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Ca^{2+} Storage Capacity of Rat Brain Mitochondria Declines During the Postnatal Development Without Change in ROS Production Capacity

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ABSTRACT

Ca^{2+} overload of mitochondria and oxidants are considered as crucial factors inducing the opening of the permeability transition pore (PTP) in mitochondria. The interdependence between permeability transition (PT), calcium retention capacity (CRC), and reactive oxygen species (ROS) generation was studied in mitochondria from immature and mature rat brain. Brain mitochondria isolated from 1-day- and 1-week-old rats are much more resistant to Ca^{2+}-triggered PT in phosphate-containing incubation medium than mitochondria from adult brain, since the CRC decreases with development. CRC of mitochondria from 1-week-old rat brain was higher than for adult rat brain (450 ± 112 vs. 175 ± 35 nmol Ca^{2+} per mg of protein). In contrast, for ROS generation there was no age difference. In immature and mature mitochondria, basal, respiratory chain-inhibited or glutathione-depleted ROS generations were similar. In addition, the extent of the Ca^{2+} load was without effect on the basal ROS generation before mitochondria underwent PT. In summary, ROS generation does not crucially affect the ability of immature mitochondria to buffer high levels of extramitochondrial Ca^{2+} without undergoing PT. However, we hypothesize that the high resistance of immature mitochondria is related to the low content of some PTP complex constituents, such as creatine kinase. *Antioxid. Redox Signal.* 9, 191–199.

INTRODUCTION

Cerebral ischemia depletes brain cells of energy-rich phosphates, which triggers a cascade of harmful cellular changes, such as excessive glutamate release, long-lasting elevation of the cytosolic Ca^{2+} level, and generation of reactive oxygen species (ROS) (for recent reviews, see Refs. 28, 30). The overload of Ca^{2+} in mitochondria is a key step of the mitochondrial route of the apoptotic cell death. Indeed, markedly swollen mitochondria with large amounts of calcium deposits were found in neuronal tissue after ischemia–reperfusion (35). These mitochondrial changes have been attributed to the occurrence of permeability transition (PT) during ischemia–reperfusion episodes (10). PT refers to the assembly of pore-forming proteins at contact sites between the inner and the outer mitochondrial membrane, thereby facilitating a sudden unselective increase of solute permeation in and out of mitochondria, depolarization of the inner membrane, and the release of apoptogenic proteins from mitochondria (5, 18, 42). Rise in matrix Ca^{2+} level and oxidative stress are considered to be potent triggers of PT in isolated mitochondria and *in vivo*. Direct evidence of PT under *in vivo* conditions came from the observation that the exposure of 7-days-old rats to hypoxia–reperfusion events resulted in a significant entrapment of [^{14}C]2-deoxyglucose 6-phosphate (2-DOG-6-P) in mitochondria (34). 2-DOG-6-P is normally not able to enter morphologically intact mitochondria.

During postnatal brain development, about 50% of the neurons are lost in certain brain regions by apoptotic cell death (36). At birth, the energy metabolism of the rat brain is in an immature state. Since during development there is ongoing mitochondrial enrichment of proteins of the oxidative phos-

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phorylation, glycolytic energy production switches to oxidative ATP generation. This developmental process takes place in the first weeks of postnatal life. The activity of various complexes of the respiratory chain increases from birth until day 60 of postnatal life about fourfold (4). These changes reflect the increasing oxygen uptake of the developing rat brain. However, it is also well established that the mitochondrial respiratory chain leaks electrons to molecular oxygen, thereby contributing to the generation of ROS. The superoxide radical ($O_2^{\bullet -}$) is mostly formed as a side-product by complexes I and III of the respiratory chain (for a recent review, see Ref. 3). Mitochondria fed with electrons derived from NAD-dependent substrates generate *in vitro* relatively low levels of $O_2^{\bullet -}$, but their $O_2^{\bullet -}$ generation increases dramatically when they are exposed to rotenone (inhibitor of complex I) or antimycin A (inhibitor of complex III). This stimulation is attributed to the increased levels of the reduced forms of redox carriers, thereby supporting a one-electron transfer to molecular oxygen, finally forming $O_2^{\bullet -}$. It is believed that the flavin centers and/or Fe–S clusters of complex I, as well as the semi-ubiquinone of complex III, are the sites that release electrons to oxygen (Fig. 1). Further details are given in Fig. 1 and the respective legend. Importantly, oxidative stress is considered to be a trigger for the opening of the PTP *in vitro* and *in vivo* (5, 10).

Energized mitochondria have the ability to store large amounts of Ca^{2+} in their matrix compartment in a reversible manner (for review, see Ref. 14). Ca^{2+} uptake is mediated by the Ca^{2+} uniporter, a ruthenium red-sensitive transport protein, and the driving force for Ca^{2+} uptake is provided by the membrane potential ($\Delta\psi_m$) at the inner mitochondrial membrane. Since high levels of cytosolic Ca^{2+} can occur after several forms of brain injury, the ability of mitochondria to buffer cytosolic Ca^{2+} levels is of crucial importance for cell survival. The responsiveness of isolated immature brain mitochondria to Ca^{2+}-dependent PT has scarcely been studied. Here, we report that immature mitochondria have a higher Ca^{2+} retention capacity (CRC) than mature mitochondria. CRC is an indicator for the resistance of mitochondria against opening of PTP by Ca^{2+} overload. However, in immature mitochondria the conversion of redox energy into the electrochemical proton gradient is less efficient than in mature mitochondria. Therefore, the cause for a larger CRC of immature mitochondria is not clear. It is still open whether differences in ROS production contribute to the different CRC of immature and mature brain mitochondria. To examine this possibility, the ROS production by immature and mature mitochondria was measured under various conditions, including those of Ca^{2+}-loading of mitochondria. We found that ROS production of immature and mature mitochondria was not significantly different in all the situations studied. In conclusion, the difference in CRC of immature and mature rat brain mitochondria is not related to the capacity of ROS generation.

MATERIALS AND METHODS

Reagents

Amplex Red and CaG5N were purchased from Molecular Probes (Eugene, OR). Safranine, FCCP, nigericin, phenylarsine oxide, and enzymes were obtained from Sigma (St. Louis, MO).

Preparation of mitochondria

Mitochondria were prepared from the brains of rats at distinct postnatal ages [1-day-old, 1-, 2-, and 3-weeks-old, 3-month-old (adult)] by an isolation procedure designed to yield a high content of mitochondria from brain tissue (24). The functional integrity of prepared mitochondria was exam-

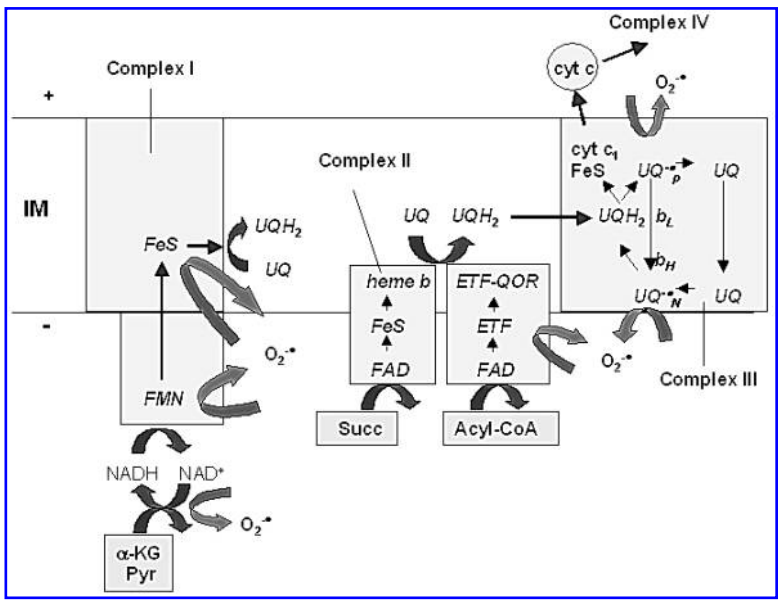

FIG. 1. Sites of superoxide generation in the respiratory chain. Complexes I and III of the mitochondrial electron transport chain are the main sites for $O_2^{\bullet -}$ generation. In complex I, flavin mononucleotide (FMN) and [Fe–S] cluster centers are considered as the sites that promote the one-electron-transfer reaction to O_2. In complex III, the half-reduced ubiquinone ($UQ^{\bullet -}$) mediates the one-electron transfer to O_2. Complex I releases $O_2^{\bullet -}$ exclusively to the matrix side, whereas from complex III $O_2^{\bullet -}$ is released to both sides (3). It is discussed that the electron transporting flavoprotein (ETF) and the flavin center of the α-ketoglutarate dehydrogenase could operate as other potential sites of $O_2^{\bullet -}$ generation (3, 13, 41). cyt c, cyt c_1: cytochrome c and c_1; b_L, b_H: cytochromes b_L and b_H; ETF-QOR: electron transfer flavoprotein quinine oxidoreductase. FAD: flavine adenine dinucleotide; IM: inner membrane; α-KG: α-ketoglutarate; Pyr: pyruvate; Succ: succinate; UQH_2: ubiquinol; UQ: ubiquinone; $UQ^{\bullet}_P$, $UQ^{\bullet}_N$: semiubiquinone at P or N side of the inner membrane. The scheme is similar to that shown in Ref. 15.

ined by the respiratory control ratio (RCR) using an oxygraph (Oroboros Oxygraph®, Bioenergetics and Biomedical Instruments, Innsbruck, Austria) to measure oxygen uptake. Oxygen uptake of mitochondria and other measurements were done in a medium (called standard incubation medium) composed of 110 m*M* mannitol, 60 m*M* KCl, 60 m*M* Tris, 10 m*M* KH_2PO_4, 5 m*M* pyruvate, 5 m*M* malate, 0.5 m*M* EGTA (pH 7.4) at 30°C. RCR is defined as the ratio of the phosphorylating respiration (also designated as active or state 3 respiration) over the nonphosphorylating respiration (also designated as resting or state 4 respiration). RCR reflects the coupling of the respiratory chain reaction and the phosphorylation of ADP by the F_OF_1-ATP synthase. For measurement of RCR, the oxygen uptake of mitochondria suspended in medium (supplied with respiratory chain substrates, such as pyruvate plus malate) is measured after addition of a limiting amount of ADP and, subsequently, after the complete phosphorylation of the added ADP. RCR values were between 3.6 ± 0.5 (1-day-old animals) and 10 ± 2.8 (adult rats). Protein contents in the stock suspensions were measured by the Biuret method using bovine serum albumin as standard.

Enzymatic activities

For further characterization of the developmental state of prepared mitochondria, the activities of citrate synthase and mitochondrial creatine kinase were measured in the mitochondrial stock suspension. Citrate synthase activity was assayed spectrophotometrically by the detection of liberated coenzyme A using 5,5′-dithiobis-2-nitrobenzoic acid (Ellman's reagent), as described by Bates *et al*. (4). The assay of creatine kinase activity is based on the reaction of creatine with ATP to creatine phosphate plus ADP (21).

Measurement of ROS generation

Respiring mitochondria release $O_2^{\bullet-}$ into the surrounding medium. The $O_2^{\bullet-}$ levels in the medium were measured after conversion of $O_2^{\bullet-}$ to H_2O_2. H_2O_2 can be detected by H_2O_2-dependent oxidation of Amplex Red (Molecular Probes) to resorufin, a stable and highly fluorescent compound. The conversion of $O_2^{\bullet-}$ to H_2O_2 is catalyzed by the added horseradish peroxidase. Briefly, mitochondria (0.2 mg of mitochondrial protein/ml) were incubated at 25°C in incubation buffer supplemented with 5 µ*M* Amplex Red plus horseradish peroxidase (2 U/ml) to detect H_2O_2, and for quantitative conversion of $O_2^{\bullet-}$ into H_2O_2 with Cu, Zn-dismutase (2 U/ml). Changes in fluorescence were monitored by a Perkin-Elmer (Boston, MA) Luminescence Spectrometer LS-50B (excitation at 560 nm, emission at 590 nm). Resorufin fluorescence was calibrated with H_2O_2.

Permeability transition

Opening of the permeability transition pore (PTP) was mostly initiated by incremental additions of Ca^{2+} to mitochondria (1 mg of protein) suspended in 1 ml of incubation medium. The onset of PT was assessed as the collapse of $\Delta\psi_m$ or the release of Ca^{2+} from mitochondria into the surrounding medium. Changes in $\Delta\Psi_m$ were monitored fluorimetrically using the cationic dye safranine (excitation at 495 nm, emission at 586 nm) as membrane potential probe (1). Ca^{2+} release was measured using Calcium green-5N (CaG5N) as membrane impermeable indicator of extramitochondrial Ca^{2+} (29).

Data analysis

Statistical analysis was performed using Prism 3.0 software (GraphPad Software, San Diego, CA). Significance was examined by the unpaired *t* test. $p < 0.05$ was considered statistically significant.

RESULTS

Developmental characterization of mitochondria during the first 3 weeks in life

Besides the respiratory control ratio (RCR), the maturational state of mitochondria prepared from rat brains at different ages was characterized by measuring the enzymatic activities of citrate synthase (CS) and mitochondrial creatine kinase (mtCK) in the mitochondrial pellet. All values obtained for mitochondria isolated from 1-day-old rat brain are low with respect to those of the adult brain. Compared to mitochondrial preparations from adult rat brain, the activities of CS and of mtCK in mitochondrial preparations from 1-week-old rat brain amounted to 88% and 32%, respectively (Fig. 2). In mitochondria prepared from the 1-week-old rat brain, RCR was 66% of that in adult animals. For mitochondria obtained from brains of rat that were 2 and 3 weeks old, the value for CS, mtCK activity, and respiratory control ratio were not significantly different from the respective values seen for adult rat brain.

Ca^{2+} buffering and permeability transition by immature mitochondria

Figure 3 shows depolarization of mitochondria from 1-week-old rat brain achieved by Ca^{2+} loading. Membrane depolarization is either indicated as increase of the fluorescence of safranine due to its release from mitochondria (traces A, B, C) or as increase of fluorescence of the Ca^{2+}–CaG5N complex due to Ca^{2+} release (traces D, E, F). Phenylarsine oxide (PhAsO), a cross-linker of vicinal thiol-groups, in Ca^{2+}-loaded mitochondria (traces A and D) initiates the opening of PTP (23). Complete depolarization of the mitochondria within the studied mitochondrial population was achieved by addition of the protonophore FCCP. The opening of PTP by PhAsO is also clearly indicated by the release of mitochondrial Ca^{2+} into the surrounding medium. Mitochondria suspended in Pi-free medium become progressively depolarized by incremental additions of Ca^{2+} (trace B). A subsequent addition of an excess of EGTA binds the added Ca^{2+}, with complexation of Ca^{2+}. This initiates the repolarization of mitochondria (given as the decrease of the safranine fluorescence in trace B), indicating the closure of PTP. In the presence of Pi (10 m*M*), mitochondria accumulate much more of the added Ca^{2+} before severe depolarization takes place (traces C and F). From these findings, it is apparent that brain mitochondria with an immature system of energy transduction exhibit already characteristic features of Ca^{2+} buffering and PT.

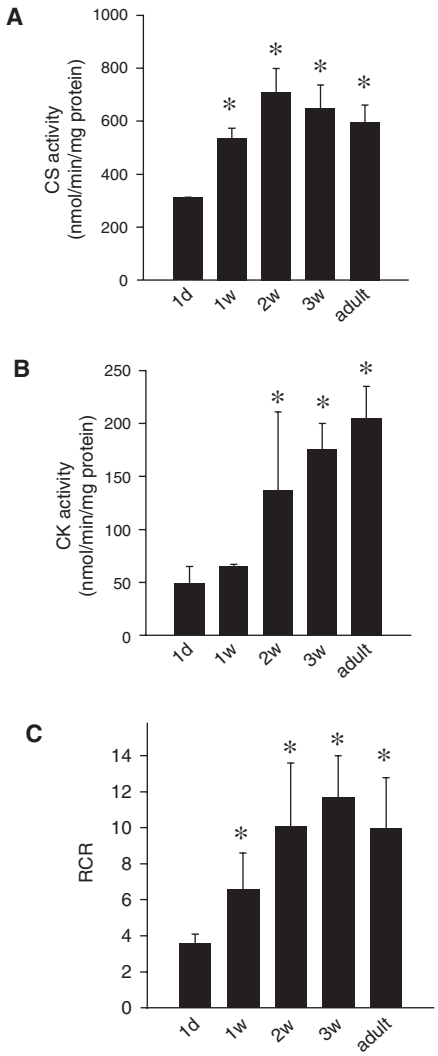

FIG. 2. Postnatal changes in the mitochondrial content of citric acid synthase, creatine kinase, and respiratory control ratio of rat brain mitochondria. Citric acid synthase (CS) and the mitochondrial creatine kinase (mtCK) were determined by measuring catalytic activity, as described in Materials and Methods. The respiratory control ratio (RCR) was measured oxygraphically. Data shown are the means ± SD of 4–6 mitochondrial preparations for animals aged 1 day, 1, 2, and 3 weeks and adult (3 months). *$p < 0.05$ compared to 1 d (1-day-old).

Calcium retention capacity

To quantify the resistance against Ca^{2+}-dependent PT in immature mitochondria, we estimated the calcium retention capacity (CRC) of brain mitochondria prepared from immature (1-day-, 1-week-, 2-week- and 3-week-old) and adult brain tissue. For practical reasons, CRC was defined as the Ca^{2+} load, which induced an increase in safranine fluorescence of 50% of the maximum value (Fig. 4). CRC was estimated in Pi-free and Pi-containing media. These measurements lead to several remarkable conclusions (Fig. 5): (a) Immature mitochondria have higher CRC than mature mitochondria; (b) the capacity to accumulate Ca^{2+} declines with postnatal maturation of mitochondria; and (c) in the absence of Pi, the CRC is much lower and does not depend on the postnatal age of the brain.

The increase of CRC in Pi-containing medium is mainly due to two activities of Pi. First, Pi is required for complexation of matrix Ca^{2+}, thereby forming a typical calcium phosphate gel (9). Second, in the presence of external Pi, the phosphate transporter exchanges $H_2PO_4^-$ for matrix OH^-, thereby lowering the matrix pH. The decrease in the ΔpH between the matrix and the medium resulted in a compensatory increase in $\Delta\Psi_m$ (Fig. 6A). In conclusion, an increase in the concentration of matrix-Pi protects mitochondria against PT by stabilization of $\Delta\Psi_m$ and complexation of accumulated Ca^{2+}. Furthermore, the high CRC of immature brain mitochondria becomes dramatically reduced by nigericin, a H^+/K^+ exchanger known to abolish the pH difference between the medium and the matrix (Fig. 6B). The latter finding clearly demonstrates that matrix alkalinization protects mitochondria against Ca^{2+}-dependent PT.

Basal and stimulated ROS production in immature and mature mitochondria

ROS are known to modify protein molecules, either by oxidation of thiol groups or by the formation of protein carbonyls. Therefore, oxidative protein modification could be a factor, which differentially affects the CRC of immature and mature brain mitochondria. To examine this possibility, we estimated first the capacity of mitochondria isolated from 1-week-old and adult rat brain to release $O_2^{\bullet-}$. Release of $O_2^{\bullet-}$ was measured as H_2O_2-dependent increase in the resorufin fluorescence. The data summarized in Fig. 7 (A and B) clearly show that the $O_2^{\bullet-}$ production of immature (1-week-old) and mature mitochondria oxidizing NAD^+-linked substrates is comparable in size. At conditions of the reverse electron transport (adjusted with succinate as substrate), both immature and mature mitochondria generate $O_2^{\bullet-}$ at a much higher rate. Then $O_2^{\bullet-}$ production was stimulated, either by enhancing the electron leakage from the respiratory chain by application of respiratory chain inhibitors (rotenone, antimycin A) or by oxidizing matrix glutathione using DCNB (1-chloro-2,4-dinitrobenzene) (22). After depletion of reduced glutathione, scavenging of H_2O_2 by the glutathione peroxidase is abolished. From Fig. 7C and D, it can be seen that the inhibition of the electron transport enhanced $O_2^{\bullet-}$ generation similarly in mature and in immature mitochondria oxidizing pyruvate plus malate. Oxidation of glutathione enhanced $O_2^{\bullet-}$ production strongly in mature and in immature mitochondria. The highest rate of $O_2^{\bullet-}$ production was found when the electron transport was blocked by rotenone and glutathione was oxidized by DCNB.

Effect of incremental calcium additions and permeability transition on ROS generation in rat brain mitochondria

Next, the effect of incremental additions of Ca^{2+} aliquots (25 nmol per addition) on mitochondrial $O_2^{\bullet-}$ production was examined. Figure 8A shows the release of $O_2^{\bullet-}$ from mitochondria (indicated as resorufin fluorescence) at the addition of increasing amounts of Ca^{2+} to the medium. It can be seen

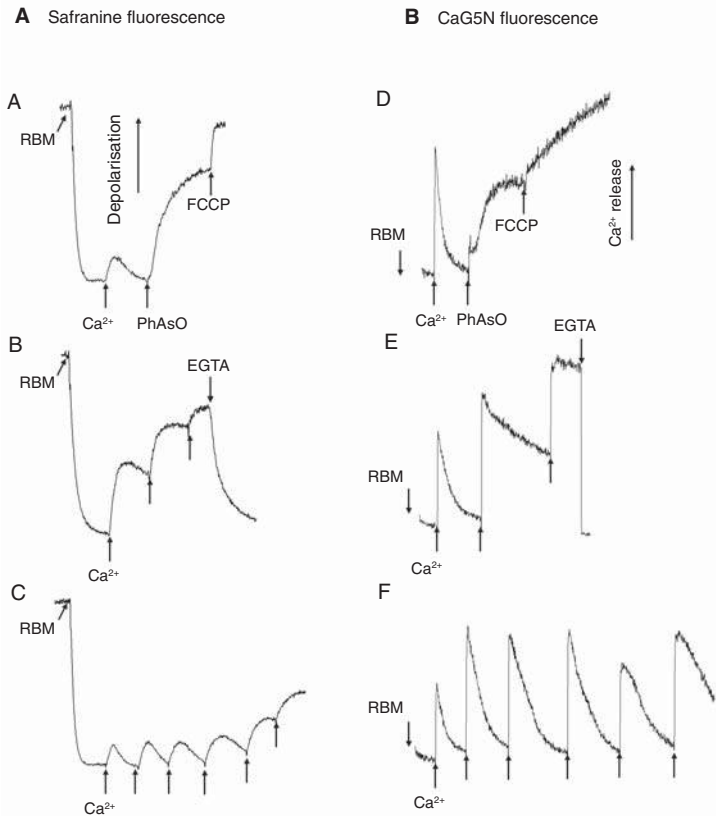

FIG. 3. Permeablity transition in brain mitochondria from newborn rats. Brain mitochondria (1 mg of protein per ml) prepared from newborn rats (1-day-old) were suspended in standard incubation media supplemented with 5 μ*M* safranine (**A**, **B**, **C**) or 0.05 μ*M* CaG5N (**D**, **E**, **F**). Ca^{2+} was added step-wise as aliquots of 50 nmol. Representative traces of the fluorescence change of the $\Delta\psi_m$ probe safranine and of CaG5N are shown. **B** and **E** show experiments with Pi-free medium. All other traces were obtained with 10 m*M* Pi in the incubation medium. Additions were 6 μ*M* phenylarsine oxide (PhAsO), 0.1 μ*M* FCCP, 1 m*M* EGTA.

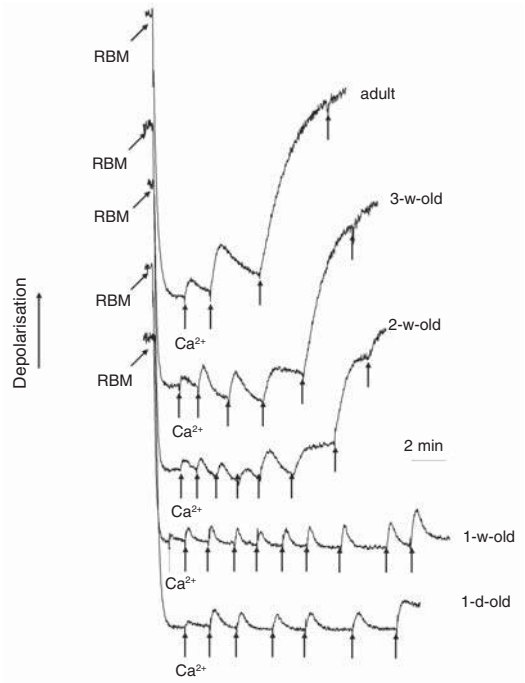

FIG. 4. Ca^{2+} storage by rat brain mitochondria at various developmental ages. Mitochondria (1 mg of protein) prepared from 1-day-, 1-, 2-, 3-weeks-old and adult brains were incubated in standard incubation medium (10 m*M* Pi) supplemented with 5 μ*M* safranine. Ca^{2+} was added as aliquots of 50 nmol. The traces show the effect of Ca^{2+} uptake on the polarization state of the inner mitochondrial membrane monitored with safranine.

that Ca^{2+} uptake by mitochondria up to 125 nmol Ca^{2+} (corresponding to 250 nmol Ca^{2+}/mg of protein) was without any effect on the release of $O_2^{\bullet-}$ by immature mitochondria (1-week-old).

Figure 8B shows the effect of the repeated additions of Ca^{2+} aliquots on the polarization state of the inner membrane (indicated as the change in the safranine fluorescence). However, when PT was triggered in Ca^{2+}-loaded mitochondria either by the addition of FCCP (trace a) or by addition of PhAsO (trace c), the release of $O_2^{\bullet-}$ increased significantly. In contrast, when Ca^{2+} loading of mitochondria was prevented by blockade of the Ca^{2+} uniporter with ruthenium red (RR), FCCP decreased the release of $O_2^{\bullet-}$ (trace b). At this condition, FCCP depolarized the inner membrane without triggering the opening of the PTP.

DISCUSSION

Rat brain mitochondria are more resistant to calcium-dependent opening of PTP than are rat liver mitochondria (2, 9, 32). Here we demonstrate that *in vitro* in the absence of external adenine nucleotides, immature rat brain mitochondria have a greater ability to buffer accumulated Ca^{2+} than mitochondria from the adult brain (Figs. 4 and 5). Interestingly, brain mitochondria from newborns (1-day-old) display much higher Ca^{2+} buffer capacity (quantified as CRC, before the opening of PTP takes place) than mitochondria from adult animals. This is a surprising finding, because the Ca^{2+} uptake is driven by $\Delta\psi_m$, the membrane potential at the inner mitochondrial membrane. However, the efficacy of 1-day-old mi-

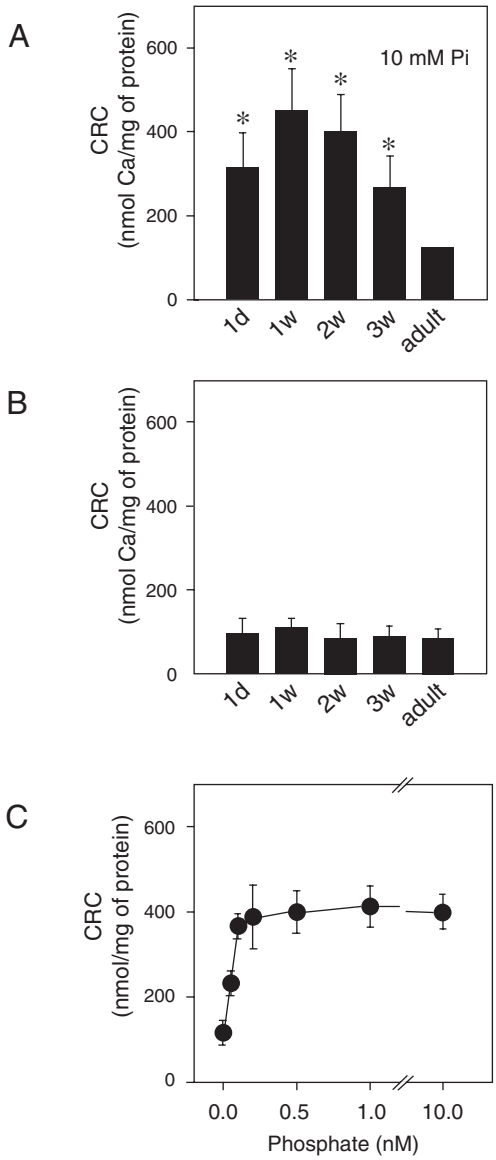

FIG. 5. Calcium retention capacity of rat brain mitochondria. Calcium retention capacity (CRC) was estimated as described in Materials and Methods. (**A**) CRC for mitochondria of different postnatal ages. Data are given as means ± S.D. obtained from 4 to 6 mitochondrial preparations of indicated ages. (**B**) CRC seen in phosphate-free medium. (**C**) CRC of 1-week-old mitochondria as function of the phosphate concentration of the incubation medium. $*p < 0.05$ compared to adult.

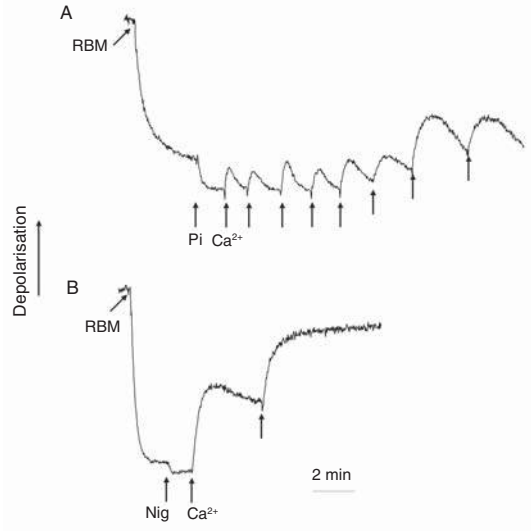

FIG. 6. Effect of phosphate and nigericin on the polarization and Ca^{2+} uptake of immature rat brain mitochondria. (**A**) Effect of Pi (1 m*M*) on the mitochondrial depolarization, followed by the subsequent addition of incremental additions of Ca^{2+} (50 nmol Ca^{2+} per addition) using safranine as $\Delta\psi_m$ probe. (**B**) Effect of adding nigericin (1 nmol per mg of protein) on mitochondrial depolarization by the Ca^{2+} pulse.

tochondria to convert redox energy into the electrochemical proton gradient or ATP synthesis is poor (see RCR in Fig. 2). Our finding that brain mitochondria of postnatal ages have a higher CRC than mitochondria from adult rats confirms a recent report according to which, in the absence of ATP, CRC from 2-week-old rat brain is higher than that of mitochondria from adults (37).

Furthermore, the ability of Ca^{2+} uptake is strongly affected by the concentration of external adenine nucleotides, especially ADP (32, 37). Adenine nucleotides (ATP, ADP) are known to decrease the probability of the opening of PTP (see Ref. 19 and references therein), thereby increasing Ca^{2+} uptake by mitochondria. The observation that the neonatal brain is less susceptible to the effects of hypoxia/ischemia than the adult brain (for a review, see Ref. 16) might be partly related to the fact that immature brain mitochondria are more resistant to Ca^{2+}-dependent opening of PTP.

Oxidative stress is considered to be an important trigger of the opening of PTP *in vitro* and *in vivo* (5, 10). Thus, enhanced $O_2^{\bullet -}$ generation by brain mitochondria treated with respiratory chain inhibitors sensitizes brain mitochondria for Ca^{2+}-dependent PT (25). Opening of PTP by ROS is probably initiated by the oxidation of the matrix GSH pool. It is further assumed that an oxidized GSH pool equilibrates with the NAD(P)H pool (10), which subsequently oxidizes the thiol groups of the inner mitochondrial membrane. Consequently, ROS may promote PT by oxidation of critical vicinal thiols at the adenine nucleotide translocase (ANT). ANT is generally believed to be a core-component of the PTP (see Refs. 5 and 6 for reviews). It is notable that the content of ANT in the inner membrane increases about threefold during postnatal development (38). In addition, matrix-ADP is assumed to inhibit the opening of PTP, presumably by stabilizing the ANT in its m-state (see Ref. 6 for review). Moreover, assuming that an oxidative modification of ANT reduces the binding of ADP to ANT, there is good reason to assume that oxidative stress can facilitate the opening of PTP (17, 27). In addition, oxidative stress could also modify cardiolipin, which is sensitive to ROS due to its high content of polyunsaturated fatty acids. Since the activity of ANT depends on the association with cardiolipin (20), it appears also likely that an oxidative damage of cardiolipin facilitates the opening of the PTP (8).

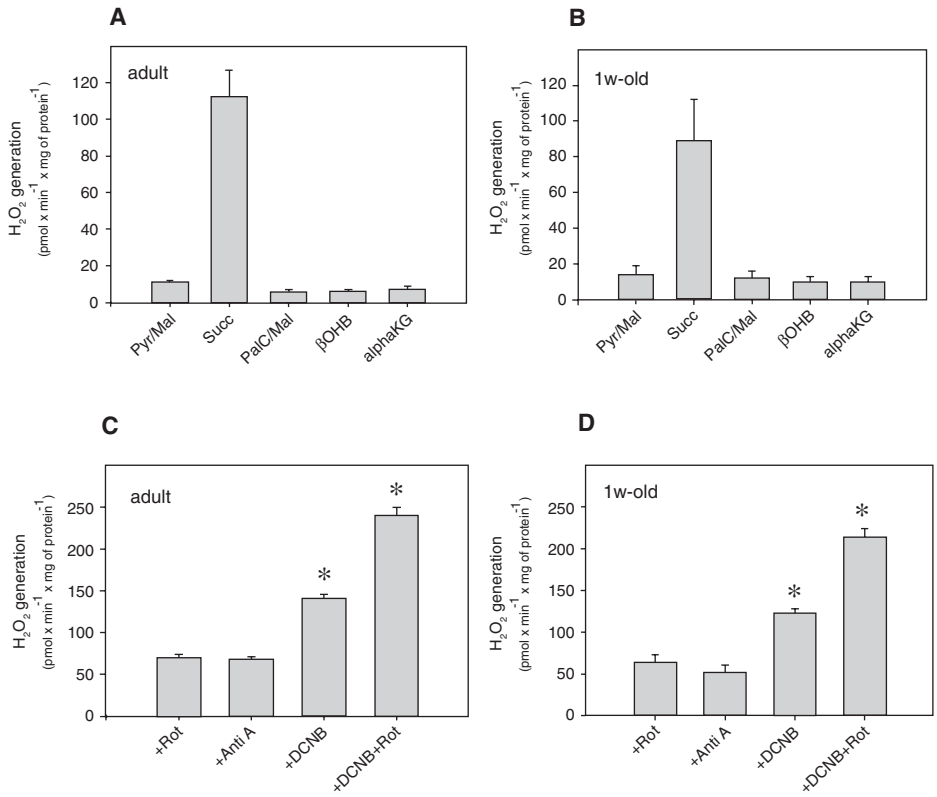

FIG. 7. ROS production by immature and mature rat brain mitochondria. Mitochondria prepared from brains of 1-week-old and adult rats were suspended in standard incubation medium (0.2 mg of protein per ml), which was supplemented with the indicated substrates (**A** and **B**). Concentrations of succinate (Succ), l-palmitoylcarnitine (PalC) plus malate, β-hydroxybutyrate (βOHB), or alpha-ketoglutarate (alphaKG) were 5 m*M*, 12.5 μ*M*/2.5 m*M*, 5 m*M,* and 5 m*M*, respectively. Superoxide was measured as described in Materials and Methods at 25°C. (**C** and **D**) mitochondria were fueled with pyruvate plus malate. Superoxide generation was stimulated by 1 μ*M* rotenone, 1 μ*M* antimycin A, or 60 μ*M* 1-chloro-2,4-dinitrobenzene (CDNB). Data are given as means ± S.D. obtained from 4 mitochondrial preparations. **p* < 0.05 vs. values in the presence of rotenone (+ Rot).

We examined whether a difference in the mitochondrial ROS generation of immature and mature brain mitochondria could partly explain the different abilities of these mitochondria to accumulate Ca^{2+} without undergoing the opening of PT. When mitochondria were incubated with saturating concentrations of various hydrogen-delivering substrates (resting state), the ROS generation by mitochondria isolated from brains of adult and 1-week-old mitochondria was comparable in size (Fig. 7A and B). In addition, in incubations with respiratory chain-inhibited mitochondria or at conditions of an oxidized glutathione pool, ROS generation by 1-week-old and adult brain mitochondria increased dramatically (Fig. 7C and D). These results reveal that the rate of ROS generation by mitochondria from 1-week-old brain is about 18% and 14% lower in glutathione-depleted and rotenone-inhibited mitochondria compared to adult brain mitochondria. However, such small differences in ROS production of immature glutathione-depleted and respiratory chain-inhibited mitochondria cannot explain the greatly different CRC values of 1-week-old and adult mitochondria, because mitochondria treated in such manner do not accumulate Ca^{2+}.

Moreover, ROS production by aged mitochondria isolated from the cortex and the hippocampus of 25-month-old rats was found to increase significantly when they were exposed to 150 μ*M* Ca^{2+} (corresponding to 150 nmol per mg of mitochondrial protein), whereas mitochondria from the cerebellum did not respond to Ca^{2+} (7). Based on this study, it was proposed that there could be region-specific and age-related alterations in mitochondrial responses to Ca^{2+}. In addition, it was found that micromolar concentrations of Ca^{2+} (>10 μ*M*) strongly stimulate the release of ROS in rotenone-treated mitochondria isolated from rat forebrain (40). This stimulation of ROS production may be specific for the rotenone-sensitive site of complex I, since with 1-methyl-4-phenylpyridinium (complex I inhibitor), no Ca^{2+}-associated ROS production was found. We also studied ROS generation under conditions of loading isolated mitochondria from the 1-week-old and adult brain with Ca^{2+}. These studies clearly showed that loading of the matrix with increasing amounts of Ca^{2+} is without effect on the basal ROS generation (shown in Fig. 8A, for 1-week-old mitochondria). However, when the opening of PTP was initiated by partial depolarization due to the protonophore FCCP or the thiol-cross-linker phenylarsine oxide, then ROS generation increased significantly (Fig. 8A, traces a and c).

In summary, our findings strongly suggest that the ROS generation capacity is not of crucial importance for the abil-

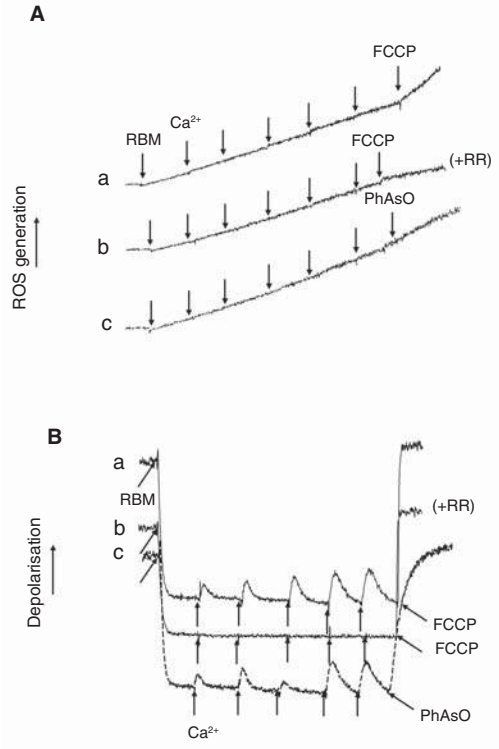

FIG. 8. Effect of incremental Ca^{2+} additions on ROS production and polarization of immature rat brain mitochondria. Release of superoxide (**A**) and polarization state of the inner membrane (**B**) of mitochondria prepared from 1-week-old rats at incremental additions of Ca^{2+} (25 nmol). Permeability transition was triggered by 1 μ*M* FCCP (trace a) or 6 μ*M* phenylarsine oxide (PhAsO; trace c). In a separate experiment (trace b), the medium was supplemented with 1 μ*M* ruthenium red (RR).

ity of immature mitochondria to buffer high levels of extramitochondrial Ca^{2+} without undergoing PT. The question arises what could be the explanation for the high CRC, and consequently, the resistance of immature mitochondria to Ca^{2+}-triggered opening of the PTP. The molecular identity of PTP is still under debate. However, it is widely believed that the voltage-dependent anion channel, ANT, and cyclophilin D are core-components of PTP. Furthermore, it is assumed that the core of PTP interacts with the mitochondrial creatine kinase (mtCK) (11), an enzyme located in the intermembrane and intercristae space that catalyzes the reversible transphosphorylation of phosphocreatine (PCr) to ATP. In addition, it has been demonstrated that the opening of PTP is inhibited due to substrates of mtCK (creatine and cyclocreatine) (12). This inhibition might be linked to direct effects of creatine on PTP (31). Because phosphate is not a substrate of mtCK, we do assume that a possible interaction of phosphate with the mtCK protein contributes to the increase in CRC in the presence of phosphate (Fig. 5C). Furthermore, we found that the enzymatic activity of mtCK is low in mitochondria prepared from the brain of newborn rats (Fig. 2B), an observation that is consistent with a low mtCK protein content reported before (39). Therefore, a low content of mtCK suggests that the assembly of PTP-constituting proteins is hampered in immature mitochondria. Taking all these facts together, we hypothesize that the resistance of immature mitochondria to Ca^{2+}-dependent opening of the PTP is associated with a low content of some components of PTP, like creatine kinase and ANT. Finally, the susceptibility of (hippocampal) neurons to excitotoxic cell death, which is related to membrane potential-driven uptake of Ca^{2+} into mitochondria (33), increases markedly during postnatal development (26). Our results suggest that the high Ca^{2+} buffer capacity of immature brain mitochondria contributes to the relatively low vulnerability of immature neurons against a NMDA-induced increase of cytosolic Ca^{2+} concentrations.

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ABBREVIATIONS

ANT, adenine nucleotide translocase; CaG5N, Calcium green-5N; mtCK, mitochondrial creatine kinase; CRC, calcium retention capacity; CS, citrate synthase; DCNB, 1-chloro-2,4-dinitrobenzene; FCCP, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone; $\Delta\Psi_{m,}$ membrane potential at the inner mitochondrial membrane; Nig, nigericin; PhAsO, phenylarsine oxide; PT, permeability transition; PTP, permeability transition pore; RBM, rat brain mitochondria; RCR, respiratory control ratio; ROS, reactive oxygen species.

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