

## Fluctuations in Mitochondrial Membrane Potential in Single Isolated Brain Mitochondria: Modulation by Adenine Nucleotides and $\text{Ca}^{2+}$

Olga Vergun and Ian J. Reynolds

Department of Pharmacology, University of Pittsburgh, Pittsburgh, Pennsylvania

**ABSTRACT** In this study we investigated fluctuations in mitochondrial membrane potential ( $\Delta\Psi_m$ ) in single isolated brain mitochondria using fluorescence imaging. Mitochondria were attached to coverslips and perfused with  $\text{K}^+$ -based buffer containing 20  $\mu\text{M}$  EDTA, supplemented with malate and glutamate, and rhodamine 123 for  $\Delta\Psi_m$  determination.  $\Delta\Psi_m$  fluctuations were triggered by mitochondrial  $\text{Ca}^{2+}$  uptake since they were inhibited by both ruthenium red, a  $\text{Ca}^{2+}$ -uniporter blocker, and by high concentrations of EGTA. A very low concentration of  $\text{Ca}^{2+}$  ( $\sim 30$  nM) was required to initiate the fluctuations. Both ATP and ADP reversibly inhibited  $\Delta\Psi_m$  fluctuations, with maximal effects occurring at 100  $\mu\text{M}$ . The effect of nucleotides could not be explained by the reversed mode of mitochondrial ATP-synthase, since oligomycin was not effective and nonhydrolysable analogs of ATP and ADP did not stop the fluctuations. The effects of adenine nucleotides were abolished by blockade of the adenine nucleotide translocator with carboxyatractyloside, but were insensitive to another inhibitor, bongkreikic acid. ATP-sensitive  $\text{K}^+$ -channels are not involved in the mechanism of  $\Delta\Psi_m$  fluctuations, since the inhibitor 5-hydroxydecanoate or the activator diazoxide did not affect dynamics of  $\Delta\Psi_m$ . We suggest  $\Delta\Psi_m$  fluctuations in brain mitochondria are not spontaneous, but are triggered by  $\text{Ca}^{2+}$  and are modulated by adenine nucleotides, possibly from the matrix side of the inner mitochondrial membrane.

### INTRODUCTION

Mitochondrial membrane potential ( $\Delta\Psi_m$ ) is a key factor for maintaining mitochondrial metabolism. It provides the energy for ATP generation, determines  $\text{Ca}^{2+}$  uptake, and produces free radicals, among other functions. Until recently it was impossible to determine heterogeneity of mitochondria within a small population because of their small size, their intrinsic motility, and the absence of appropriate equipment and analytical reagents. Typically,  $\Delta\Psi_m$  has been evaluated as a single overall estimate from all mitochondria present in a given cell or tissue. However, a number of recent studies in which  $\Delta\Psi_m$  was measured in individual mitochondria have revealed differences in the value and dynamics of  $\Delta\Psi_m$  within a cell. Spontaneous changes in  $\Delta\Psi_m$  were first observed in living cells (neuroblastoma cells) by Loew et al. (1993). After that, transient changes in  $\Delta\Psi_m$  have been shown in different cell types, including cardiomyocytes (Duchen et al., 1998; Aon et al., 2003), astrocytes (Grover and Garlid, 2000; Jacobson and Duchen, 2002), neurons (Buckman and Reynolds, 2001), smooth muscle cells (O'Reilly et al., 2003, 2004), and pancreatic B-cells (Krippeit-Drews et al., 2000). 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 permeability transition (MPT) (Ichas et al., 1997). Others reported the involvement

of the  $\text{F}_1\text{F}_0\text{ATPase}$  (Buckman and Reynolds, 2001),  $\text{Ca}^{2+}$ -influx through mitochondrial  $\text{Ca}^{2+}$ -uniporter (Duchen et al., 1998), mitochondrial membrane anion channels (O'Rourke, 2000; Aon et al., 2003), and free radicals released from the matrix side of mitochondria (Aon et al., 2003). It is still not clear how change in  $\Delta\Psi_m$  in a small number of mitochondria might influence the overall function of the cell.

Several recent studies have reported that mitochondria isolated from different tissues also show heterogeneous changes in  $\Delta\Psi_m$ . Specifically, imaging of individual mitochondria isolated from Ehrlich cells (Ichas et al., 1997), heart (Huser et al., 1998; Huser and Blatter, 1999; Nakayama et al., 2002), and brain (Vergun et al., 2003) showed transient changes in  $\Delta\Psi_m$ . This simple model offers some advantages in comparison with intact cells, such as the ability to rapidly and reversibly deliver substrates and drugs to mitochondria, and the elimination of the complications related with the local cellular environment.

In a previous study (Vergun et al., 2003), we analyzed the dynamics of  $\Delta\Psi_m$  fluctuations in single isolated brain mitochondria. We concluded there that the fluctuations in  $\Delta\Psi_m$  reflect an intermediate unstable state of mitochondria which may lead to or reflect mitochondrial dysfunction. We have also shown that these fluctuations were not the consequence of oxidative stress and/or the high conductance MPT. In this series of experiments, we focused on the role of mitochondrial ATP-synthase, adenine nucleotide translocator (ANT), and  $\text{Ca}^{2+}$  in the mechanisms underlying  $\Delta\Psi_m$  fluctuations. We report that this phenomenon is triggered by  $\text{Ca}^{2+}$  and can be potently inhibited by adenine nucleotides.

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Address reprint requests to Ian J. Reynolds, Dept. of Pharmacology, University of Pittsburgh, W1351 Biomedical Science Tower, Pittsburgh, PA 15261. Tel.: 412-648-2134; Fax: 412-624-0794; E-mail: ianmmda@pitt.edu.

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## MATERIALS AND METHODS

### Materials

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

### 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 (1991) 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  $31,000 \times g$ , 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. 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 protein concentration in each preparation was determined by the Biuret method using a plate reader.

### Experimental solutions and 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, EDTA 0.02, malate 5, glutamate 5, pH 7.0. Mitochondria were added to this buffer at a final concentration of 0.75–1 mg protein/ml immediately before each experiment. Thirty-one mm glass coverslips were washed with 70% ethanol, then with  $H_2O$ , and dried before use. A 20  $\mu$ l drop of mitochondrial suspension was placed in the middle of the coverslip for 5–7 min. The coverslips with the mitochondrial suspension were placed into a 700- $\mu$ l perfusion chamber that was then mounted onto a microscope fitted for fluorescence imaging as described below. The mitochondria were perfused with KCl buffer at 7 ml/min; after 1 min of perfusion, mitochondria which had not attached 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, ~2000 mitochondria were present in a field observed with a 100 $\times$  objective.

For  $\Delta\Psi_m$  measurements, we used the potentiometric probe Rh123 (Molecular Probes, Eugene, OR) at nonquenching concentrations. Rh123 (200 nM) were present in the perfusion medium during the experiment. For fluorescence recording, we used a BX50WI Olympus Optical (Tokyo, Japan) microscope fitted with an Olympus Optical LUM PlanFI 100 $\times$  water immersion quartz objective. The fluorescence was monitored using excitation light provided by a 75W 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). The captured field was  $640 \times 512$  pixels and digitized to 8-bit resolution; fluorescence intensity was measured on scale of 0–255 arbitrary units. 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, Brattleboro, VT). Fluorescence data was acquired and analyzed using Simple PCI software (Compix, Cranberry,

PA). Fluorescence was measured in 100–150 individual mitochondria for each coverslip. Objects smaller than 0.3  $\mu$ m were not analyzed. Background fluorescence, determined from 3–4 mitochondrion-free regions of the coverslip, was subtracted from all signals. All experiments were repeated 4–6 times using mitochondria from different preparations.

### Oxygen consumption measurements

Mitochondrial respiration was monitored using a Clark oxygen electrode (PP Systems; Hansatech Instruments, Haverhill, MA) at 37°C in the same media we used for fluorescence measurements. Mitochondria were added to the medium at the concentration of 0.2 mg protein/ml. ADP was added at a final concentration of 1 mM.

### Calculation of divalent ion concentration

Free  $Ca^{2+}$  in the medium, containing divalent ions chelators, ATP and ADP, was calculated using the Max-Chelator program (Webmaxc Standard by C. Patton, <http://www.stanford.edu/cpatton/maxc.html>)

### Statistics

Statistical analysis was performed using Prism 3.0 (Graph Pad Software, San Diego, CA). All the data are presented as mean  $\pm$  SE. Comparisons were made using Student's *t*-test, with *P* values of  $<0.05$  taken as significant.

## RESULTS

Rh123 accumulated in ~70–80% of single isolated mitochondria attached to the coverslip (Fig. 1). Nonfluorescent mitochondria were presumably depolarized and were excluded from further analysis; ~70% of polarized mitochondria showed spontaneous changes in  $\Delta\Psi_m$  when perfused with HBS containing 20  $\mu$ M EDTA. During the fluctuations  $\Delta\Psi_m$  changed from maximal levels (40–100 fluorescence units) to minima near background, which reflects complete mitochondrial depolarization. To describe the dynamics of  $\Delta\Psi_m$  we calculated the frequency of fluctuations (the mean number of fluctuations per minute in both oscillating and stable mitochondria). Decreases or increases in fluorescence with minimum amplitude of 8–10

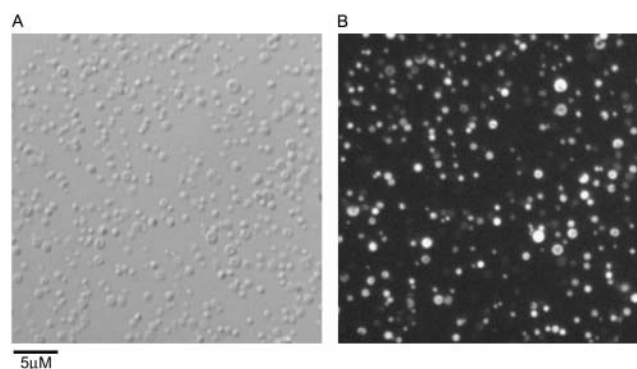


FIGURE 1 Phase-contrast (A) and fluorescence (B) images of single isolated brain mitochondria attached to the coverslip. Mitochondria were incubated with HBS containing 200 nM Rh123.

fluorescence units were counted as a single event. We did not find any correlation between the amplitude and the frequency of fluctuations.

### Adenine nucleotides stabilize $\Delta\Psi_m$

First we determined the effect of adenine nucleotides on the dynamics of the  $\Delta\Psi_m$  fluctuations. As shown in Fig. 2, *A* and *B*, the fluctuations in  $\Delta\Psi_m$  stopped completely after 30–60 s of adenine nucleotides application. The fluctuations then resumed upon the removal of ATP or ADP from the medium. An evaluation of the concentration-dependency of ATP and ADP is shown in Fig. 2 *C*. Both ATP and ADP were effective at the same concentrations and had a profound effect even at low concentrations (5  $\mu\text{M}$ ). The inhibitory effect was saturated at 75–100  $\mu\text{M}$ . The fact that both ATP and ADP were effective at the same concentrations rules out the possibility that contamination of the ATP-containing medium by small amounts of ADP induced the effect shown in Fig. 2 *A*. Thus, both ATP and ADP can stabilize  $\Delta\Psi_m$  in a concentration-dependent manner.

### Properties of adenine nucleotide modulation of $\Delta\Psi_m$ fluctuations

We next sought to characterize the modulatory effect of ATP and ADP on the fluctuations in  $\Delta\Psi_m$ . We first examined the effects of other nucleotides by testing GTP, TTP, UTP, and AMP. None of these nucleotides had any effect on the frequency of  $\Delta\Psi_m$  fluctuations (Table 1). We also compared the effects of natural nucleotides to nonhydrolysable analogs of ATP and ADP. These agents are transported by the ANT (Vignais et al., 1985), but cannot be further metabolized. We used the ATP analog adenosine 5'-( $\beta,\gamma$ -imido)triphosphate tetralithium salt hydrate (AMP-PNP), and the ADP analog adenosine 5'-[ $\alpha,\beta$ -methylene]diphosphoric acid (AMP-CP). Because these modified nucleotides exhibit a lower affinity and velocity with respect to the natural nucleotides (i.e., AMP-PNP is transported at 40% the rate of ATP) (Yount, 1975), they were used at concentrations three times higher than for ATP and ADP. Fig. 3, *A* and *B*, show examples illustrating these experiments; the summarized data are presented in Fig. 3 *D*. Application of 300  $\mu\text{M}$  of AMP-PNP or AMP-CP had no effect on the  $\Delta\Psi_m$  fluctuations, whereas subsequent ATP or ADP addition stopped the fluctuations in the same mitochondria. As the nonhydrolysable analogs could not substitute for ATP or ADP, it is possible that the effects of the latter are due to an interaction with the ATP synthase. We next inhibited ATP synthase by oligomycin (Fig. 3 *C*). Inhibition of mitochondrial ATP-synthase did not change the frequency of  $\Delta\Psi_m$  fluctuations. It is also interesting that oligomycin did not modify the effect of adenine nucleotides: ATP (Fig. 3 *C*) and ADP (not shown) had the same stabilizing effect on  $\Delta\Psi_m$  in mitochondria with either active or inhibited ATP synthase.

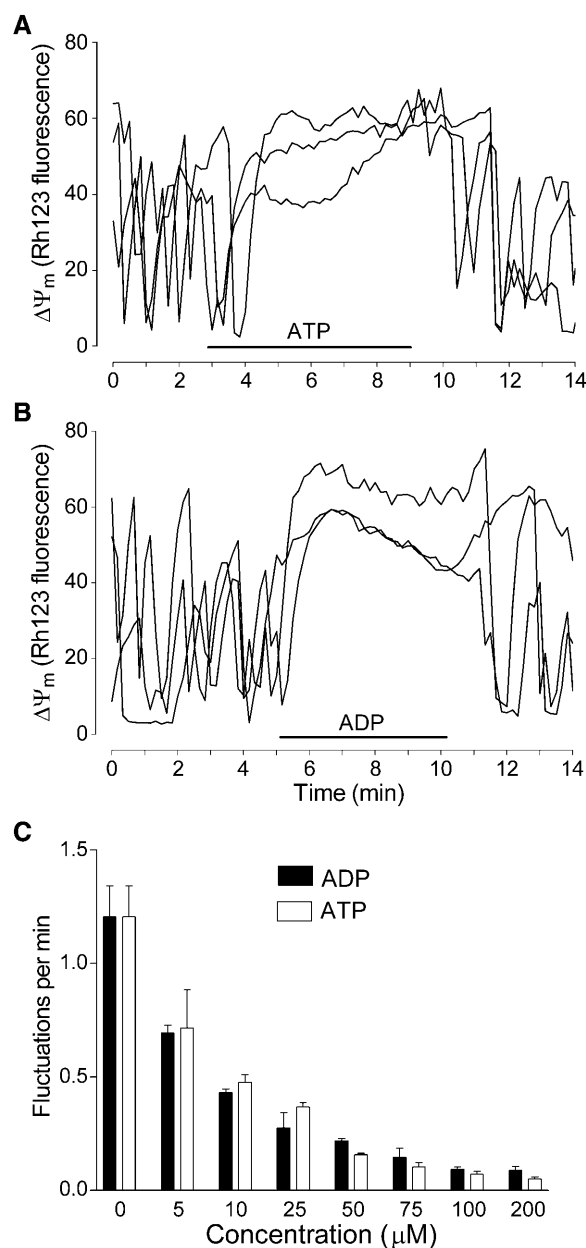


FIGURE 2 Stabilizing effect of adenine nucleotides on  $\Delta\Psi_m$ . (*A* and *B*) Representative traces of three individual mitochondria show changes in Rh123 fluorescence in control and during application of 100  $\mu\text{M}$  ATP (*panel A*) or ADP (*panel B*). (*C*) Concentration dependence of adenine nucleotide effects. Each histogram represents the mean ( $\pm$  SE) of four experiments, with  $\sim 150$  mitochondria analyzed in each experiment.

These data show that mechanisms of fluctuations in  $\Delta\Psi_m$  not related to ATP synthase activity but may require nucleotide hydrolysis or a structural feature of ATP and ADP lost in the nonhydrolysable analogs.

### Role of ANT in the effect of adenine nucleotides

Since the previous series of experiments ruled out the involvement of mitochondrial ATP-synthase in the effect of

**TABLE 1** Effect of different nucleotides on the frequency of  $\Delta\Psi_m$  fluctuations

Nucleotides	Fluctuations per minute
Control	1.20 $\pm$ 0.10
ATP	0.21 $\pm$ 0.02
GTP	1.03 $\pm$ 0.18
TTP	1.13 $\pm$ 0.13
UTP	1.17 $\pm$ 0.22
AMP	1.00 $\pm$ 0.18

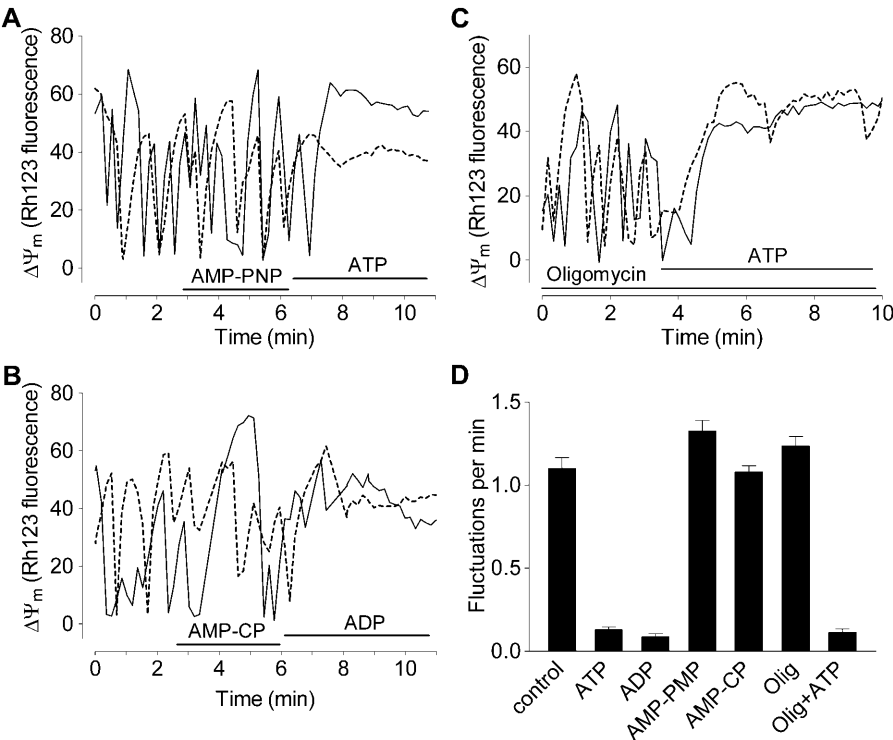
Nucleotides were added at 100  $\mu$ M each. Data are shown as mean  $\pm$  SE,  $n$  = 3 experiments with 100 individual mitochondria analyzed in each experiment.

adenine nucleotides, we next investigated whether adenine nucleotides have to be transported to the matrix side of mitochondria to stabilize  $\Delta\Psi_m$ . To inhibit the transport of nucleotides, two blockers of ANT were used: CA, which inhibits ANT from the cytosolic side, and BA, which functions as a matrix-side inhibitor of ANT. As shown in Fig. 4, CA did not change the frequency of  $\Delta\Psi_m$  fluctuations by itself, but it completely abolished the effect of adenine nucleotides. The traces presented in Fig. 4 A *i* illustrates an experiment where 2  $\mu$ M CA prevented the subsequent effect of ATP, whereas Fig. 4 A *ii* demonstrates that CA reverses the stabilizing effect of ATP when applied after the nucleotide. The same effect of CA was observed in experiments with ADP (not shown). The summarized data from these experiments are presented in Fig. 4 A *iv*. Surprisingly, however, BA was not effective in either preventing or reversing the actions of ATP. Fig. 4 A *iii*

shows that BA (2  $\mu$ M) slightly hyperpolarized mitochondrial membrane when applied with ADP, but did not initiate fluctuations in  $\Delta\Psi_m$ . The summarized data of experiments showing the effect of BA alone and in the combination with ATP and ADP are provided in Fig. 4 A *iv*. To ensure that CA and BA were active in brain mitochondria, we monitored oxygen consumption (Fig. 4 B). These experiments show that the same concentrations of BA, which was ineffective in the previous experiment, completely blocked mitochondrial respiration measured by oxygen consumption. In these experiments BA had the same effect as CA. It is therefore unlikely that BA's inability to prevent the effect of ATP and ADP is due to lack of inhibition of ANT in brain mitochondria.

**Mitochondrial  $K_{ATP}$  channels are not involved in  $\Delta\Psi_m$  fluctuations**

Another ATP-dependent mechanism in mitochondria, which can affect  $\Delta\Psi_m$ , is mitochondrial ATP-sensitive  $K^+$ -channels (mt $K_{ATP}$ -channels). mt $K_{ATP}$  channels located in the inner mitochondrial membrane (Inoue et al., 1991) can regulate  $\Delta\Psi_m$  (Holmuhamedov et al., 1998).  $K^+$  flux through mt $K_{ATP}$  channels depolarizes mitochondria when ATP in matrix is deficient (Inoue et al., 1991). To check the hypothesis that mt $K_{ATP}$  are involved in the mechanisms of the  $\Delta\Psi_m$  fluctuations, we used the activator of mt $K_{ATP}$  channels diazoxide and the selective antagonist of mt $K_{ATP}$  5-hydroxydecanoate. We used a relatively low concentration of diazoxide that has been suggested to preferentially inhibit



**FIGURE 3** Adenine nucleotide analogs and inhibition of mitochondrial ATP-synthase have no effect on  $\Delta\Psi_m$  dynamics. (A and B) Representative traces of individual mitochondria exposed to 300  $\mu$ M AMP-PNP and 100  $\mu$ M ATP (A); 300  $\mu$ M AMP-CP and 100  $\mu$ M ADP (B); (C) application of 100  $\mu$ M ATP in the presence of 2  $\mu$ M oligomycin; and (D) Summarized data show the frequency of  $\Delta\Psi_m$  fluctuations at the experimental conditions illustrated in A–C. Each histogram represents the mean ( $\pm$  SE) of four experiments with 100–150 mitochondria analyzed in each experiment.

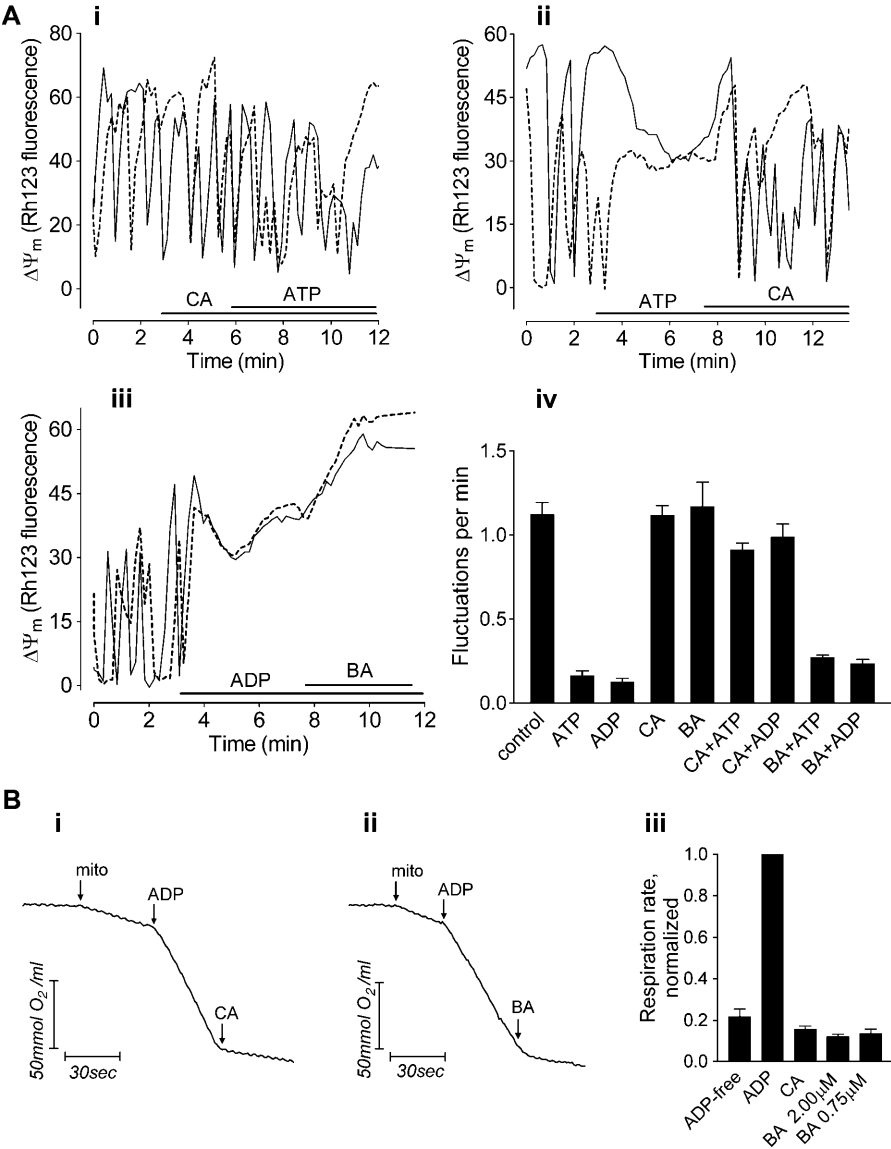


FIGURE 4 Effect of ANT inhibition on ΔΨ<sub>m</sub> dynamics and mitochondrial respiration. (A *i* and *ii*) representative traces of ΔΨ<sub>m</sub> measured in individual mitochondria exposed to 2 μM CA before and after ATP (100 μM) application. (A *iii*) an example of ΔΨ<sub>m</sub> dynamics in individual mitochondria exposed to 2 μM BA against a background of 100 μM ADP. (A *iv*) Summarized data shows the frequency of ΔΨ<sub>m</sub> fluctuations in mitochondria treated with 100 μM ATP or ADP, 2 μM CA or BA and the combinations of these drugs. Each bar represents the mean value of 5–8 experiments (± SE), 100–150 mitochondria were analyzed in each experiment. (B) The rate of respiration measured by Clark electrode (as described in Methods section) in mitochondria stimulated by ADP. The effect of 2 μM CA and BA is shown on the records from the individual experiments (*i* and *ii*) and as mean (± SE) respiration rate calculated from five experiments (*iii*). The rate of oxygen consumption was normalized to that measured at ADP application.

mtK<sub>ATP</sub> channels (15 μM) as well as a relatively high concentration that is often used in intact cell experiments (300 μM) (Grover and Garlid, 2000). As shown in Table 2, neither 5-hydroxydecanoate nor diazoxide either alone nor

with ATP changed the frequency or amplitude of ΔΨ<sub>m</sub> fluctuations. These data show that K<sub>ATP</sub> channels are not involved in the ΔΨ<sub>m</sub> fluctuations.

TABLE 2 Effect of mtK<sub>ATP</sub> channel modulators on the frequency of ΔΨ<sub>m</sub> fluctuations

	Fluctuations per minute
Control	1.09 ± 0.04
5-Hydroxydecanoate (500 μM)	1.09 ± 0.08
Diazoxide 15 μM	1.16 ± 0.14
Diazoxide 300 μM	0.97 ± 0.12
ATP	0.12 ± 0.02
ATP + diazoxide 15 μM	0.15 ± 0.03
ATP + diazoxide 300 μM	0.13 ± 0.01

Data are shown as mean ± SE, *n* = 3 experiments with 100 individual mitochondria were analyzed in each experiment.

### Ca<sup>2+</sup>-dependency of ΔΨ<sub>m</sub> fluctuations

We next investigated the role of Ca<sup>2+</sup> in regulating fluctuations. We previously showed that Ca<sup>2+</sup> loads can depolarize mitochondria and thus abolish fluctuations in this preparation (Vergun et al., 2003). However, given that the mitochondria are prepared in EDTA and that the perfusion buffer also contains a low concentration of EDTA, we did not anticipate a role of calcium in the baseline properties of the mitochondria. Surprisingly, when the mitochondrial Ca<sup>2+</sup> uniporter was blocked with 2 μM ruthenium red (RuRed), the ΔΨ<sub>m</sub> fluctuations were essentially abolished

(Fig. 5 A). To exclude the possibility that this was an unexpected nonspecific effect of RuRed, we also added EGTA at a higher concentration than in normal HBS to more aggressively buffer any residual  $\text{Ca}^{2+}$ . Fig. 5 B demonstrates that addition of 150  $\mu\text{M}$  EGTA to HBS inhibited  $\Delta\Psi_m$  fluctuations to the same extent as RuRed. This suggests that normal HBS (containing 0.02 mM EDTA) contained residual  $\text{Ca}^{2+}$ , which triggered the fluctuations. Further evidence confirming this conclusion is presented in Fig. 6. After the fluctuations were stopped with a high concentration of EGTA (230  $\mu\text{M}$ ), the addition of a small amount of  $\text{Ca}^{2+}$  (15  $\mu\text{M}$ ) initiates the fluctuations again. In fact, the concentration of free  $\text{Ca}^{2+}$  required to initiate the fluctuations was very low, only 30 nM as calculated by Max-Chelator (see Methods section). The observation that addition of  $\text{Ca}^{2+}$  into the EGTA containing medium triggered the  $\Delta\Psi_m$  fluctuations, also confirms that the stabilizing effect of EGTA was not attributable to a non-specific effect, but was a result of chelation of external  $\text{Ca}^{2+}$ . The experiment illustrated in Fig. 6 also demonstrates that  $\text{Ca}^{2+}$ -induced fluctuations can be stopped by addition of 100  $\mu\text{M}$  ADP. ATP had the same effect on  $\text{Ca}^{2+}$ -induced oscillation (data not shown). The adenine nucleotides in the concentration we used did not change significantly free  $[\text{Ca}^{2+}]$ ; addition 100  $\mu\text{M}$  ADP or ATP decreased free  $[\text{Ca}^{2+}]$  from 30.65 nM to 30.58 and 30.57 nM, respectively.

Although the most of the experiments were performed using malate and glutamate as a substrate for complex I, we also observed the  $\text{Ca}^{2+}$ -dependent fluctuations in  $\Delta\Psi_m$  with respiration supported by succinate in the presence of rotenone. The frequency and amplitude of these fluctuations had similar characteristics to those observed in malate/glutamate (data not shown).

Finally, it has been proposed that mitochondrial chloride channels may be involved in the regulation of  $\Delta\Psi_m$  (see Kicinska et al., 2000, for a review). Typically the experiments on isolated mitochondria are performed using KCl-based media, which has a  $\text{Cl}^-$  concentration that is higher than the physiological range. We reduced the  $\text{Cl}^-$  concentration to 20 mM ( $\text{Cl}^-$  was substituted by  $\text{SO}_4^{2-}$ ). Decreasing the  $\text{Cl}^-$  concentration did not change the amplitude or frequency of  $\Delta\Psi_m$  fluctuations. The mean frequency of fluctuations was  $1.23 \pm 0.09$  per min in control and  $1.32 \pm 0.09$  in low  $\text{Cl}^-$  ( $n = 4$ , difference is not significant). A complete substitution of  $\text{Cl}^-$  for  $\text{SO}_4^{2-}$  also did not affect the frequency of fluctuations (data not shown).

## DISCUSSION

This study has provided several novel insights into the phenomenon of oscillatory changes in  $\Delta\Psi_m$  in mitochondria isolated from rat brain. Previously described as “spontaneous” fluctuations, we have now established that this behavior is actually triggered by very low concentrations

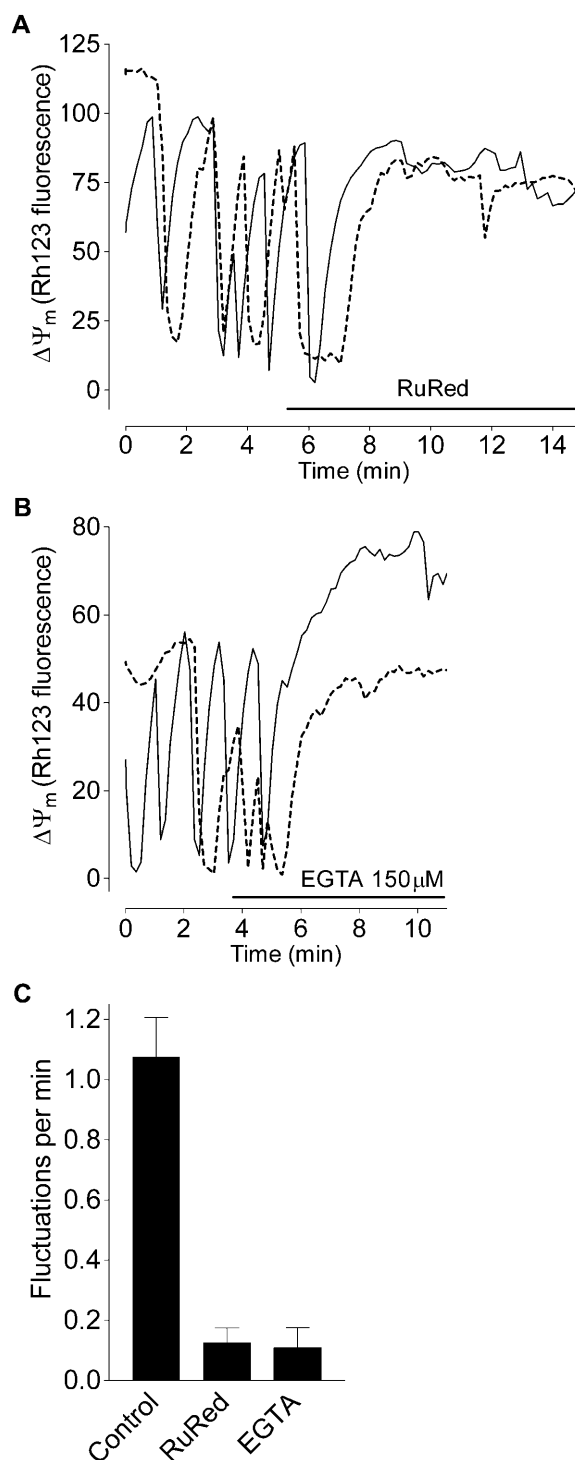


FIGURE 5 Effect of inhibition of mitochondrial  $\text{Ca}^{2+}$  uptake on  $\Delta\Psi_m$  fluctuations. (A and B) the changes in Rh123 fluorescence in the individual mitochondria in response to 2  $\mu\text{M}$  RuRed and 150  $\mu\text{M}$  EGTA. (C) Summarized data of the experiments illustrating in A and B; the mean ( $\pm$  SE) frequency of fluctuations was calculated from four experiments, 100 individual mitochondria were analyzed in each experiment.

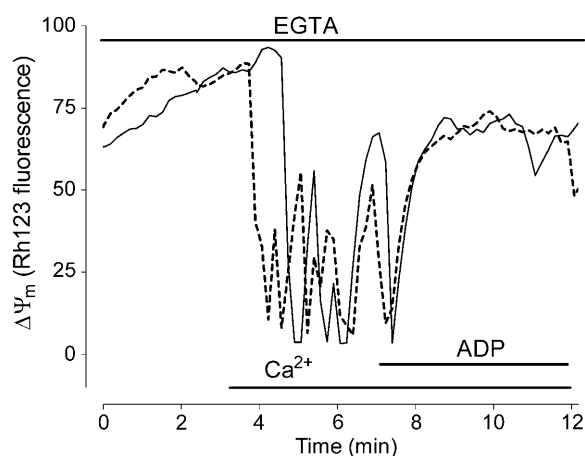


FIGURE 6 ADP stops Ca<sup>2+</sup>-triggered fluctuations in  $\Delta\Psi_m$ . EGTA (230  $\mu$ M), Ca<sup>2+</sup> (15  $\mu$ M), and ADP (100  $\mu$ M) were added to normal HBS; the free Ca<sup>2+</sup> concentrations calculated by using Max-Chelator program (see Methods) were 30.65 nM in EGTA + Ca<sup>2+</sup> and 30.58 nM in EGTA + Ca<sup>2+</sup> + ADP.

of Ca<sup>2+</sup>, because the transient depolarizations are blocked by an inhibitor of the Ca<sup>2+</sup> uniporter and by aggressive chelation of extra-mitochondrial Ca<sup>2+</sup>. We have also established that the Ca<sup>2+</sup>-induced fluctuations are completely inhibited by relatively low concentrations of both ATP and ADP, but not by nonhydrolysable analogs of adenine nucleotides or other nucleotides. Although the mechanism of the Ca<sup>2+</sup>-mediated depolarizations remains unknown, these studies provide important insights into the properties of the  $\Delta\Psi_m$  fluctuations and exclude some possible mechanisms, as will be discussed below.

Our prior studies with isolated mitochondria (Vergun et al., 2003) showed that larger Ca<sup>2+</sup> loads could profoundly depolarize mitochondria studied in this manner, and thus occlude fluctuations. This set of experiments reveals quite a different property of Ca<sup>2+</sup>, in that the fluctuations appear to be triggered by very low Ca<sup>2+</sup> concentrations. These actions of Ca<sup>2+</sup> were inhibited when the mitochondria were either exposed to RuRed in the absence of added Ca<sup>2+</sup>, or when the perfusion buffer was supplemented with additional EGTA beyond the 20  $\mu$ M EDTA normally present. The effects of RuRed suggest that the actions of Ca<sup>2+</sup> occur on the matrix side of the inner membrane. Our calculations indicate that a remarkably low concentration of Ca<sup>2+</sup>, ~30 nM free ion, is sufficient to activate the fluctuations. This is surprising given that the uniporter is typically thought to transport Ca<sup>2+</sup> when mitochondria are exposed to relatively high concentrations of the ion (Gunter et al., 1994). Thus, although a recent study found that Ca<sup>2+</sup> binds to the uniporter with a low nanomolar affinity and blocks Na<sup>+</sup> currents (Kirichok et al., 2004), net Ca<sup>2+</sup> accumulation occurs when extra-mitochondrial Ca<sup>2+</sup> exceeds the set point, which is typically near 500 nM (Gunter et al., 1994). It is unlikely that the fluctuations reflect Na<sup>+</sup> currents carried by the uniporter because these currents

would be blocked by Ca<sup>2+</sup> addition, and because the Na<sup>+</sup> concentration in the buffer is very low (Kirichok et al., 2004). Gunter and colleagues have identified a rapid mode of Ca<sup>2+</sup> uptake that results in matrix Ca<sup>2+</sup> accumulation after relatively small pulses of Ca<sup>2+</sup> via a RuRed-sensitive pathway (Gunter et al., 1998). This mechanism is poorly characterized in brain mitochondria, and it is not clear that the mechanism operates at such low concentrations. It is also unlikely that the Ca<sup>2+</sup> is being accumulated by another pathway, such as by the reversed mode of the Na<sup>+</sup>-dependent and independent Ca<sup>2+</sup> efflux pathways, given the RuRed sensitivity of the effect, even though this may be a route of Ca<sup>2+</sup> entry into mitochondria (Griffiths, 1999). This forces the conclusion that the fluctuations are mediated by a matrix Ca<sup>2+</sup> elevation induced by an unexpectedly low concentration of extra-mitochondrial Ca<sup>2+</sup> that is apparently transported into mitochondria by the uniporter. The low concentrations of Ca<sup>2+</sup> required to activate fluctuations imply that there is sufficient Ca<sup>2+</sup> present in resting cells to support fluctuations in  $\Delta\Psi_m$ .

A second key finding of this study is the sensitivity of the fluctuations to adenine nucleotides. Both ATP and ADP are effective inhibitors of the fluctuations at relatively low concentrations relative to normal cellular ATP levels. This inhibitory effect is quite selective, in that it is not mimicked by any of the other nucleotides tested, and is also not recapitulated by nonhydrolysable analogs. The lack of effect of GTP is noteworthy, because this excludes the possibility that the fluctuations are carried by an uncoupling protein, given that uncoupling protein-mediated transport is quite sensitive to GTP (Boss et al., 1998; Klingenberg and Huang, 1999; Nicholls, 2001). We speculated that the inhibitory effects of ATP were mediated by hydrolysis of the nucleotide by the F<sub>1</sub>F<sub>0</sub>-ATPase, which would generate a membrane potential, potentially occluding the depolarizations that we observed. This is unlikely given that the ATP effect was mimicked by ADP, and was also completely insensitive to oligomycin. Another way to approach this question is to use nonhydrolysable forms of ATP and ADP. These agents are transported into mitochondria by ANT, albeit at a somewhat slower rate (Vignais et al., 1985). We compensated for this limitation by exposing the mitochondria to a higher concentration of the analogs. Even so, they had no effect. This leaves open two possibilities; that the nucleotide effects depend on either hydrolysis or exchange of phosphate groups, or that there is specificity to the binding of ATP that is not adequately mimicked by the analogs.

We used the ANT inhibitor CA to determine the site of action of ATP and ADP. CA does not alter the fluctuations by itself, however, CA was very effective in blocking the inhibitory effects of ATP and ADP. The immediate and obvious conclusion is that the adenine nucleotides operate on the matrix side of the membrane to modulate the fluctuations, and that they are transported to the matrix by ANT. However, this conclusion is at variance with the results of

the experiments with BA, which did not affect the fluctuations nor prevent the adenine nucleotide effects. This is despite the fact that both drugs were clearly effective in inhibiting ADP transport in brain mitochondria as judged by their ability to convert state 3 respiration into state 4 in the continued presence of ADP. Perhaps the most conservative conclusion from these observations is that the adenine nucleotides are being transported into the matrix via a CA-sensitive carrier that is distinct from ANT. There are many metabolite carriers in mitochondria that lack full characterization, and some of the recently identified carriers for molecules, such as nucleosides, do show sensitivity to CA (Dolce et al., 2001), so this remains a possibility. Unfortunately, the pharmacology of ANT is relatively limited, so the opportunity to use a range of CA mimetics to establish the relatively potency of the effect described here to authentic ANT inhibition does not exist. Previous studies have reported  $\text{Ca}^{2+}$ -induced currents mediated by ANT in reconstituted carriers (Brustovetsky and Klingenberg, 1996; Brustovetsky et al., 1996). However, these currents were directly inhibited by CA and required much higher concentrations of  $\text{Ca}^{2+}$ , and are thus unlikely to be the phenomenon under study here.

ADP and BA are known as inhibitors of mitochondrial permeability transition (MPT), and CA is known as a MPT activator (Crompton, 1999). Based on these data we could speculate that the CA-induced oscillatory activity and the inability of BA to induce fluctuations in the presence of adenine nucleotides were due to action of these drugs on MPT. However, our previous findings showed that the MPT blocker cyclosporin A does not stop the  $\Delta\Psi_m$  fluctuations, and calcein is retained in mitochondria undergoing  $\Delta\Psi_m$  fluctuations (Vergun et al., 2003). Furthermore, 2-aminoethoxydiphenyl borate, which blocked cyclosporine A-insensitive MPT in neurons (Chinopoulos et al., 2003), did not have an effect on the  $\Delta\Psi_m$  dynamics in our conditions (data not shown). These data together do not provide strong evidence to conclude that MPT is involved in the mechanism of  $\Delta\Psi_m$  fluctuations.

It is important to note that the concentrations of adenine nucleotides that block fluctuations in  $\Delta\Psi_m$  are quite low relative to concentrations one would normally expect to find in intact cells. It is difficult to imagine circumstances in which the ATP and ADP levels would drop below 100  $\mu\text{M}$ , in fact. This raises the question of whether the phenomenon under study here has any physiological or pathophysiological relevance. This question remains difficult to conclusively address. The characteristics of the reversible and repeated depolarizations of  $\Delta\Psi_m$  are very similar to those seen in several different cell types, as noted above, and it seems unreasonable to propose that there are several completely distinct depolarization mechanisms that operate separately in the various model systems studied. It is perhaps more reasonable to suggest that there are additional, as yet unidentified, modulators that alter the sensitivity to ATP and

ADP such that the depolarizations can still occur at higher adenine nucleotide concentrations. Calcium might be one such modulator, although it is likely that there are others, too.

It has been suggested that mitochondria are endowed with  $\text{mtK}_{\text{ATP}}$  channels, which are located in the inner mitochondrial membrane and can be reversibly inactivated by ATP (Inoue et al., 1991). It has been shown that activation of  $\text{mtK}_{\text{ATP}}$  channels depolarizes mitochondrial membrane in cardiac mitochondria (Holmuhamedov et al., 1998). We hypothesized that a transient opening of such channels can represent a mechanism for the  $\Delta\Psi_m$  fluctuations. However, the modulators of  $\text{mtK}_{\text{ATP}}$  channels activity we used did not affect the dynamics of  $\Delta\Psi_m$ , thus eliminating a potential role of these channels in the  $\Delta\Psi_m$  fluctuations. It is also unlikely that mitochondrial  $\text{Cl}^-$  channels, which are involved in the regulation of  $\Delta\Psi_m$  in some cell types (see for review Kicinska et al., 2000), are responsible for the  $\Delta\Psi_m$  fluctuations, since reducing or completely removing  $\text{Cl}^-$  from the medium did not change significantly the frequency of fluctuations.

On the basis of these results we can exclude the possibility that the depolarizations occur as a result of the activation of an uncoupling protein. However, we cannot exclude the possibility that the depolarizations are the result of proton or phosphate currents. Studies are currently underway to assess the contribution of these mechanisms.

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