

# Calcium-induced precipitate formation in brain mitochondria: composition, calcium capacity, and retention

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## Abstract

Both isolated brain mitochondria and mitochondria in intact neurons are capable of accumulating large amounts of calcium, which leads to formation in the matrix of calcium- and phosphorus-rich precipitates, the chemical composition of which is largely unknown. Here, we have used inhibitors of the mitochondrial permeability transition (MPT) to determine how the amount and rate of mitochondrial calcium uptake relate to mitochondrial morphology, precipitate composition, and precipitate retention. Using isolated rat brain (RBM) or liver mitochondria (RLM) Ca<sup>2+</sup>-loaded by continuous cation infusion, precipitate composition was measured *in situ* in parallel with Ca<sup>2+</sup> uptake and mitochondrial swelling. In RBM, the endogenous MPT inhibitors adenosine 5'-diphosphate

(ADP) and adenosine 5'-triphosphate (ATP) increased mitochondrial Ca<sup>2+</sup> loading capacity and facilitated formation of precipitates. In the presence of ADP, the Ca/P ratio approached 1.5, while ATP or reduced infusion rates decreased this ratio towards 1.0, indicating that precipitate chemical form varies with the conditions of loading. In both RBM and RLM, the presence of cyclosporine A in addition to ADP increased the Ca<sup>2+</sup> capacity and precipitate Ca/P ratio. Following MPT and/or depolarization, the release of accumulated Ca<sup>2+</sup> is rapid but incomplete; significant residual calcium in the form of precipitates is retained in damaged mitochondria for prolonged periods.

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Brain mitochondria both *in situ* and *in vitro* are capable of accumulating and retaining large amounts of Ca<sup>2+</sup> – up to 1.5 μmoles/mg mitochondrial protein – because of formation in the matrix of calcium- and phosphorus-rich precipitates (Lehninger *et al.* 1967; Nicholls 1978; for review see Nicholls and Chalmers 2004). While the precise composition of these precipitates remains uncertain, recent evidence (Chalmers and Nicholls 2003; Panov *et al.* 2004) indicates that they are composed primarily of tribasic calcium phosphate [Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>] and/or dibasic calcium phosphate (CaHPO<sub>4</sub>), as originally proposed nearly 40 years ago (Lehninger *et al.* 1967; Lehninger 1970). Precipitate formation plays an important role in mitochondrial Ca<sup>2+</sup> homeostasis. This process buffers matrix free Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>m</sub>) with remarkable efficiency, maintaining [Ca<sup>2+</sup>]<sub>m</sub> at an essentially constant level while the amount of Ca<sup>2+</sup> accumulated ranges over several orders of magnitude (Zoccarato and Nicholls 1982; Chalmers and Nicholls 2003). Nonetheless, the maximum amount of Ca<sup>2+</sup> that mitochondria can take up, i.e. the Ca<sup>2+</sup> loading capacity (CLC), is finite and typically

limited by opening of a large conductance pore, the mitochondrial permeability transition (MPT) pore, in the inner membrane (for review see Bernardi 1999; Crompton 1999). Mitochondrial CLC is increased in the presence of MPT inhibitors (Kristian *et al.* 2000, 2002; Brustovetsky *et al.* 2003; Chalmers and Nicholls 2003; Brown *et al.* 2006); so too, presumably, is the abundance of Ca-sequestering

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**Abbreviations used:** ADP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; BSA, bovine serum albumin; [Ca<sup>2+</sup>]<sub>e</sub>, extramitochondrial free calcium; CLC, Ca<sup>2+</sup> loading capacity; CsA, cyclosporine A; FCCP, carbonyl cyanide 4-(trifluoro-methoxy)phenylhydrazone; MPT, mitochondrial permeability transition; RBM, rat brain mitochondria; P<sub>i</sub>, inorganic phosphate; RLM, rat liver mitochondria.

precipitates. Whether this affects precipitate composition, however, is unknown, as are the relationships, if any, between composition and CLC.

The mitochondrial permeability transition is accompanied by mitochondrial depolarization, swelling, and a massive release of accumulated  $\text{Ca}^{2+}$  (Bernardi 1999). Given the dissipation of the mitochondrial membrane potential and the acidic pH shift after pore opening, it is generally considered that essentially all accumulated  $\text{Ca}^{2+}$  should be released shortly after MPT. There is, however, evidence to the contrary. For example, addition of the pore-forming antibiotic alamethicin releases a substantial amount of residual  $\text{Ca}^{2+}$  that is retained in isolated mitochondria after MPT (Kushnareva *et al.* 2005). Furthermore, high-Ca precipitates were found in swollen isolated mitochondria  $\text{Ca}^{2+}$ -overloaded in the presence of adenosine 5'-triphosphate (ATP) (Kristian *et al.* 2002), in swollen mitochondria of cultured hippocampal neurons exposed to toxic levels of NMDA (Pivovarova *et al.* 2004), and even *in vivo* in swollen, structurally damaged mitochondria of ischemic,  $\text{Ca}^{2+}$ -overloaded neuronal cells (Solenski *et al.* 2002).

Here, we have employed a continuous infusion technique to load isolated brain and liver mitochondria with  $\text{Ca}^{2+}$  while spectroscopically monitoring the medium free  $\text{Ca}^{2+}$  concentration. At various  $\text{Ca}^{2+}$  loading rates and in the presence of various MPT inhibitors that modify mitochondrial CLC, samples were rapidly frozen for analytical and structural electron microscopy. This analysis allowed us to demonstrate, in both brain and liver mitochondria, that changing the conditions of precipitate formation affects the composition of mitochondrial precipitates. After  $\text{Ca}^{2+}$  loading, neither MPT induction nor depolarization of mitochondria led to complete release of accumulated  $\text{Ca}^{2+}$ , suggesting that  $\text{Ca}^{2+}$  mobilization from precipitates, even from damaged mitochondria, is a slow process. As it is generally agreed that mitochondrial  $\text{Ca}^{2+}$  uptake and release significantly modifies cellular responses to changing cytosolic  $\text{Ca}^{2+}$  levels, it seems likely that prolonged release of precipitate  $\text{Ca}^{2+}$  will influence  $\text{Ca}^{2+}$  signaling during post-stimulus recovery periods.

## Materials and methods

### Reagents

Mannitol, sucrose, EGTA, HEPES, Tris, dibasic potassium phosphate ( $\text{K}_2\text{HPO}_4$ ), magnesium chloride ( $\text{MgCl}_2$ ), malate, glutamate, oligomycin, rotenone, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), adenosine 5'-diphosphate (ADP), ATP, hexokinase, glucose, fatty acid free bovine serum albumin (BSA), glutaraldehyde, and sodium cacodylate trihydrate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Potassium chloride (KCl) suprapure, and sodium chloride (NaCl) suprapure were purchased from EM Sciences (Fort Washington, PA, USA).

### Isolation of mitochondria

Non-synaptic brain mitochondria were isolated using a Percoll gradient centrifugation as described previously (Kristian *et al.* 2002, 2006). Brains of male Sprague-Dawley rats (300–350 g of weight) were homogenized in isolation medium (225 mmol/L mannitol, 75 mmol/L sucrose, 5 mmol/L HEPES, 1 mmol/L EGTA, pH 7.4 at 4°C). The homogenate was centrifuged at 1300 g for 3 min, and the pellet was re-suspended and centrifuged again at 1300 g for 3 min. The pooled supernatants were centrifuged at 21 200 g for 10 min, the crude mitochondrial pellet was re-suspended in 15% Percoll, and layered on a pre-formed gradient of 40% and 24% Percoll. After centrifugation at 31 700 g for 8 min the mitochondria were collected from the interface of the lower two layers, diluted with isolation medium and centrifuged at 16 700 g for 10 min. The purified non-synaptic mitochondrial pellet was diluted in isolation medium and BSA (10 mg/mL) was added to bind free fatty acids. Following centrifugation at 6800 g for 10 min the pelleted mitochondria were re-suspended in 50  $\mu\text{L}$  of isolation medium without EGTA. Brain mitochondria isolated by this method exhibit high rates of respiration and excellent respiratory control (Kristian *et al.* 2006).

Rat liver mitochondria (RLM) were isolated by differential centrifugation methods according to Lapidus and Sokolove (1993). Protein concentration of mitochondrial samples was determined using a Lowry DC kit (Bio-Rad, Hercules, CA, USA). BSA was used as the protein concentration standard.

### Mitochondrial calcium uptake and light scattering

Calcium uptake by mitochondria was monitored using the pentapotassium salt of the ratiometric calcium-sensitive dye Fura 6F (Molecular Probes, Eugene, OR, USA). Mitochondria (0.25 mg/mL) were re-suspended in a medium containing 100 mmol/L NaCl, 25 mmol/L KCl, 2 mmol/L  $\text{K}_2\text{HPO}_4$ , 1 mmol/L  $\text{MgCl}_2$ , 1  $\mu\text{mol/L}$  EGTA, 20 mmol/L HEPES-Tris, pH 7.0 at 37°C, which resembles the ion composition of cytosol under excitotoxic conditions (Kiedrowski *et al.* 1994; Pivovarova *et al.* 2004). A conventional KCl-based medium (125 mmol/L KCl, 2 mmol/L  $\text{K}_2\text{HPO}_4$ , 1 mmol/L  $\text{MgCl}_2$ , 1  $\mu\text{mol/L}$  EGTA, 20 mmol/L HEPES, pH 7.0) was used in phosphate removal and depletion experiments. Mitochondria were energized with the complex-I linked substrates malate and glutamate. Experiments were performed in a Hitachi 2500 (Hitachi, Tokyo, Japan) fluorescence spectrometer, with excitation wavelengths of 340/380 nm and an emission wavelength of 510 nm. Using the slow cation infusion technique of Chalmers and Nicholls (2003),  $\text{Ca}^{2+}$  was added to the cuvette by means of a CMA/102 microdialysis pump (CMA/microdialysis, North Chelmsford, MA, USA). Mitochondrial light scattering was measured at 540 nm using the Hitachi 2500 spectrofluorimeter.

### Depletion of endogenous mitochondrial phosphate

To deplete endogenous phosphate, mitochondria were incubated for 5 min in the KCl-based medium described above; the medium also contained 5 mmol/L malate, 5 mmol/L glutamate, 1 unit/mL of hexokinase, 1 mmol/L glucose and 3 mmol/L ATP (Zoccarato and Nicholls 1982). Prior to calcium infusion, oligomycin (1  $\mu\text{g/mL}$ ) was added to inhibit inorganic phosphate ( $\text{P}_i$ ) generation by reverse operation of the ATP synthase.

### Electron microscopy and electron probe X-ray microanalysis

For conventional electron microscopy, mitochondrial pellets were fixed overnight with 4% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.4) at 4°C, post-fixed with 1% osmic acid in 0.1 mol/L cacodylate buffer, dehydrated, and embedded in LX-112 epoxy resin. Alternatively, pellets were high-pressure frozen (Moor 1987; Studer *et al.* 2001) by means of a Baltec HM10 high-pressure freezing machine (Boeckeler Instruments, Tucson, AZ, USA) and freeze-substituted using an AFS freeze-substitution device (Leica, Bannockburn, IL, USA). Thin sections were viewed in a JEM-1200 transmission electron microscope (JEOL, Peabody, MA, USA) equipped with an XR-100 CCD camera (AMT, Danvers, MA, USA).

For electron probe X-ray microanalysis (EPMA), mitochondrial pellets were rapidly frozen, processed, and analyzed as previously described for cell suspensions (Yagodin *et al.* 1999). Briefly, pellets were plunge-frozen in liquid ethane and cryosections prepared at  $-160^{\circ}\text{C}$  by means of a Leica Ultracut S/FCS ultracryomicrotome. Frozen-hydrated sections were mounted on carbon/Formvar-coated grids and cryotransferred into an EM912 Omega microscope (Carl Zeiss SMT, Thornwood, NY, USA), where they were freeze-dried prior to imaging and analysis at  $-170^{\circ}\text{C}$ . X-ray spectra were recorded with electron probes focused on mitochondrial precipitates or matrix and processed by established procedures (Buchanan *et al.* 1993; Pivovarova *et al.* 1999). EPMA-derived concentrations are given in mmol/kg dry weight.

### Statistical analysis

Statistical differences ( $p < 0.05$ ) among experimental groups were determined using one-way ANOVA followed by *post hoc* Bonferroni

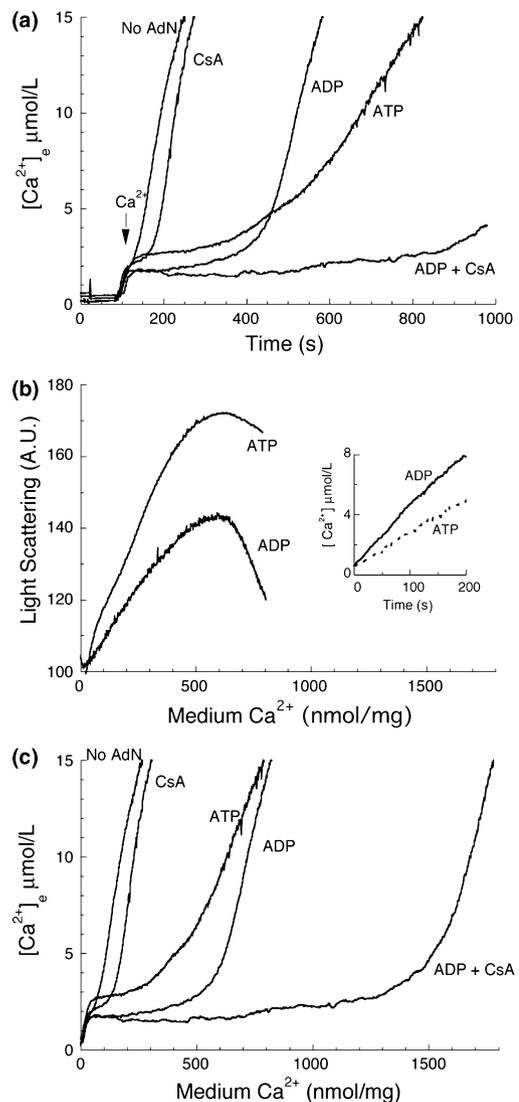
**Fig. 1** Mitochondrial permeability transition (MPT) inhibitors increase calcium-loading capacity in brain mitochondria. Rat brain mitochondria in a suspension were infused with a buffered  $\text{CaCl}_2$  solution at 100 nmol  $\text{Ca}^{2+}$ /mg/min without MPT inhibitors or in the presence of 1  $\mu\text{mol/L}$  cyclosporine A (CsA), 0.2 mmol/L adenosine 5'-diphosphate (ADP) plus 1  $\mu\text{g/mL}$  oligomycin, ADP/oligomycin plus 1  $\mu\text{mol/L}$  CsA, or 3 mmol/L adenosine 5'-triphosphate (ATP). Fura-6F (250 nmol/L) was present, as appropriate. (a) Traces of medium free  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_e$ ) as a function of time. Infusion begins at the arrow. The time to onset of MPT – taken as the sharp upward inflection of  $[\text{Ca}^{2+}]_e$  – reveals a substantial protective effect for the endogenous MPT inhibitors ADP and ATP. CsA, which is ineffective when added alone, greatly amplifies the protective effect of ADP. (b) *Inset*: In the absence of mitochondria, the actual rate of  $[\text{Ca}^{2+}]_e$  increase in the presence of ATP is  $\sim 70\%$  of that for ADP because ATP has a significantly higher  $\text{Ca}^{2+}$  binding affinity. *Main panel*: Traces of light scattering intensities plotted as a function of the amount of available free  $\text{Ca}^{2+}$  by correcting for the effective slower infusion rate in the presence of ATP. Increased light scattering during  $\text{Ca}^{2+}$  infusion reflects mitochondrial  $\text{Ca}^{2+}$  uptake and precipitate formation. Maxima in these traces correlate well with the inflection points in panel (c) and coincide with MPT. (c) Traces of  $[\text{Ca}^{2+}]_e$  as a function the amount of added free  $\text{Ca}^{2+}$ .  $\text{Ca}^{2+}$  loading capacities, estimated as the cumulative amount of infused  $\text{Ca}^{2+}$  at the inflection point, are  $\sim 400$  nmol/mg and  $\sim 600$  nmol/mg in the presence of ATP and ADP, respectively. CsA strongly amplifies the protective effect of ADP, increasing the CLC to  $\sim 1500$  nmol/mg.

multiple comparisons test for unbalanced data. Data are given as mean  $\pm$  SEM. Statistical analyses were carried out using InStat software (GraphPad, San Diego, CA, USA).

## Results

### Effect of adenine nucleotides and CsA on mitochondrial calcium uptake, light scattering, and ultrastructure

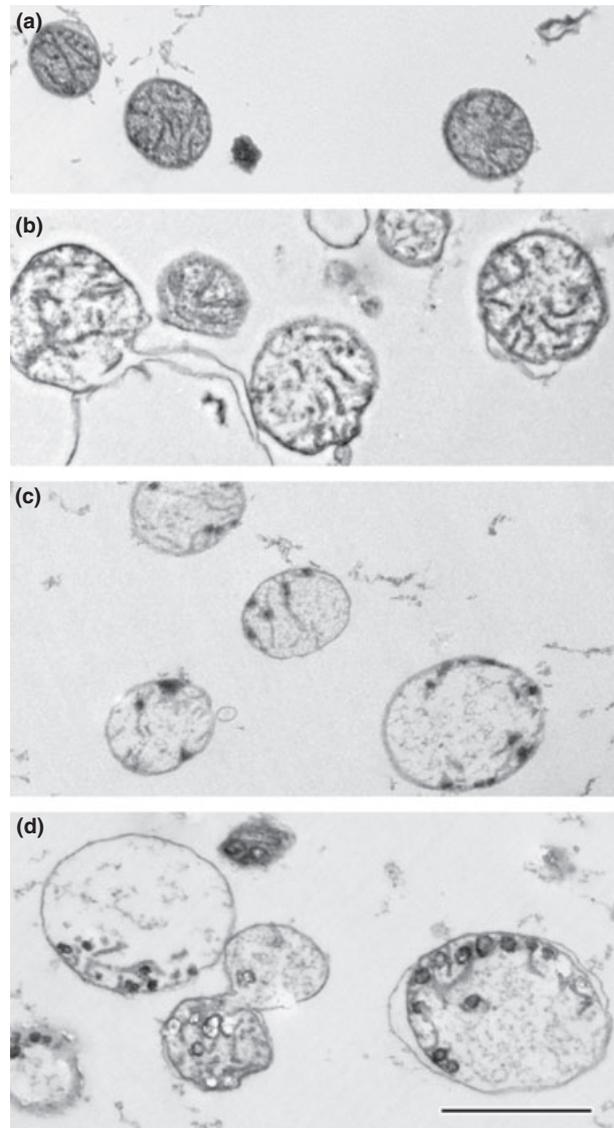
A continuous cation infusion technique (Chalmers and Nicholls 2003) was used to load isolated rat brain mitochondria (RBM) in suspension with calcium. Almost immediately after initiating  $\text{Ca}^{2+}$  infusion, the extramitochondrial free calcium concentration ( $[\text{Ca}^{2+}]_e$ ) increases enough to activate mitochondrial  $\text{Ca}^{2+}$  uptake (Fig. 1a, arrow). From this point on, and unless MPT occurs immediately, the rate of mitochondrial  $\text{Ca}^{2+}$  accumulation matches the infusion rate and in the presence of MPT inhibitors  $[\text{Ca}^{2+}]_e$  remains constant for a period of time determined by the CLC of



mitochondria. When  $\text{Ca}^{2+}$  uptake becomes compromised because of mitochondrial dysfunction, a second, much larger increase in  $[\text{Ca}^{2+}]_e$  is observed as infused  $\text{Ca}^{2+}$  remains in the medium (Fig. 1a). While in suspensions without adenine nucleotides the second increase follows almost immediately upon the first, MPT inhibitors as previously demonstrated (Kristian *et al.* 2000, 2002; Brustovetsky *et al.* 2003; Chalmers and Nicholls 2003), significantly delay the failure of mitochondrial  $\text{Ca}^{2+}$  uptake; that is, they increase mitochondrial CLC. The endogenous inhibitors ADP and ATP, at typical intracellular concentrations of 0.2 and 3 mmol/L, respectively, are much more effective than the pharmacological inhibitor cyclosporine A (CsA) (Fig. 1a).

As reported previously (Kristian *et al.* 2002; Chalmers and Nicholls 2003), an increase in light scattering is observed as mitochondria accumulate  $\text{Ca}^{2+}$  in the presence of ATP or ADP. This increase, which is thought to represent enhanced scattering by refractile, Ca-rich precipitates that form in the matrices of functional, intact mitochondria is followed by a decline as an increasing number of mitochondria undergo MPT and swell (Fig. 1b). The light scattering traces in Fig. 1b are plotted as a function of the amount of available, as opposed to total, free  $\text{Ca}^{2+}$  added to the suspension. This conversion takes into account the relatively stronger  $\text{Ca}^{2+}$  buffering strength of ATP solutions. The need for a correction is illustrated by the  $\sim 30\%$  reduction, relative to a 0.2 mmol/L ADP solution, in the amount of  $\text{Ca}^{2+}$  available when  $\text{Ca}^{2+}$  is infused into a mitochondria-free solution containing 3 mmol/L ATP (Fig. 1b, inset). When  $[\text{Ca}^{2+}]_e$  traces are also plotted as a function of available added  $\text{Ca}^{2+}$  (Fig. 1c), the upstroke in the  $[\text{Ca}^{2+}]_e$  records well matches the maxima in the light scattering traces, allowing one to pinpoint with confidence the onset of mitochondrial dysfunction (compare Figs 1b and c). The data show that the presence of the endogenous MPT inhibitors, ATP and ADP, increases mitochondrial CLC by about 400 and 600 nmol/mg mitochondrial protein, respectively (Fig. 1c). CsA alone is minimally effective, but increases the CLC by  $\sim 1500$  nmol/mg when present together with ADP.

To examine the structural effects of inhibitor-enhanced mitochondrial Ca accumulation at comparable times near the onset of deregulation, mitochondrial suspensions were rapidly frozen just after the upstroke in  $[\text{Ca}^{2+}]_e$  traces. High-pressure techniques (Moor 1987; Studer *et al.* 2001) followed by freeze-substitution were used to avoid structural distortions inherent in chemical fixation protocols, and to preserve any electron dense precipitates that might form (see Materials and methods for details). In fact, electron-dense precipitates were detected only when MPT inhibitors were present (Fig. 2). Mitochondria that accumulated Ca in the absence of MPT inhibitors were markedly swollen, with damaged cristae, but precipitates were rarely seen. The presence of ATP or ADP in the medium resulted in precipitate formation in almost all mitochondria, including



**Fig. 2** Mitochondrial calcium accumulation leads to precipitate formation. Digital transmission electron micrographs of high-pressure frozen rat brain mitochondria suspensions, freeze-substituted by means of a protocol that preserves calcium phosphate precipitates. (a) Control mitochondria are structurally normal. They are spherical and unswollen, with a homogeneous dense matrix and normal cristae; membranes are smooth and unbroken. (b) Mitochondria frozen after  $\text{Ca}^{2+}$  infusion for 200 s without mitochondrial permeability transition (MPT) inhibitors are swollen and damaged, but precipitates are rare. (c) In mitochondria frozen after 400 s  $\text{Ca}^{2+}$  infusion in the presence of 0.2 mmol/L adenosine 5'-diphosphate (ADP) with 1  $\mu\text{g}/\text{mL}$  oligomycin, multiple precipitate foci are present in almost all mitochondria, which are also generally swollen. (d) Mitochondria frozen after 400 s  $\text{Ca}^{2+}$  infusion in the presence of 5 mmol/L adenosine 5'-triphosphate appear generally similar to those infused in an ADP medium. In all cases, mitochondria were frozen at times just after the presumed onset of MPT, as indicated by an increase in  $[\text{Ca}^{2+}]_e$  (see Fig. 1). Bar (all panels), 1  $\mu\text{m}$ .

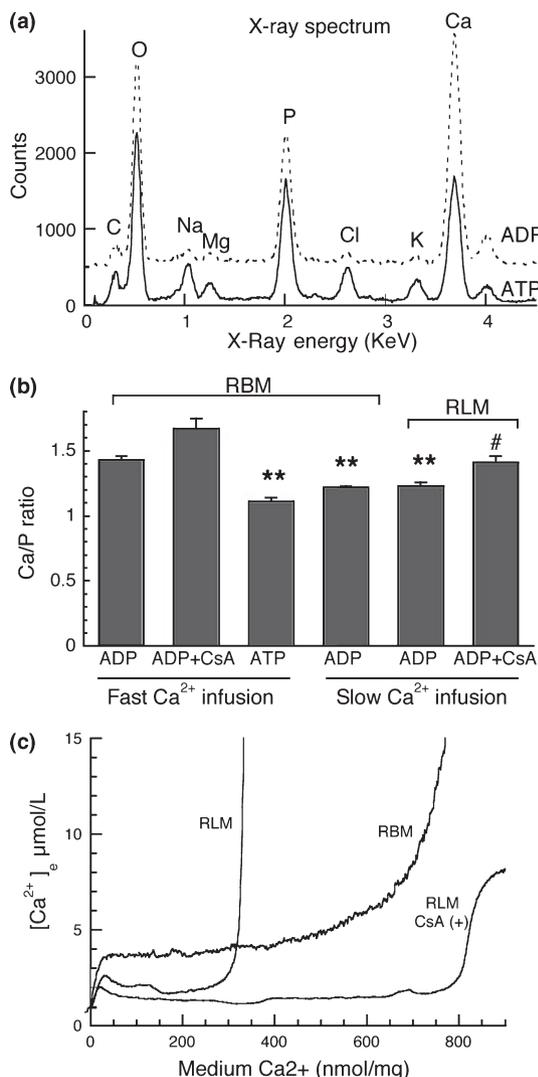
the subpopulation that was swollen and damaged (Fig. 2, lower panels).

### Composition of intramitochondrial precipitates depends on the calcium accumulation rate

Electron probe microanalysis was used to determine the elemental composition of mitochondria in parallel preparations rapidly frozen at comparable time points. Elemental analysis indicates that, as expected, the major elements present in precipitates are carbon, oxygen, phosphorus, and calcium, with a significant concentration of magnesium (Fig. 3a, Table 1). More interestingly, quantification of EPMA spectra shows that the calcium-to-phosphorus (Ca/P) ratio depends on the conditions under which mitochondria accumulate Ca. For example, at relatively high infusion rates (100 nmol Ca<sup>2+</sup>/min/mg protein) in the presence of ADP – conditions under which the extended period of active Ca<sup>2+</sup> accumulation corresponds to a high

CLC (Fig. 1c) – the Ca/P ratio is  $1.43 \pm 0.03$  (Fig. 3b and Table 1). Ratios approaching 1.5 suggest the predominance of tribasic calcium phosphate, Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>. Conditions that increase the CLC even further, e.g. ADP plus CsA at the same infusion rate (Fig. 1c), further increased the Ca/P ratio (Fig. 3b and Table 1). In contrast, mitochondrial Ca<sup>2+</sup> uptake in a medium containing ATP gave precipitates with a Ca/P ratio close to 1.0 ( $1.11 \pm 0.03$ ; Fig. 3b and Table 1), which suggests the predominance of dibasic calcium phosphate, CaHPO<sub>4</sub>. Since, as shown above, the ATP-containing medium lowers free Ca<sup>2+</sup> available for mitochondrial uptake, effectively reducing the infusion rate to ca. 70 nmol Ca<sup>2+</sup>/min/mg protein, Ca<sup>2+</sup> uptake experiments in the ADP-containing medium were repeated at this slower infusion rate. The precipitate Ca/P ratio under these conditions ( $1.22 \pm 0.01$ ; Fig. 3b and Table 1) was similar to that found with the ATP medium.

Rat liver mitochondria are capable of accumulating significant amounts of Ca<sup>2+</sup> during slow Ca<sup>2+</sup> infusion in the presence of ADP, although the tolerated load is much less than for RBM (Fig. 3c) (RLM deregulate immediately at faster infusion rates). Nonetheless, RLM also exhibit a correlation between precipitate composition and CLC. RLM precipitates formed in the presence of only ADP have a low Ca/P ratio ( $1.23 \pm 0.03$ ) that rises towards 1.5 ( $1.41 \pm 0.05$ )



**Fig. 3** Calcium capacity and accumulation rates influence precipitate composition. (a) Representative energy-dispersive X-ray spectra recorded from mitochondrial precipitates formed in the presence of adenosine 5'-triphosphate (ATP) (solid line) or adenosine 5'-diphosphate (ADP) (dashed line). Elements corresponding to the major characteristic K-line X-ray peaks are identified. Calcium and phosphorus are the major inorganic elements in precipitates, but the molar Ca/P ratio (approximately reflected by the ratio of areas under their characteristic X-ray peaks) is higher in the presence of ADP than with ATP. For clarity, the x-axis of the ADP spectrum is offset by +500 counts. (b) In rat brain mitochondria (RBM), stronger mitochondrial permeability transition (MPT) inhibition, e.g. ADP or ADP + cyclosporine A (CsA) and faster Ca<sup>2+</sup> infusion favors relatively high (~1.5) Ca/P precipitate ratios, while slower infusion, e.g. ATP or ADP at 70 nmol/mg/min, leads to formation of precipitates with lower (~1.2) ratios. See also Table 1. Rat liver mitochondria (RLM) fail immediately at standard infusion rates (not shown), but at slow infusion rates in the presence of ADP they form precipitates that have relatively low Ca/P ratios. Ratios are higher if MPT is strongly inhibited by CsA. Symbols indicate a significant decrease relative to fast Ca<sup>2+</sup> infusion of an RBM suspension in the presence of ADP (*leftmost bar*; \*\**p* < 0.001) or an increase in the presence of CsA and ADP, relative to ADP only, for RLM (#*p* < 0.05). (c) Traces of extramitochondrial free calcium ([Ca<sup>2+</sup>]<sub>e</sub>) as a function of the amount of free Ca<sup>2+</sup> added at 50 nmol/mg/min in the presence of ADP show that the Ca<sup>2+</sup> capacity of RLM is much less than RBM under comparable conditions. Similar to RBM, however, the higher Ca<sup>2+</sup> capacity when CsA is added correlates with a higher Ca/P ratio (panel b).

**Table 1** Effect of mitochondrial permeability transition inhibitors on Ca, P, and Mg concentrations and Ca/P ratio in precipitates of isolated mitochondria following calcium infusion

	Infusion time (s)	n	Ca	P	Mg	Ca/P ratio <sup>a</sup>	CLC <sup>b</sup> (nmol/mg)
			(mmol/kg dry weight)				
RBM matrix							
Control, no Ca <sup>2+</sup> infusion	–	15	5 ± 1	282 ± 30	18 ± 3	–	–
Control, fast Ca <sup>2+</sup>	400	21	41 ± 12	246 ± 22	16 ± 2	–	<50
RBM precipitates							
ADP, fast Ca <sup>2+</sup>	400	76	1658 ± 46	1160 ± 24	79 ± 4	1.43 ± 0.03	600
ADP, slow Ca <sup>2+</sup>	700	48	1577 ± 39	1303 ± 33*	79 ± 3	1.22 ± 0.01**	600
ATP, fast Ca <sup>2+</sup>	500	80	1230 ± 49*	1111 ± 32	202 ± 12**	1.11 ± 0.03**	400
ADP + CsA, fast Ca <sup>2+</sup>	700	23	1624 ± 74	1003 ± 55*	75 ± 3	1.67 ± 0.08*	1500
RLM precipitates							
ADP, very slow Ca <sup>2+</sup>	400	25	1528 ± 77	1242 ± 54	135 ± 7**	1.23 ± 0.03**	300
ADP + CsA, very slow Ca <sup>2+</sup>	800	37	1506 ± 47	1098 ± 45 <sup>#</sup>	71 ± 3	1.41 ± 0.05 <sup>#</sup>	800

Electron probe X-ray microanalysis data from at least two experiments under each condition are given as mean ± SEM, where column 'n' indicates the total number of mitochondria analyzed. Mitochondria suspended in a high-Na<sup>+</sup> medium (see Materials and methods) were infused with a buffered Ca<sup>2+</sup> solution at a rate of 100 (fast), 70 (slow), or 50 (very slow) nmol/min/mg and were frozen immediately after the upward inflection in [Ca<sup>2+</sup>]<sub>e</sub>. Symbols indicate significant differences relative to fast Ca<sup>2+</sup> infusion in the presence of ADP in RBM (\*p < 0.05; \*\*p < 0.001) or to very slow infusion in RLM (<sup>#</sup>p < 0.05). ANOVA with *post hoc* Bonferroni multiple comparisons test for unbalanced data.

<sup>a</sup>Mean ratios were calculated from the ratios of individual paired measurements.

<sup>b</sup>Ca<sup>2+</sup> loading capacity is approximated as the cumulative amount of Ca<sup>2+</sup> infused at inflection points in corresponding Ca<sup>2+</sup> traces (Figs 1c and 3c).

ADP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; [Ca<sup>2+</sup>]<sub>e</sub>, extramitochondrial free calcium; CsA, cyclosporine A; RBM, rat brain mitochondria; RLM, rat liver mitochondria.

(Fig. 3b and Table 1) when CsA, which greatly increases the CLC of RLM (Fig. 3c), was present together with ADP.

### Production of phosphate by ATP hydrolysis is sufficient to support precipitate formation

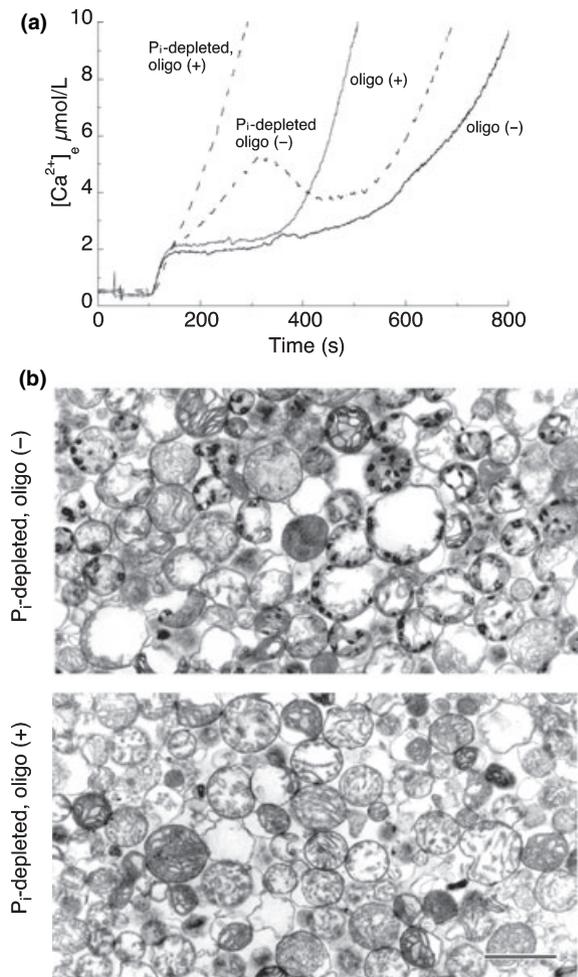
An important and outstanding question concerns the effect of phosphate availability on precipitate formation. To reduce available phosphate, mitochondria were exposed to Ca<sup>2+</sup> in a medium containing ATP but with no added phosphate. Plots of medium Ca<sup>2+</sup> concentration versus time were similar to those obtained when the medium did contain added phosphate, indicating that removal of exogenous phosphate did not appreciably affect the CLC (compare Fig. 4a, rightmost solid trace, oligo (–) to Fig. 1a). In a parallel infusion experiment carried out in the presence of oligomycin, which was added to inhibit ATP hydrolysis and eliminate P<sub>i</sub> production, the CLC was halved [Fig. 4a, middle solid trace, oligo (+)]. This indicates that P<sub>i</sub> arising from ATP hydrolysis by reversal of the ATP synthase was a significant source of counterions for Ca<sup>2+</sup> buffering.

To completely remove available phosphate from both incubation media and mitochondria we used a method described by Zoccarato and Nicholls (1982). Mitochondria were incubated in the presence of substrate, ATP, hexokinase, and glucose. During a 5-min incubation, hexokinase utilizes ATP to produce glucose-6-phosphate. The ADP generated in

this reaction is converted to ATP by oxidative phosphorylation, thereby consuming endogenous P<sub>i</sub>. Following the 5-min incubation, oligomycin is added to stop ATP production, as well as to inhibit ATP hydrolysis. Calcium infusion after this P<sub>i</sub> depletion step resulted in a rapid, continuous increase in [Ca<sup>2+</sup>]<sub>e</sub> (Fig. 4a, leftmost dashed trace) and no precipitate formation (Fig. 4b, lower panel). If oligomycin addition was omitted before Ca<sup>2+</sup> infusion, however, mitochondria Ca<sup>2+</sup> uptake persisted for ~500 s (Fig 5a, middle dashed trace) while precipitates formed in the matrix (Fig. 4b, upper panel). The results establish that precipitate formation can be supported by ATP hydrolysis and endogenous mitochondrial P<sub>i</sub> in the absence of added P<sub>i</sub>.

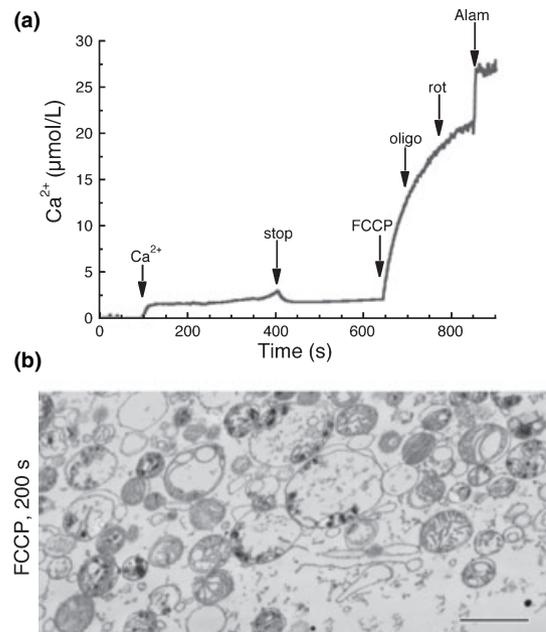
### Precipitates are retained in dysfunctional mitochondria for a prolonged time period

The release of accumulated mitochondrial Ca<sup>2+</sup> after stimulus-induced Ca<sup>2+</sup> uptake has important regulatory effects on Ca<sup>2+</sup> signaling (reviewed by Rizzuto and Pozzan 2006). Beyond Ca<sup>2+</sup> release driven by activity of the classical Na<sup>+</sup>/Ca<sup>2+</sup> exchanger, release is also triggered by depolarization (Brocard *et al.* 2001), and facilitated by an acidic shift in intramitochondrial pH, which promotes solubilization of generally insoluble precipitate components such as Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> (Chalmers and Nicholls 2003). To determine the extent of Ca<sup>2+</sup> release from maximally loaded mitochondria,



**Fig. 4** Precipitate formation is partially supported by adenosine 5'-triphosphate (ATP) hydrolysis. (a) Records of extramitochondrial free calcium ( $[Ca^{2+}]_e$ ) versus time for rat brain mitochondria (RBM) suspended in a  $K^+$ -based buffer containing ATP but no added phosphate reveal that the  $Ca^{2+}$  loading capacity (CLC) of RBM is reduced from  $\sim 400$  to  $\sim 250$  nmol  $Ca^{2+}$ /mg protein in the presence of oligomycin (compare solid curves,  $\pm$  oligomycin). This indicates that inorganic phosphate ( $P_i$ ) derived from ATP hydrolysis supports  $Ca^{2+}$  sequestration. Further reduction of phosphate availability by depleting endogenous  $P_i$  (cf. text for rationale and Materials and methods for procedure) further reduces RBM  $Ca^{2+}$  capacity (compare dotted curves). The effects are additive, so that  $Ca^{2+}$  uptake and sequestration are essentially eliminated when both  $P_i$  sources are removed (leftmost curve). (b) Electron micrographs confirm that precipitates are evident when ATP hydrolysis alone supplies phosphate ( $P_i$ -depleted, oligo (-); top panel), but are not formed in the complete absence of phosphate ( $P_i$ -depleted, oligo (+); bottom panel). Electron micrographs are from conventional preparations. Bar, 1  $\mu$ m.

$Ca^{2+}$  infusion in ATP-containing medium was stopped at the time when the uptake rate began to decline, i.e. when  $[Ca^{2+}]_e$  began to rise (Fig. 5a).  $[Ca^{2+}]_e$  remained stable for at least 300 s, indicating minimal net  $Ca^{2+}$  unloading during this



**Fig. 5** Only part of the mitochondrial calcium load is rapidly released upon pore opening and depolarization. (a) Trace of extramitochondrial free calcium ( $[Ca^{2+}]_e$ ) versus time for rat brain mitochondria (RBM)  $Ca^{2+}$ -loaded in the presence of adenosine 5'-triphosphate (ATP). Infusion was terminated at the onset of mitochondrial permeability transition (MPT) (*stop*). Depolarization with 1  $\mu$ mol/L carbonyl cyanide 4-(trifluoro-methoxy)phenylhydrazone (FCCP), added  $\sim 250$  s later, elicited a large but incomplete burst of  $Ca^{2+}$  release, which was unaffected by the addition of 5  $\mu$ g/mL oligomycin or 2  $\mu$ mol/L rotenone at the points indicated. Subsequent application of alamethicin, 40  $\mu$ g/mg protein, released a significant amount of residual  $Ca^{2+}$ . (b) Digital transmission electron micrograph of high-pressure frozen, freeze-substituted RBM incubated as in panel (a) and frozen 200 s after application of FCCP (no other drugs) confirms that residual precipitates are retained in both swollen and non-swollen mitochondria. Bar, 1  $\mu$ m.

period when  $Ca^{2+}$  released from dysfunctional mitochondria is accumulated by still functional neighbors. Subsequent depolarization induced a rapid, massive efflux of calcium (Fig. 5a), which was independent of the type of nucleotide, ATP or ADP, present in the medium (not shown). However, even several minutes of FCCP treatment did not liberate all the accumulated Ca, as indicated by electron micrographs showing abundant precipitates in both swollen and unswollen mitochondria after FCCP (Fig. 5b). As a further indication of the size of the residual, FCCP-insensitive mitochondrial  $Ca^{2+}$  pool, subsequent addition of the non-selective pore-forming compound alamethicin induced an additional large bolus of  $Ca^{2+}$  release (Fig. 5a). Electron microscopy revealed that mitochondria did not contain any precipitates after alamethicin treatment (not shown). The basis for incomplete release by FCCP was not because of a residual mitochondrial membrane potential, as neither rote-

none nor oligomycin affected FCCP-induced calcium efflux (Fig. 5a). Considering that extant driving forces would seem to favor complete release of  $\text{Ca}^{2+}$  from depolarized and/or permeabilized mitochondria, it is perhaps surprising that a significant fraction of accumulated  $\text{Ca}^{2+}$  is retained inside dysfunctional mitochondria for extended periods of time. It will be interesting to establish the factor(s) determining the delayed release of this sequestered  $\text{Ca}^{2+}$  pool.

## Discussion

In both isolated and *in situ* brain mitochondria, increasing the extramitochondrial  $\text{Ca}^{2+}$  concentration above the 'set point' leads to robust  $\text{Ca}^{2+}$  accumulation and the formation of electron dense precipitates within the mitochondrial matrix (Lehninger 1970; Nicholls 1978). Elemental analysis of brain mitochondria, both isolated and *in situ*, has shown that these precipitates contain remarkably high levels of Ca and P (Kristian *et al.* 2002; Pivovarova *et al.* 2004). These earlier analyses of mitochondrial precipitates prompted this study, which aimed to more fully characterize the chemical composition and stability of precipitates, as well as to explore the factors that affect composition and the impact of any such changes on mitochondrial CLC and resistance to Ca overload-induced injury.

An important aspect in this study is the use of cryotechniques to preserve the composition and structure of mitochondrial precipitates. Structural electron microscopy showed directly that the presence of MPT inhibitors, either endogenous (ADP or ATP) or pharmacological (CsA), was required for the formation of precipitates. Further, precipitates formation was obligatorily coupled to the inhibitor-dependent increase in mitochondrial CLC. The morphology of the precipitates in high-pressure frozen material – more-or-less round with smooth contours and soft edges – strongly suggests that the precipitates are non-crystalline.

This work was informed by recent biochemical studies that deduced, by measuring the ratio of protons extruded to  $\text{Ca}^{2+}$  ions taken up in the presence of excess  $\text{P}_i$  (Lehninger *et al.* 1967), that  $\text{Ca}^{2+}$  accumulated by RBM is sequestered as a mixture of the salts  $\text{CaHPO}_4$  and  $\text{Ca}_3(\text{PO}_4)_2$ , apparently in variable proportions (Chalmers and Nicholls 2003; Panov *et al.* 2004). These findings are generally reminiscent of earlier chemical analyses of precipitates prepared or isolated in the context of biomineralization; crystallographic and morphological data from that field show that formed precipitates were structurally amorphous and compositionally heterogeneous (for review see Eanes 2001). Consistent with these reports, it appears that mitochondrial precipitates are similarly complex. In the present experiments, we find Ca/P ratios ranging from 1.0 to 1.65. This range covers all likely stoichiometric compounds of Ca and P, such as  $\text{CaHPO}_4$  (Ca/P ratio 1.0), octacalcium phosphate (1.33),  $\text{Ca}_3(\text{PO}_4)_2$  (1.5), and apatite (1.67), as well as chemically defined but

non-stoichiometric species, the best characterized of which is 'amorphous calcium phosphate' (Ca/P ratio  $\sim 1.45$ ) (Eanes 2001). Many of these compounds are known to interconvert with time, and, in addition, there is the possibility of mixtures of compounds or adulteration with moieties like magnesium ions or carbonate. Evidence for the latter includes significantly elevated  $\text{Mg}^{2+}$  under some conditions (Table 1), which would tend to reduce the Ca/P ratio, and Ca/P ratios  $>1.5$ , which could be explained by the presence of carbonate or related carboxylate-based anions. Of all these possibilities, apatite and octacalcium phosphate are the only species that can be ruled out, since, as noted, electron microscopy indicates that mitochondrial precipitates are not crystalline. Interestingly, many mitochondrial precipitates bear a strong morphological resemblance to amorphous calcium phosphate (Eanes 2001).

Regardless of the precise chemistry, two questions stand out: What determines the Ca/P ratio and what, if anything, is the impact of the compositional differences implied by higher or lower ratios? One important factor affecting Ca/P ratios is the rate of mitochondrial  $\text{Ca}^{2+}$  accumulation. To illustrate the rate effect, the precipitate Ca/P ratio under our conditions was  $\sim 1.5$  when RBM accumulated Ca in the presence of ADP, whereas it was  $\sim 1.1$  with ATP. The experimental  $\text{Ca}^{2+}$  infusion rates were the same for both ATP and ADP, but as ATP (at 3 mmol/L) binds calcium better than ADP (0.2 mmol/L), the actual infusion rate of available  $\text{Ca}^{2+}$  is 30% lower in the ATP medium; consequently, the actual rate of increase in  $[\text{Ca}^{2+}]_e$  and therefore, the mitochondrial calcium uptake rate are correspondingly slower. This observation implies that the stoichiometry of calcium phosphate precipitates generated in mitochondria depends on the rate of  $\text{Ca}^{2+}$  accumulation, with faster uptake rates favoring higher Ca/P ratios. This conclusion was tested by reducing the rate of  $\text{Ca}^{2+}$  infusion into a mitochondrial suspension with ADP by 30%, which lowered the Ca/P ratio from  $\sim 1.5$  to  $\sim 1.2$ . A similar dependency can be inferred for RLM. Thus, the CLC of RLM, which is very low in the absence of MPT inhibitors, increases significantly in the presence of ADP, but only at very slow infusion rates. In this case, precipitates with a Ca/P ratio of  $\sim 1.0$  are formed.

The CLC also appears to correlate with precipitate composition. This can be seen most clearly by the effect of CsA on ADP-containing suspensions of mitochondria. In both RBM and RLM, CsA increases precipitate Ca/P ratio in parallel with  $\text{Ca}^{2+}$  capacity (Table 1). The higher CLC and Ca/P ratio observed in ADP medium as compared with ATP medium further supports this correlation. Taken together, these data suggest that the conditions of  $\text{Ca}^{2+}$  loading determine the nature of precipitates formed, and that this in turn may affect the CLC. This possibility prompts the question: What is the relationship, if any, between mitochondrial precipitates and MPT? We have observed here, and in other contexts (Pivovarova *et al.* 2004), that precipitates are

often localized in close proximity to the inner surface of the inner mitochondrial membrane (Fig. 2), which is also where the MPT pore is thought to be located (Bernardi *et al.* 2006). Although precipitate formation maintains free matrix  $\text{Ca}^{2+}$  at a constant level over a remarkably large range of  $\text{Ca}^{2+}$  accumulation (Chalmers and Nicholls 2003), MPT induction may only require the elevation of free  $\text{Ca}^{2+}$  in microdomains in the vicinity of inner mitochondrial membrane pore components. In this scenario, solubility differences between various calcium phosphate species would likely affect the CLC. The occurrence of different forms of calcium phosphate has recently been proposed to contribute to CLC differences between synaptic and non-synaptic mitochondria (Brown *et al.* 2006). On the other hand, our data do not rule out the possibility that precipitate composition is independent of CLC, and simply a function of the rate and conditions of  $\text{Ca}^{2+}$  accumulation. In this view, CLC is mainly determined by soluble factors – for example, the concentration and efficacy of various endogenous (ADP, ATP, and cyclophilins) or exogenous (CsA) MPT inhibitors, or even of free mitochondrial  $\text{Ca}^{2+}$  itself – so that precipitate composition is only a passive indicator of the efficacy of precipitate formation under various conditions.

So far this discussion has focused on calcium, but the role of phosphates must also be considered. Another way to view the correlation of low Ca/P ratios with slow  $\text{Ca}^{2+}$  delivery argues that reducing the  $\text{Ca}^{2+}$  delivery rate is equivalent to decreasing the instantaneous Ca/P ratio of solvated (as opposed to precipitated) ions in the mitochondrial matrix. Such an effective increase in phosphate availability would be expected to favor precipitates with a low Ca/P ratio, as observed and consistent with previous observations (Chalmers and Nicholls 2003). The importance of phosphate availability is indicated by the reduction in CLC – and, ultimately, the abolition of  $\text{Ca}^{2+}$  accumulation and the disappearance of precipitates – under  $\text{P}_i$ -depleted conditions. It is interesting to note that complete  $\text{P}_i$  depletion required the presence of oligomycin to block  $\text{P}_i$  generation by ATP hydrolysis. The implication that  $\text{P}_i$  from a variety of sources is capable of supporting precipitate formation and increasing the CLC could in principle have profound physiological significance if, as seems likely, a general increase in mitochondrial CLC improves a cell's resistance to insults like ischemia. As an example, the stronger intracellular, and presumably intramitochondrial,  $\text{Ca}^{2+}$  buffering associated with the maintenance of higher ATP levels in hypoglycemic coma produces minimal brain damage, whereas the same duration of forebrain ischemia leads to nearly complete ATP depletion and significant neuronal death (for review see Kristian 2004).

The solubility of mitochondrial precipitates is strongly dependent on intramitochondrial pH (Chalmers and Nicholls 2003). Thus, an acid shift in matrix pH by depolarization, or because of MPT pore opening, facilitates the

dissolution of calcium phosphate compounds, even nominally insoluble ones like  $\text{Ca}_3(\text{PO}_4)_2$ , leading to the release of accumulated Ca. It appears, however, that not all sequestered calcium is released from swollen mitochondria following MPT or depolarization. It has been known for some time that neurons retain accumulated calcium for relatively long times when their bioenergetic metabolism is significantly compromised (for reviews see Kristian and Siesjö 1998; Silver and Erecinska 1998; Kristian 2004). Much evidence indicates that the mitochondrion is the reservoir for this residual  $\text{Ca}^{2+}$ . For example, mitochondria of cortical neurons contain deposits, presumably calcium phosphate precipitates, several hours after transient focal ischemia (Solenski *et al.* 2002), while isolated mitochondria of cerebellar granule neurons retain a significant fraction of calcium accumulated during glutamate exposure (Ward *et al.* 2005). Retention of accumulated calcium by brain mitochondria after depolarization in the presence of ATP has also been reported (Andreyev and Fiskum 1999). These results are consistent with our own previous observations on isolated RBM (Kristian *et al.* 2002) and on *in situ* mitochondria in NMDA-treated cultured hippocampal neurons (Pivovarova *et al.* 2004), as well as with present observations on RBM after MPT induction (Fig. 2). These data indicate the presence of precipitates not only in normal, intact mitochondria but also in swollen mitochondria with damaged membranes and matrices.

Although we observed massive  $\text{Ca}^{2+}$  efflux upon FCCP exposure several minutes after mitochondrial  $\text{Ca}^{2+}$  loading, only ~50% of intramitochondrial Ca was released (Fig. 5a). Furthermore, electron microscopy revealed that precipitates were still present in mitochondria that displayed typical characteristics of swelling and damage (Fig. 5b). The addition of the pore-forming antibiotic alamethicin after FCCP induced essentially complete liberation of intramitochondrial calcium. A similar effect of alamethicin was reported by Kushnareva *et al.* (2005) for mitochondria isolated from glutamate-stimulated cultured neurons. These authors suggested that alamethicin facilitates the additional release of calcium because the large pores formed throughout the mitochondrial membranes further expose the matrix to the external medium, thereby promoting faster solubilization of calcium phosphate complexes.

Given that MPT pore formation, which permeabilizes the inner mitochondrial membrane, removes any driving force for  $\text{Ca}^{2+}$  retention and allows the free passage of low molecular weight solutes between the matrix and extramitochondrial fluids (for reviews see Halestrap and Brennerb 2003; Bernardi *et al.* 2006), it is interesting to ask why damaged mitochondria retain  $\text{Ca}^{2+}$ . One possibility proposes compartmentalization of the mitochondrial matrix into several 'micro-compartments', with restricted diffusion between them such that asynchronous MPT might promote selective swelling and  $\text{Ca}^{2+}$  release from some micro-compartments,

but not from others (Frey and Mannella 2000; Uchino *et al.* 2002). An alternative possibility considers that the thermodynamics or kinetics of precipitate resolubilization may dictate a slow process. Mitochondrial precipitates appear to be compositionally complex, consisting of mixtures or non-stoichiometric forms of calcium phosphate. Very little is known about the properties of such solids, but it may be relevant that the significant carbon and magnesium content found here (Table 1) again recapitulates biomineralization chemistry, where it has been shown that organic components and Mg, as well as ATP and ADP, greatly stabilize calcium phosphate-based precipitates (Eanes 2001). Regardless of precise mechanisms, it is clear that not all the accumulated calcium is released immediately upon breaching the mitochondrial membrane barrier. Indeed, it appears that the release process can last for many minutes.

In summary, our results suggest that differences in the conditions of formation affect the chemical composition of mitochondrial precipitates, and that this in turn affects  $\text{Ca}^{2+}$  sequestration capacity and, possibly, precipitate mobilization during recovery. In this case, certain conditions will favor the long-term retention of significant amounts of calcium as precipitates within mitochondria, while other conditions will favor rapid release. If, as seems likely, mitochondrial precipitates serve as a  $\text{Ca}^{2+}$  reservoir, affecting the magnitude and time course of cellular responses to accumulated challenges or stress, then the conditions of precipitate formation will play an important role in normal processes such as  $\text{Ca}^{2+}$  signaling, as well as in pathological processes such as those induced by ischemia.

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