activate mPTP, but instead competitively inhibits the mPTP [36]. We have shown here that the depolarization, which is not associated with mPTP, is different from that induced by Ca^{2+} in both brain and liver mitochondria. Sr^{2+}-induced depolarization was characterized by slow kinetics, did not show the oscillatory activity, and was not sensitive to adenine nucleotides or CsA. The time-course of Sr^{2+}-induced depolarization was similar with the slow phase of depolarization observed in liver mitochondria treated with CsA or adenine nucleotides. This observation supports the suggestion that the slow phase of Ca^{2+}-induced depolarization is not related to mPTP activation.

Finally, the similarities in the Ca^{2+}-induced depolarization in brain and liver mitochondria suggest that the oscillatory depolarization in brain mitochondria, as well as the non-oscillatory depolarization in liver mitochondria, is associated with the transformation of ANT to the non-selective pore. However, the foremost conclusion from this work is that Ca^{2+}-induced depolarization in brain mitochondria is different from that in liver mitochondria. Depolarization of brain mitochondria at the early stage is reversible and transient. $\Delta\Psi_m$ can be restored completely by the removal of Ca^{2+} during the $\Delta\Psi_m$ oscillations [33]. On the contrary, liver mitochondria do not show the intermediate state of depolarization; the removal of Ca^{2+} cannot restore $\Delta\Psi_m$ (data not shown). We suggest that Ca^{2+} induces conformational change of ANT which can be transient in brain mitochondria, reflecting a 'low conductance' state of mPTP. In liver mitochondria, this transformation of ANT from antiporter to a non-selective pore is non-reversible, suggesting an important difference in the functional consequences of Ca^{2+} loading.

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References

[1] P. Mitchell, J. Moyle, Chemiosmotic hypothesis of oxidative phosphorylation, Nature 213 (1967) 137–139.
[2] M. Erecinska, I.A. Silver, Ions and energy in mammalian brain, Prog. Neurobiol. 43 (1994) 37–71.
[3] R.J. White, I.J. Reynolds, Mitochondria and Na^+/Ca^{2+} exchange buffer glutamate-induced calcium loads in cultured cortical neurons, J. Neurosci. 15 (1995) 1318–1328.
[4] B. Khodorov, V. Pinelis, T. Storozhevykh, O. Vergun, N. Vinskaya, Dominant role of mitochondria in protection against a delayed neuronal Ca^{2+} overload induced by endogenous excitatory amino acids following a glutamate pulse, FEBS Lett. 393 (1996) 135–138.
[5] S.L. Budd, D.G. Nicholls, A reevaluation of the role of mitochondria in neuronal Ca^{2+} homeostasis, J. Neurochem. 66 (1996) 403–411.
[6] G.J. Wang, S.A. Thayer, Sequestration of glutamate-induced Ca^{2+} loads by mitochondria in cultured rat hippocampal neurons, J. Neurophysiol. 76 (1996) 1611–1621.
[7] J.M. Dubinsky, S.M. Rothman, Intracellular calcium concentrations during "chemical hypoxia" and excitotoxic neuronal injury, J. Neurosci. 11 (1991) 2545–2551.
[8] S.L. Budd, D.G. Nicholls, Mitochondria, calcium regulation, and acute glutamate excitotoxicity in cultured cerebellar granule cells, J. Neurochem. 67 (1996) 2282–2291.
[9] A.K. Stout, H.M. Raphael, B.I. Kanterewicz, E. Klann, I.J. Reynolds, Glutamate-induced neuron death requires mitochondrial calcium uptake, Nat. Neurosci. 1 (1998) 366–373.
[10] B. Sengpiel, E. Preis, J. Krieglstein, J.H. Prehn, NMDA-induced superoxide production and neurotoxicity in cultured rat hippocampal neurons: role of mitochondria, Eur. J. Neurosci. 10 (1998) 1903–1910.
[11] M. Zoratti, I. Szabo, The mitochondrial permeability transition, Biochim. Biophys. Acta 1241 (1995) 139–176.
[12] M. Crompton, The mitochondrial permeability transition pore and its role in cell death, Biochem. J. 341 (Pt. 2) (1999) 233–249.
[13] P. Bernardi, Mitochondrial transport of cations: channels, exchangers, and permeability transition, Physiol. Rev. 79 (1999) 1127–1155.
[14] A.P. Halestrap, S.J. Clarke, S.A. Javadov, Mitochondrial permeability transition pore opening during myocardial reperfusion—A target for cardioprotection, Cardiovasc. Res. 61 (2004) 372–385.
[15] P. Bernardi, S. Vassanelli, P. Veronese, R. Colonna, I. Szabo, M. Zoratti, Modulation of the mitochondrial permeability transition pore. Effect of protons and divalent cations, J. Biol. Chem. 267 (1992) 2934–2939.
[16] F. Ichas, L.S. Jouaville, J.P. Mazat, Mitochondria are excitable organelles capable of generating and conveying electrical and calcium signals, Cell 89 (1997) 1145–1153.
[17] L.M. Loew, R.A. Tuft, W. Carrington, F.S. Fay, Imaging in five dimensions: time-dependent membrane potentials in individual mitochondria, Biophys. J. 65 (1993) 2396–2407.

[18] C.P. Fall, J.P. Bennett Jr., Visualization of cyclosporin A and Ca^{2+}-sensitive cyclical mitochondrial depolarizations in cell culture, Biochim. Biophys. Acta 1410 (1999) 77–84.

[19] M.R. Duchen, A. Leyssens, M. Crompton, Transient mitochondrial depolarizations reflect focal sarcoplasmic reticular calcium release in single rat cardiomyocytes, J. Cell Biol. 142 (1998) 975–988.

[20] M.A. Aon, S. Cortassa, E. Marban, B. O'Rourke, Synchronized whole cell oscillations in mitochondrial metabolism triggered by a local release of reactive oxygen species in cardiac myocytes, J. Biol. Chem. 278 (2003) 44735–44744.

[21] G.J. Grover, K.D. Garlid, ATP-Sensitive potassium channels: a review of their cardioprotective pharmacology, J. Mol. Cell Cardiol. 32 (2000) 677–695.

[22] J. Jacobson, M.R. Duchen, Mitochondrial oxidative stress and cell death in astrocytes—Requirement for stored Ca^{2+} and sustained opening of the permeability transition pore, J. Cell Sci. 115 (2002) 1175–1188.

[23] J.F. Buckman, I.J. Reynolds, Spontaneous changes in mitochondrial membrane potential in cultured neurons, J. Neurosci. 21 (2001) 5054–5065.

[24] C.M. O'Reilly, K.E. Fogarty, R.M. Drummond, R.A. Tuft, J.V. Walsh Jr., Quantitative analysis of spontaneous mitochondrial depolarizations, Biophys. J. 85 (2003) 3350–3357.

[25] C.M. O'Reilly, K.E. Fogarty, R.M. Drummond, R.A. Tuft, J.V. Walsh Jr., Spontaneous mitochondrial depolarizations are independent of SR Ca^{2+} release, Am. J. Physiol.: Cell Physiol. 286 (2004) C1139–C1151.

[26] P. Krippeit-Drews, M. Dufer, G. Drews, Parallel oscillations of intracellular calcium activity and mitochondrial membrane potential in mouse pancreatic B-cells, Biochem. Biophys. Res. Commun. 267 (2000) 179–183.

[27] B. O'Rourke, Pathophysiological and protective roles of mitochondrial ion channels, J. Physiol. 529 (Pt. 1) (2000) 23–36.

[28] J. Huser, C.E. Rechenmacher, L.A. Blatter, Imaging the permeability pore transition in single mitochondria, Biophys. J. 74 (1998) 2129–2137.

[29] J. Huser, L.A. Blatter, Fluctuations in mitochondrial membrane potential caused by repetitive gating of the permeability transition pore, Biochem. J. 343 (Pt. 2) (1999) 311–317.

[30] S. Nakayama, T. Sakuyama, S. Mitaku, Y. Ohta, Fluorescence imaging of metabolic responses in single mitochondria, Biochem. Biophys. Res. Commun. 290 (2002) 23–28.

[31] T. Hattori, K. Watanabe, Y. Uechi, H. Yoshioka, Y. Ohta, Repetitive transient depolarizations of the inner mitochondrial membrane induced by proton pumping, Biophys. J. 88 (2005) 2340–2349.

[32] O. Vergun, T.V. Votyakova, I.J. Reynolds, Spontaneous changes in mitochondrial membrane potential in single isolated brain mitochondria, Biophys. J. 85 (2003) 3358–3366.

[33] O. Vergun, I.J. Reynolds, Fluctuations in mitochondrial membrane potential in single isolated brain mitochondria: modulation by adenine nucleotides and Ca^{2+}, Biophys. J. 87 (2004) 3585–3593.

[34] N.R. Sims, Selective impairment of respiration in mitochondria isolated from brain subregions following transient forebrain ischemia in the rat, J. Neurochem. 56 (1991) 1836–1844.

[35] A.P. Halestrap, C. Brennerb, The adenine nucleotide translocase: a central component of the mitochondrial permeability transition pore and key player in cell death, Curr. Med. Chem. 10 (2003) 1507–1525.

[36] I. Szabo, P. Bernardi, M. Zoratti, Modulation of the mitochondrial megachannel by divalent cations and protons, J. Biol. Chem. 267 (1992) 2940–2946.

[37] J.E. Kokoszka, K.G. Waymire, S.E. Levy, J.E. Sligh, J. Cai, D.P. Jones, G.R. MacGregor, D.C. Wallace, The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore, Nature 427 (2004) 461–465.