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Selective Cytochrome *c* Displacement by Phosphate and Ca^{2+} in Brain Mitochondria

Morena Buratta[1], Lucia Piccotti[1], Silvia Giannini[2], Paolo Gresele[2], Rita Roberti[1], Lanfranco Corazzi[1]
[1]Laboratory of Biochemistry, Department of Internal Medicine, University of Perugia, Via del Giochetto, 06122 Perugia, Italy
[2]Division of Internal and Cardiovascular Medicine, Department of Internal Medicine, University of Perugia, 06122 Perugia, Italy

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Abstract. In brain mitochondria, phosphate- and Ca^{2+}-dependent cytocrome *c* (cyt *c*) release reveals pools that interact differently with the inner membrane. Detachment of the phosphate-dependent pool did not influence the pool released by Ca^{2+}. Cyt *c* pools were also detected in a system of cyt *c* reconstituted in cardiolipin (CL) liposomes. Gradual binding of cyt *c* (1 nmol) to CL/2–[12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]dodecanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine ($NBDC_{12}$-HPC) liposomes (10 nmol) produced NBD fluorescence quenching up to 0.4 nmol of added protein. Additional bound cyt *c* did not produce quenching, suggesting that cyt *c*-CL interactions originate distinct cyt *c* pools. Cyt *c* was removed from CL/$NBDC_{12}$-HPC liposomes by either phosphate or Ca^{2+}, but only Ca^{2+} produced fluorescence dequenching and leakage of encapsulated 8-aminonaphthalene-1,3,6-trisulfonic acid/*p*-xylene-bis-pyridinium bromide. In mitochondria, complex IV activity and mitochondrial membrane potential ($\Delta\psi_m$) were not affected by the release of the phosphate-dependent cyt *c* pool. Conversely, removal of cyt *c* by Ca^{2+} caused inhibition of complex IV activity and impairment of $\Delta\psi_m$. In a reconstituted system of mitochondria, nuclei and supernatant, cyt *c* detached from the inner membrane was released outside mitochondria and triggered events leading to DNA fragmentation. These events were prevented by enriching mitochondria with exogenous CL or by sequestering released cyt *c* with anti-cyt *c* antibody.

Key words: Brain mitochondria — Cytochrome *c* — Cardiolipin — Liposome — Mitochondrial membrane potential

Correspondence to: Lanfranco Corazzi; email: corazzi@unipg.it

Abbreviations: S/H, sucrose/HEPES; HEPES, 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid; TH ESIT, dodecylpoly (ethylenglycolether)$_9$; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; $NBDC_{12}$-HPC, 2-(12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)dodecanoyl-1-h exadecanoyl-*sn*-glycero-3-phosphocholine; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid, disodium sa lt; DPX, *p*-xylene-bis-pyridinium bromide; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; CsA, Cyclosporin A; CL, cardiolipin; $\Delta\psi_{m,}$ mitochondrial membrane potential; PTP, permeability transition pore; IGFL, integral of fluorescence

Introduction

Cytochrome *c* (cyt *c*) is an essential component of the inner mitochodrial membrane involved in the respiratory chain. Under physiological conditions, cyt *c* is present also in the intermembrane space, where it acts as an electron shuttle between the outer and inner mitochondrial membrane (Bernardi & Azzone, 1981). In recent years, the relevance of this protein has rapidly increased due to the ability to leave mitochondria and direct the cell toward a controlled metabolism in the cytosol, leading to proteolytic enzyme activation and apoptosis (Wang, 2001). Furthermore, it has been reported that cyt *c* not only enters the cytoplasm but also is released from the cytosol into the extracellular medium, as monitored in the serum of patients with hematological malignancies in the course of cancer chemotherapy (Renz et al., 2001). Lack of mitochondrial apoptotic pathways may contribute to oncogenesis in ovarian cancer cells defective in cyt *c*-dependent caspase activation (Wolf et al., 2001). A block of the cyt *c* mitochondrial pathway has been observed in breast cancer cells

(Gao et al., 2001). In epidermoid carcinoma cells, photodynamic therapy triggers reactive oxygen species formation, followed by mitochondrial swelling, cyt *c* release and cell death (Lam, Oleinick & Nieminen, 2001).

Cyt *c* release from mitochondria is also seen in certain neuropathological conditions and is indicative of apoptosis. In amyotrophic lateral sclerosis, the pathway of cell death triggered by mitochondrial release of cyt *c* is a likely cause of damage and death of the motor neurons (Guégan et al., 2001). There is evidence that dopaminergic neurons die by apoptosis in Parkinson's disease (Hartmann et al., 2001). Although apoptosis could occur through two general pathways, i.e., receptor-mediated and mitochondria-initiated (Budihardjo et al., 1999), release of cyt *c* is a common event initiated by the detachment of the protein from the inner mitochondrial membrane.

The interaction of cyt *c* with the inner mitochondrial membrane has been interpreted in a cyclic model where cyt *c* binds to the surface of the inner membrane in at least two different membrane-bound conformations that equilibrate with a weakly bound protein conformation released back into the intermembrane space of mitochondria (Cortese, Voglino & Hackenbrock, 1998). A model of hydrophobic protein-lipid interaction involves the extension of the two lipid tails in the opposite directions from the headgroup and further accommodation of one acyl chain within the nonpolar cavity of cyt *c*, without cyt *c* penetration in the hydrocarbon region of the membrane (Tuominen, Wallace & Kinnunen, 2002). However, recent reports claim that the tightly bound conformation of the protein is accompanied by a loosening of the tertiary structure and that tail lipid extensions are very unlikely to occur without a partial penetration of cyt *c* in the bilayer (Gorbenko, 1999; Gorbenko & Domanov, 2003). Soluble cyt *c* pool is released outside mitochondria following Bax-dependent outer mitochondrial membrane permeabilization (Ott et al., 2002). We found that the pool size of the solubilized form of cyt *c* may be regulated through the mitochondrial membrane potential ($\Delta\psi_m$) (Piccotti et al., 2004a). Moreover, the quantitative interactions of cyt *c* with cardiolipin (CL) may also be important in determining the extent of cyt *c* release. In fact, in a condition of metabolic decline of CL synthesis, the decrease of CL and the release of the protein are directly correlated (Ostrander et al., 2001).

Although many studies have focused on the release of cyt *c* and downstream activated processes, little attention has been given to the interaction of cyt *c* with the inner mitochondrial membrane and to the possible role of cyt *c* pools interacting differently with the membrane. We observed a phosphate-dependent release of cyt *c* from mitochondria (Piccotti et al., 2002). Inorganic phosphate accumulation and alterations in intracellular Ca^{2+} homeostasis accompany the decrease in adenosine triphosphate (ATP) levels occurring under certain pathological conditions (Crompton, 1999). For instance, elevated phosphate concentrations were detected in brains of patients with Alzheimer's disease (Brown et al., 1989).

Previous results from our laboratory indicated that exposure of mitochondria to 5 mM potassium phosphate produced slow mitochondrial swelling and permeability transition pore (PTP). Opening of PTP was not accompanied by cyt *c* release, which was observed only for phosphate concentrations higher than 10 mM (Piccotti et al., 2002). The relationship between Ca^{2+} uptake, PTP activation and cyt *c* release in brain mitochondria has been investigated (Andreyev, Fahy & Fiskum, 1998; Brustovetsky et al., 2002; Schild et al., 2001).

In this work, phosphate and Ca^{2+} loading of mitochondria was mimicked *in vitro* and the effect on cyt *c* detachment and release is reported. Different cyt *c* pools are involved when mitochondria are treated with either phosphate or Ca^{2+}. Cyt *c* pools are also evidenced in a model system of cyt *c* reconstituted in CL liposomes. In mitochondria, the effects of phosphate on some functional parameters, such as complex IV activity and membrane potential formation and consumption, were compared with that produced by Ca^{2+}.

Materials and Methods

Chemicals

5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1); 2-[12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]dodecanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine ($NBDC_{12}$-HPC); 8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt (ANTS); and *p*-xylene-bis-pyridinium bromide (DPX) were from Molecular Probes (Gottingen, Germany). Digitonin, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), cyclosporin A (CsA), 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (HEPES), propidium iodide (PI), nigericin, 3,3′-diexyloxacarbocyanine iodide ($DiOC_6[3]$) and cyt *c* were from Fluka (Buchs, Switzerland). Ruthenium red, adenosine diphosphate (ADP, K^+ salt), pyruvic acid, malic acid, CL and other substrates were obtained from Sigma (Milan, Italy). Pyruvic acid and malic acid solutions were adjusted to pH 7.0 with KOH. Mouse anti-cyt *c* monoclonal immunoglobulin G (IgG), goat anti-mouse horseradish peroxidase (HRP)-conjugated IgG, rabbit anti-ANT polyclonal antibody (AAC1 isoform) and goat anti-rabbit HRP-conjugated IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-cyclic adenosine monophosphate-response element binding protein (CREB) polyclonal antibody was a kind gift of Dr. G. Servillo (University of Perugia, Perugia, Italy).

Preparation of Rat Brain Cortex Subfractions

Mitochondria were prepared from rat brain cortex (CD, 2 months old; Charles River, Como, Italy) as previously reported (Monni et al., 2000) and then resuspended in a medium containing 5 mM

HEPES (pH 7.4), 2 mM KCl and 0.32 M sucrose (S/H buffer). The S/H buffer makes the system sensitive to changes in ionic strength and then suitable to study the hydrophilic interaction component responsible for cyt *c* binding to the inner membrane of mitochondria. Highly pure preparations were obtained, as checked by testing both positive (complex IV activity) and negative (NADPH-cyt *c* reductase, endoplasmic reticulum; Na^+,K^+-ATPase, plasma membrane; arylsulfatase A, lysosomes) marker enzymes (Monni et al., 2000). Oxygen uptake was determined using a Clark-type microelectrode equipped with a fiber-optic oxygen monitor (Instech, Horsham, PA). The outer mitochondrial membrane was permeabilized by digitonin treatment (0.3 mg/mg mitochondrial protein, 10 min at 0°C) following a described procedure (Monni et al., 2000). The integrity and functionality of mitochondria after digitonin treatment were assessed through analysis of CL in the postdigitonin supernatant and by respiration measurements. The cytosolic fraction was prepared from the postmitochondrial supernatant by centrifugation at 105,000 × *g* for 60 min.

For nuclei preparation, brain cortex was homogenized in S/H buffer plus 0.5 mM $MgCl_2$. The homogenate was centrifuged at 2,000 × *g* and the pellet resuspended in the same buffer and centrifuged as above. The washed pellet was resuspended in 2.1 M sucrose plus 2 mM HEPES (pH 7.4) and 0.5 mM $MgCl_2$, layered on a cushion of the same buffer and centrifuged at 64,000 × *g* for 30 min. Pelletted nuclei were recovered in S/H buffer, and integrity was tested by measuring the leakage of nuclear CREB by Western blotting with rabbit anti-CREB polyclonal antibodies.

Treatment of Mitochondria

Mitochondria were incubated in the absence (deenergized) or presence of respiratory substrates (1.5 mM pyruvate, 3.0 mM malate and 5 mM potassium phosphate, state 4; the same plus 0.8 mM ADP, state 3) at 37°C or at room temperature for the indicated times. In some experiments, mitochondria were pretreated with the indicated concentrations of potassium phosphate or Ca^{2+} and recovered by centrifugation prior to incubation. When needed, mitochondria were preincubated 5 min at 37°C in the presence of 5 μM ruthenium red and 2 μM CsA before incubation with Ca^{2+}.

In cyt *c* release experiments, mitochondria (about 0.3 mg protein) resuspended in S/H buffer were treated with 20 mM potassium phosphate or 4 μM Ca^{2+} for 5 min at room temperature. In some experiments, phosphate-pretreated mitochondria were recovered by centrifugation and subjected to Ca^{2+} or phosphate treatment. After centrifugation (9,000 × *g* for 10 min) supernatant (S) was recovered and pellets were resuspended in S/H buffer and treated with digitonin as described above to permeabilize the outer mitochondrial membrane. After centrifugation for 10 min at 9,000 × *g*, the pellet (M) and the postdigitonin supernatant (I) were recovered. Western blot analysis of cyt *c* was performed in M, S and I fractions using anti-cyt *c* IgG.

Complex IV Activity

The assay of complex IV activity in mitochondria that had been incubated for 20 min at 37°C in different experimental conditions was carried out, incubating about 15 μg of mitochondrial protein in 1 ml of 80 mM sucrose, 5 mM HEPES (pH 7.4) and 25 μM reduced cyt *c*. The activity was measured by following the decrease of reduced cyt *c* absorbance at 550 nm.

Flow-Cytometric Analysis

Mitochondria were pretreated with either 20 mM phosphate or 4 μM Ca^{2+} (5 min at room temperature) and centrifuged for 10 min at 9,000 × g. Aliquots (about 0.2 mg protein) of mitochondria were then incubated for 10 min at room temperature in the presence of respiratory substrates (state 3). Samples were then loaded with JC-1 probe (1 μM) and incubated for 5 min at room temperature. Evaluation of changes in $\Delta\psi_m$ was performed by flow-cytometric analysis using a FACScan flow cytometer (Epics XL-MCL; Beckman Coulter, High Wycombe, UK) equipped with a focused argon laser. The merits as well as limitations of the use of JC-1 in $\Delta\psi_m$ measurement have been reviewed (Cossarizza, Ceccarelli & Masini, 1996). For complete depletion of $\Delta\psi_m$ (positive control), the mitochondrial uncoupler CCCP (5 μM) was used. Data were analyzed and stored in a data management system (System II software, Beckman, Coulter, UK). JC-1 fluorescence of mitochondria was detected as described (Cossarizza et al., 1996), and red/green fluorescence emission (FL1/FL2) of particles was reported as a dot plot.

Particular care was taken in the use of JC-1. In our experimental conditions, the respiratory control ratio of mitochondria was not affected by JC-1 and the probe showed a single fluorescent peak (λ_{exc} 490 nm, λ_{em} 539 nm, monomeric form, green fluorescence). The aggregated form of the probe (λ_{em} 597 nm, orange fluorescence) was observed only after energization of mitochondria. In addition, phosphate and respiratory substrates did not interfere with the orange fluorescence, indicating that JC-1 fluorescence is a good indicator of $\Delta\psi_m$ changes.

Mitochondrial preparations were routinely checked for their ability to modulate $\Delta\psi_m$ in the absence of substrates (deenergized), with 5 mM phosphate, in state 3 or in the presence of other factors (5 μM nigericin, 5 μM CCCP). After incubation for 10 min at room temperature, the membrane potential-sensitive probe $DiOC_6(3)$ (40 nM) was added. $DiOC_6(3)$ green fluorescence (FL1) was plotted on a logarithmic scale *vs.* the frequency of events, and the mean value of the integral of fluorescence (IGFL) was evaluated.

Preparation of Liposomes

CL liposomes were prepared by resuspending the lipid in S/H (about 0.5 μmol lipid/ml), and the solution was sonicated with a tip sonicator to clearing. Metal particles from the sonicator tip and multilamellar liposomes were removed from the preparation by centrifugation at 100,000 × g for 20 min. For the resonance energy transfer studies, CL liposomes labeled with $NBDC_{12}$-HPC were prepared following the same procedure by mixing CL with 1% fluorescent probe. In leakage experiments, CL (about 0.5 μmol lipid/ml) was hydrated with 12.5 mM ANTS, 45 mM DPX, 20 mM NaCl and 10 mM Tris-HCl (pH 7.4) and unilamellar vesicles were prepared by reverse-phase evaporation (Szoka & Papahadjopoulos, 1978). Liposomes were extruded successively through polycarbonate Unipore membranes (0.1 μm pore size; Millipore, Bedford, MA). The unencapsulated material was separated from liposomes through a Sephadex G-50 column (Pharmacia, Uppsala, Sweden) using 100 mM NaCl, 3 mM HEPES and 2 mM L-histidine (pH 7.4) as eluent buffer. Liposomes encapsulating ANTS/DPX probes were collected in the void volume. Phospholipid concentration of liposomal preparations was determined by phosphate analysis (Bartlett, 1959).

Binding and Release of Cyt *C* in Liposomes

Reconstitution of cyt *c* in CL liposomes was performed by mixing liposomes (10 nmol lipid in 200 μl of S/H buffer) with cyt *c* (1 nmol). The solutions were incubated for 10 min at room temperature, and unbound cyt *c* was removed by filtering through Microcon YM-100 (Amicon, Danvers, MA) and washing with S/H buffer. In some experiments, cyt *c*-CL liposomes were incubated in the presence of 100 mM phosphate or 1 mM Ca^{2+}. After the incubation, released cyt *c* was filtered through Microcon YM-100 with repeated S/H buffer washings. Filtered and retained fractions were recovered and

analyzed by immunoblotting for the detection of cyt *c* by Western blotting. Individual band densities of immunoblots were integrated using Quantiscan software (Biosoft, Cambridge, UK).

Fluorescence Measurements

Liposomal Content Leakage Assay

Aqueous content leakage from CL liposomes was determined by the ANTS/DPX assay, as described (Ellens, Bentz & Szoka, 1985). The fluorescence measurements were performed in 2 ml of 100 mM NaCl, 3 mM HEPES and 2 mM L-histidine (pH 7.4) solution at 25°C. Aliquots of ANTS/DPX-loaded CL liposomes were added to a final concentration of 5 μM lipid, and the fluorescence was recorded (λ_{exc}, 360 nm; λ_{em}, 530 nm). Leakage of liposomes caused by the addition of increasing amounts of cyt *c* or Ca^{2+} or phosphate was measured as fluorescence increase. The fluorescence scale was calibrated taking the fluorescence of ANTS-DPX liposomes as 0% and the fluorescence of the same solution after addition of 20 μl of 1% dodecylpoly(ethyleneglycolether)$_9$ (THESIT) as 100%.

Binding of Cyt c with CL Liposomes

The binding of cyt *c* with CL liposomes was assessed by monitoring the resonance energy transfer between $NBDC_{12}$-HPC and the heme of cyt *c*, following the interaction of the protein with $NBDC_{12}$-HPC/CL liposomes. Aliquots of $NBDC_{12}$-HPC/CL liposomes were added to 2 ml of S/H buffer to a final concentration of 5 μM lipid, and the fluorescence was recorded (λ_{exc}, 470 nm; λ_{em}, 523 nm).

Enrichment of Mitochondria with Exogenous CL

Fusion of CL with mitochondria was performed as described (Piccotti et al., 2002), by incubating mitochondria (0.4 mg protein) with CL liposomes (2.5 nmol lipid) for 5 min at room temperature in the presence of 5 mM potassium phosphate (pH 7.4), ADP, pyruvate and malate (0.8, 1.5 and 3.0 mM, respectively). Nonfused lipid was eliminated by centrifugation on 0.5 ml of 0.6 M sucrose (Piccotti et al., 2002). In the experiments performed to test complex IV activity, CL liposomes were fused after mitochondria had been incubated for 20 min at 37°C in the presence of respiratory substrates.

DNA Degradation Assays

For determination of nuclear DNA degradation, nuclei (0.8 mg protein) were mixed with mitochondria (0.3 mg) in the presence of cytosol (0.1 mg protein) in a final volume of 0.3 ml. Incubation was carried out at 37°C in the presence of 20 mM phosphate. Treatment with phosphate was also performed in CL-enriched mitochondria or in the presence of mouse anti-cyt *c* monoclonal IgG (5 μg). Degradation of nuclear DNA was evaluated by PI staining of DNA. At fixed times, aliquots of the mixtures were added to a cuvette containing S/H buffer (2 ml). PI probe was added to a final concentration of 10 μM, and fluorescence was monitored at 604 nm (λ_{exc}, 535 nm).

Results

Characterization of Mitochondrial Preparations

Biochemical Test

Monoamino oxidase and cyt c oxidase (mitochondrial enzymes) were enriched four and six times, respectively, in mitochondria compared to the homogenate, whereas Na^+,K^+-ATPase and arylsulfatase A were not detected in mitochondria. Very weak contamination of NADPH:cyt c reductase (microsomal marker) was detected in mitochondria (Monni et al., 2000).

Respiratory Activity

Mitochondria were metabolically active with a respiratory control ratio (state 3 to state 4) in the range of 5–6, a value that is in agreement with data reported in the literature (Lai, Rex Sheu & Carlson, 1985). Functionality of mitochondrial preparations was routinely assayed by monitoring the sensitivity of $\Delta\psi_m$ to several factors. A calibrated scale of $\Delta\psi_m$ for $DiOC_6(3)$ probe is shown in Figure 1. Complete $\Delta\psi_m$ collapse and a maximal $\Delta\psi_m$ value were obtained in the presence of CCCP and nigericin, respectively (0% and 100% in the potential value scale). $\Delta\psi_m$ was sensitive to respiratory substrates. The lowest value was measured in the deenergized state. $\Delta\psi_m$ increased upon addition of phosphate, whereas addition of ADP, malate, pyruvate and phosphate produced a decrease of $\Delta\psi_m$ compared to phosphate alone, due to the consumption of membrane potential in state 3.

Treatment of mitochondria with digitonin was performed to permeabilize the outer mitochondrial membrane and to favor the release of cyt *c* restricted in the intermembrane space of mitochondria and not bound to the inner membrane. In our experimental conditions, digitonin acted on the outer mitochondrial membrane without significantly affecting the inside of mitochondria. Only 4–5% of total CL and 14–15% of total mitochondrial phospholipids were solubilized and recovered in the postdigitonin supernatant. Respiratory activity was maintained and mitochondria were sensitive to respiratory substrates.

Phosphate- and Ca^{2+}-Dependent Cyt *C* Release from Mitochondria

Mitochondria, control or treated with phosphate or Ca^{+2}, were recovered by centrifugation and treated with digitonin, and cyt *c* was analyzed by Western blotting (Fig. 2). In control mitochondria, no cyt *c* was found in the extramitochondrial medium (S). A small cyt *c* pool, very likely representing a soluble pool restricted to the intermembrane space of mitochondria, was found in the postdigitonin supernatant (I), while the largest amount of cyt *c* was associated with the inner mitochondrial membrane (M). Treatment of mitochondria with 20 mM phosphate resulted in the release of cyt *c* outside mitochondria. Treatment of mitochondria with 4 μM Ca^{2+} also produced cyt *c* release outside mitochondria. In the experiments performed by pretreating mitochondria with

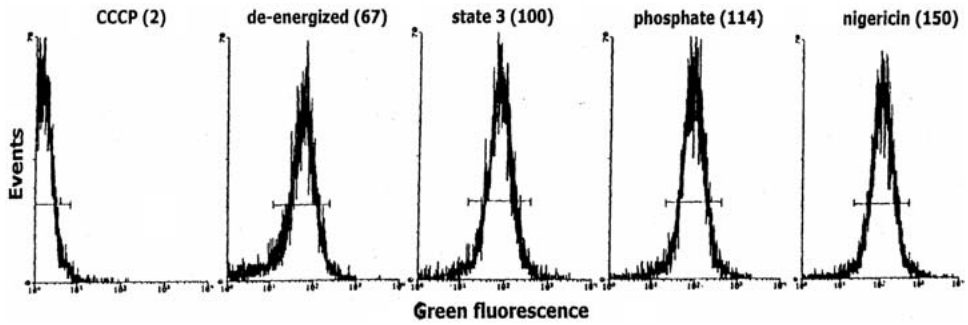

Fig. 1. $\Delta\psi_m$ calibration scale. Mitochondria (0.2 mg protein) were incubated for 10 min at room temperature in different respiratory conditions (see Materials and Methods) and then loaded with $DiOC_6(3)$ before flow-cytometric analysis. Frequency histograms are reported. Numbers in parentheses indicate IGFL values.

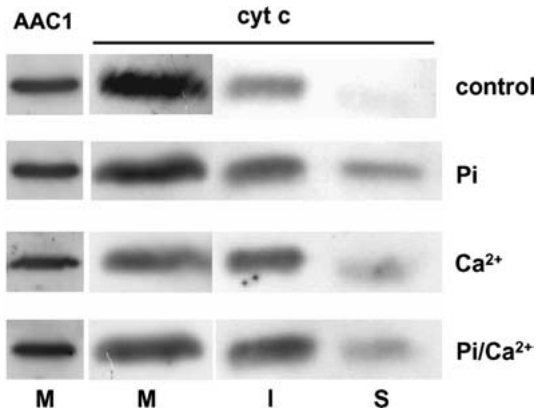

Fig. 2. Phosphate- and Ca^{2+}-dependent cyt *c* release from mitochondria. Freshly prepared mitochondria (0.3 mg protein) resuspended in S/H buffer were treated with 20 mM phosphate or 4 μM Ca^{2+}. Ca^{2+} treatment was also performed in phosphate-pretreated mitochondria. After centrifugation (9,000 x *g* for 10 min) supernatant (S) was recovered for Western blot analysis of released cyt *c*. Mitochondrial pellets were resuspended in S/H buffer and treated with digitonin (0.3 mg/mg of mitochondrial protein, 10 min at 0°C). The pellet (M) and the postdigitonin supernatant were recovered by centrifugation as above and analyzed for cyt *c*. Cyt *c* in the postdigitonin supernatant was taken as the protein present in the intermembrane space (I). Western blot of AAC1 protein in M fractions is reported. A representative experiment of three is shown.

phosphate, besides the cyt *c* pool already detached by phosphate, subsequent Ca^{2+} treatment removed an additional cyt *c* pool (Fig. 2). The extent of this pool was similar to that produced by treating mitochondria with Ca^{2+} alone, suggesting that Ca^{2+}-dependent cyt *c* release is not influenced by phosphate pretreatment or by previous phosphate-dependent cyt *c* release. It is worth noticing that no cyt *c* release was observed when phosphate-pretreated mitochondria were reincubated with 20 mM phosphate (*not shown*). The AAC1 isoform of the ADP/ATP carrier, a protein spanning the inner mitochondrial membrane (Gawaz, Douglas & Klingemberg, 1990), taken as a housekeeping marker, did not change in M fractions following the different treatments (Fig. 2).

Cyt *C* Pools in Cyt *C*-Cl Liposomes

Since cyt *c* is bound to the inner mitochondrial membrane by its association with CL, the hypothesis of different cyt *c* pools was investigated in a model system of cyt *c* reconstituted in CL liposomes using phosphate and Ca^{2+} to interfere with cyt *c*-CL associations.

First, the perturbing effects of cyt *c*, phosphate and Ca^{2+} on CL liposomes were revealed by monitoring the leakage of encapsulated ANTS-DPX probes. Leakage was registered as increase of ANTS fluorescence due to dilution of the DPX quencher. Treatment of vesicles with 0–10 mM Ca^{2+} caused extensive leakage of the probes, whereas neither the binding of cyt *c* nor the addition of phosphate to CL liposomes caused leakage of the probes (Fig. 3). Moreover, the phosphate-dependent removal of cyt *c* from cyt *c*-CL liposomes also occurred without leakage (*not shown*). These data indicate that cyt *c* and phosphate do not alter the lamellar phase of CL liposomes whereas Ca^{2+} induces the formation of hexagonal phases (Epand et al., 2002), resulting in leakage of the liposomal content.

In our previous work, the complete binding of cyt *c* (1 nmol) to CL liposomes (10 nmol) and the localization of the protein on the external surface of liposomes were demonstrated (Piccotti et al., 2004a). Experiments were performed by gradually adding cyt *c* to CL liposomes (10 nmol), and the binding of cyt *c* to the lipid phase was assessed by monitoring the resonance energy transfer (i.e., dipole-dipole coupling) between $NBDC_{12}$-HPC inserted in CL liposomes and the heme of cyt *c*. The efficiency of quenching of NBD fluorescence, measured as the slope of fluorescence decrease in function of added cyt *c*, exhibits biphasic behavior (Fig. 4). Fluorescence quenching was detected up to 0.4 nmol of added cyt *c*. The binding of additional cyt *c* to CL (up to 1 nmol), verified by filtering the reconstituted vesicles, did not produce fluorescence quenching, indicating that this amount of cyt *c* does not interfere

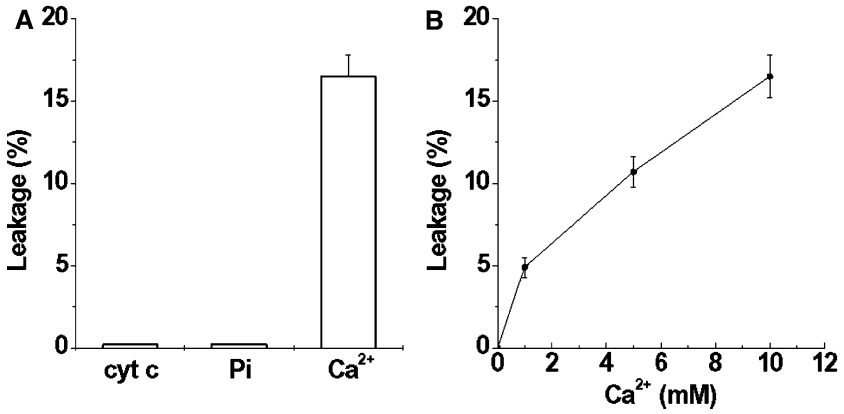

Fig. 3. Leakage of encapsulated ANTS/DPX from CL liposomes: effect of phosphate, Ca^{2+} and cyt *c*. (***A***) Aliquots of ANTS/DPX-loaded CL liposomes (10 nmol lipid) were added to 2 ml of 100 mM NaCl, 3 mM HEPES and 2 mM L-histidine (pH 7.4) and fluorescence was recorded (λ_{exc}, 360 nm; λ_{em}, 530 nm) following addition of 1 μg cyt *c*, 60 mM phosphate or 10 mM Ca^{2+}. (***B***) Ca^{2+} dependence of leakage. We evaluated 0% and 100% leakage by measuring fluorescence before and after addition of THESIT (see Materials and Methods). Data are means ± standard deviation of four experiments.

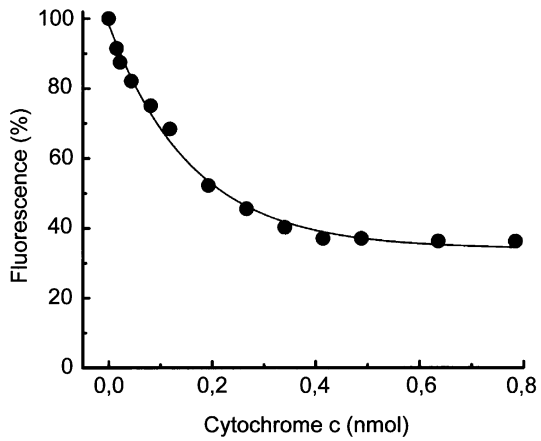

Fig. 4. Binding of cyt *c* to CL liposomes and $NBDC_{12}$-HPC fluorescence quenching. Aliquots of $NBDC_{12}$-HPC/CL liposomes (10 nmol lipid) were added to 2 ml of S/H buffer and fluorescence was recorded (λ_{exc}, 470 nm; λ_{em}, 523 nm) following increasing cyt *c* additions. A representative experiment of three is shown.

with the fluorescent probe. As a positive control, the fluorescence quencher sodium hydrosulfite abolished NBD fluorescence completely (*not shown*), proving that the quenching curve really represents a biphasic response and not the result of the saturation of NBD quenching. These results suggest that, in liposomes, cyt *c*-CL binding originates distinct pools, characterized by hydrophobic and hydrophilic interactions.

Detachment of cyt *c* from cyt *c*-CL liposomes was also investigated. In our previous work, the phosphate-dependent removal of cyt *c* from cyt *c*-CL liposomes was investigated (Piccotti et al., 2004a). For phosphate concentrations higher than 100 mM, a plateau was reached, producing displacement of about 45% of total cyt *c*. In this work, we found that Ca^{2+} is also able to remove cyt *c*. By treating both phosphate-pretreated and untreated cyt *c*-CL liposomes with 1 mM Ca^{2+}, 14–17% of total cyt *c* was discharged (Fig. 5A), indicating that cyt *c* released by phosphate may represent a pool that can be distinguished by the pool removable by 1 mM Ca^{2+}. The gradual displacement of cyt *c* from CL/$NBDC_{12}$-HPC liposomes, performed by treating the reconstituted vesicles with increasing phosphate concentrations, occurred without fluorescence release (Fig. 5B), suggesting that phosphate-dependent detachment of cyt *c* acts by breaching the hydrophilic lipid-protein interactions. On the contrary, Ca^{2+}, due to the demonstrated destabilization properties toward the bilayer structure, produced cyt *c* removal and fluorescence development (Fig. 5B).

Perturbing Effects of Phosphate and Ca^{2+} on Mitochondrial Functions

The model of cyt *c*-CL liposomes supports the finding that cyt *c* molecules organize and interact differently with the inner membrane of mitochondria. Experiments were carried out to detach cyt *c* from the inner membrane through the perturbing effects of either phosphate or Ca^{2+}, and some mitochondrial functions were investigated.

Complex IV Activity

First, mitochondria were preincubated for 20 min at 37°C in the absence or presence of respiratory substrates, and complex IV activity was tested as described in Materials and Methods. A significant decrease of complex IV activity was found in mitochondria in state 3 compared to deenergized mitochondria (Fig. 6A). The fusion of exogenous CL to the inner mitochondrial membrane resulted in the recovery of complex IV activity. Second, mitochondria were pretreated with up to 30 mM phosphate,

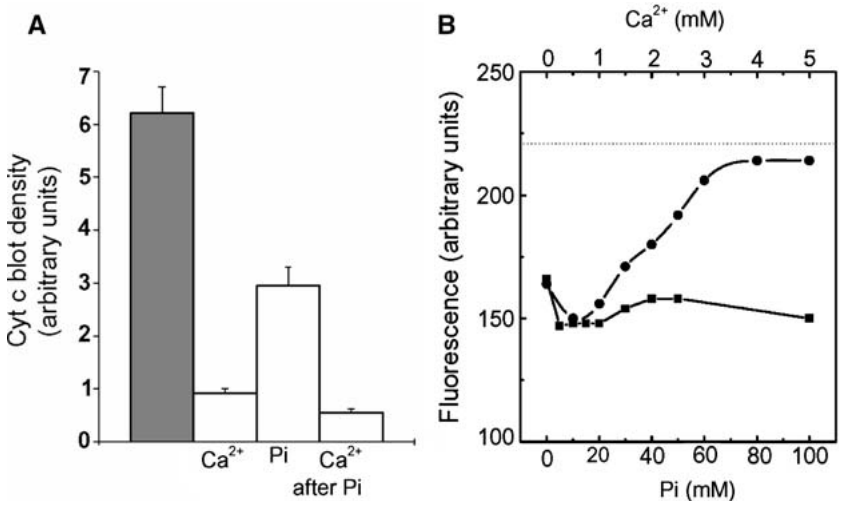

Fig. 5. Detachment of cyt *c* from cyt *c*-CL liposomes. (**A**) Reconstituted cyt *c*-CL liposomes (10 nmol lipid, 1 nmol cyt *c*) were incubated with 1 mM Ca^{2+} or 100 mM phosphate for 10 min at room temperature. Samples were then filtered through Microcon YM-100, and Western blot analysis of released cyt *c* was performed in the filtrate. In some experiments, phosphate treatment preceded Ca^{2+} treatment. *Solid column* is total bound cyt *c*. Data are means ± standard deviation of four experiments. (**B**) Cyt *c* reconstituted $NBDC_{12}$-HPC/CL liposomes were added to 2 ml of S/H buffer and fluorescence was recorded (λ_{exc}, 470 nm; λ_{em}, 523 nm) after addition of phosphate (■-■) or Ca^{2+} (●-●). A representative experiment of three is shown.

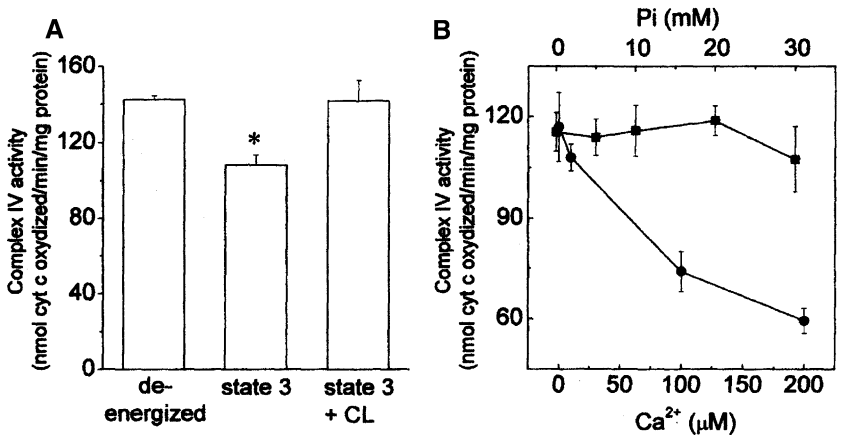

Fig. 6. Complex IV activity. (**A**) Mitochondria were preincubated for 20 min at 37°C in the absence (deenergized) or presence (state 3) of respiratory substrates. The enrichment of mitochondria with CL after incubation in state 3 was performed as described in Materials and Methods. (**B**) Mitochondria were pretreated with phosphate (■-■) or Ca^{2+} (●-●) before incubation in state 3 as above. Complex IV activity was measured by following the oxidation of reduced cyt *c* at 550 nm, as described in Materials and Methods. Data are means ± standard deviation of four experiments. *Significance reached, $P < 0.01$ (Student's test).

and cyt *c*-impoverished mitochondria (Piccotti et al., 2002) were recovered by centrifugation and incubated for 20 min at 37°C in state 3. No influence on complex IV activity was observed following phosphate-dependent cyt *c* removal, whereas a decrease of activity was obtained by pretreating mitochondria with up to 200 μM Ca^{2+}(Fig. 6B). When mitochondria were preincubated for 5 min at 37°C in the presence of 5 μM ruthenium red and 2 μM CsA before addition of Ca^{2+}, complex IV activity was not inhibited.

Membrane Potential

$\Delta\psi_m$ was determined using JC-1. Freshly prepared mitochondria were pretreated for 5 min at room temperature in the presence of either 20 mM phosphate or 4 μM Ca^{2+} and incubated for 10 min at room temperature in the presence of respiratory substrates (state 3). Samples were then loaded with 1 μM JC-1 and incubated for 5 min at room temperature before flow-cytometric analysis. Control mitochondria appeared as heterogenous population

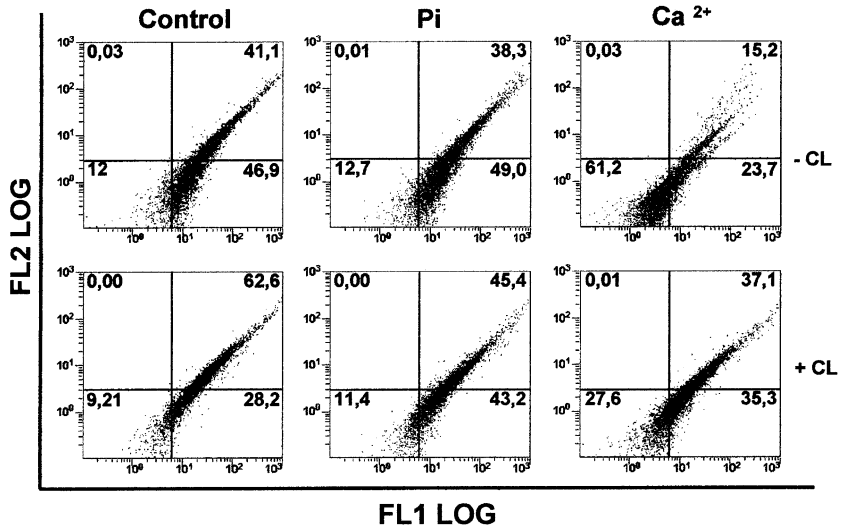

Fig. 7. Flow-cytometric analysis of $\Delta\psi_m$ of single mitochondria stained with JC-1. Mitochondria were pretreated with either 20 mM phosphate or 4 μM Ca^{2+} and centrifuged for 10 min at 9,000 × *g*. Aliquots (about 0.2 mg protein) of mitochondria were then incubated for 10 min at room temperature in the presence of respiratory substrates (state 3). Samples were then loaded with 1 μM JC-1 and incubated for 5 min at room temperature before flow-cytometric analysis. The percentage values of mitochondria within prefixed energy sectors are reported. A representative experiment of three is shown.

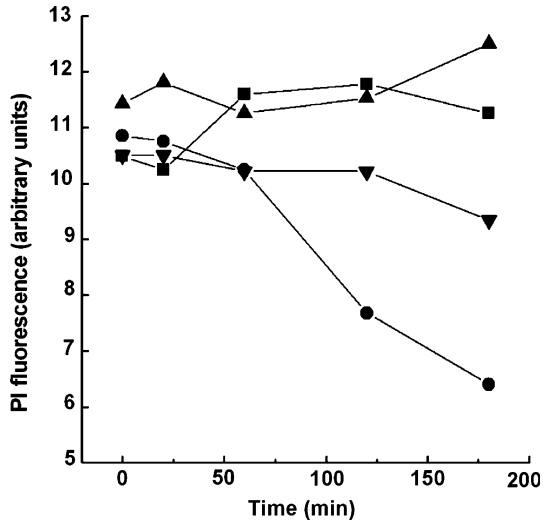

Fig. 8. DNA-bound PI fluorescence. Nuclei (0.8 mg protein), mitochondria (0.3 mg protein) and cytosol (0.1 mg protein) were mixed in a final volume of 0.3 ml and incubated at 37°C in the conditions specified below. At fixed times, aliquots of the mixtures were added to a cuvette containing S/H buffer (2 ml). PI probe was added to 10 μM final concentration and fluorescence decrease was monitored at 604 nm (λ_{exc}, 535 nm). ■-■, Control; ●-●, 20 mM phosphate; ▲-▲, 20 mM phosphate, CL-enriched mitochondria; ▼-▼, 20 mM phosphate plus 5 μg of mouse anti-cyt *c* monoclonal IgG. DNA degradation was evaluated by the extent of fluorescence decline. A representative experiment of three is shown.

particles, most of them with high energy (41.1% orange and 46.9% green fluorescence). Cyt c removal by pretreatment with phosphate did not significantly affect the energetic distribution of particles. Contrarily, $\Delta\psi_m$ distribution of Ca^{2+}-treated mitochondria showed a trend to lower values compared to control mitochondria, with 61.2% particles at low energy state (Fig. 7). The $\Delta\psi_m$ of particles was affected by CL. Enrichment of control or treated mitochondria with exogenous CL increased $\Delta\psi_m$ with the production of more homogeneous mitochondrial populations (Fig. 7). In Ca^{2+}-treated mitochondria, the number of low-energy particles decreased from 61.2% to 27.6% after CL enrichment.

Effect of CL on Cyt *C* Release and Nuclear DNA Fragmentation

In order to verify whether cyt c detached from the inner membrane is also released outside mitochondria and possibly involved in the apoptotic cascade, nuclear DNA integrity was evaluated during coincubations of isolated nuclei, mitochondria and cytosol in the presence of phosphate. PI was used as a marker of DNA integrity as it enters nuclei and develops fluorescence by binding intact DNA. Figure 8 shows a consistent decrease of fluorescence during incubation with phosphate at 37°C, indicating that DNA fragmentation occurs in these conditions. However, when CL was previously fused to mitochondria, no effects were exerted by phosphate. The involvement of phosphate-dependent cyt c release in nuclear DNA fragmentation was demonstrated by the inhibition of fluorescence decrease observed in the presence of anti-cyt c antibody. In control experiments, phosphate did not influence PI fluorescence when isolated nuclei were incubated in the absence of mitochondria or cytosol.

Discussion

A model system of cyt *c* reconstituted in CL liposomes demonstrates that cyt *c* localizes on the external surface of vesicles and organizes in pools anchored to the lipid matrix through different interactions. This model supports experimental data indicating the presence of cyt *c* pools that interact differently with the inner mitochondrial membrane.

The release of cyt *c* outside mitochondria is a necessary upstream event in the activation of metabolic steps leading to caspase activation (Wang, 2001). Many factors are able to trigger the release of the protein, with the common feature of weakening protein-membrane interactions. Anchorage of the protein is mainly due to CL, and any cellular event perturbing the stationary state of the lipid may influence the stability and the amount of anchored protein (Ostrander et al., 2001; Piccotti et al., 2002). According to the literature (Bernardi & Azzone, 1981; Ott et al., 2002), mitochondria contain a small amount of free cyt *c* localized in the intermembrane space (Fig. 2). Membrane potential and cyt *c*-CL interactions modulate the amount of free cyt *c* (Piccotti et al., 2004a). Early studies also indicated that increase of ionic strength depresses the binding of cyt *c* to the inner membrane (Jacobs & Sanadi, 1960). We found that phosphate can enter mitochondria and interfere with electrostatic interactions between cyt *c* and the inner mitochondrial membrane (Piccotti et al., 2002), thus triggering the release of the protein (Fig. 2). The entrance of Ca^{2+} in mitochondria occurs through a well-defined mechanism (Crompton, 1999). Once inside, Ca^{2+} promotes cyt *c* detachment (Fig. 2), independently of a previous phosphate treatment of mitochondria, suggesting that phosphate-dependent cyt *c* release and Ca^{2+}-dependent cyt *c* release are supported by different cyt *c* pools.

Cyt *c* exhibits high binding capability toward CL liposomes (Piccotti et al., 2004a). Binding occurs without leakage (Fig. 3) but produces the quenching of inserted $NBDC_{12}$-HPC, suggesting an interaction with the lipid phase of the membrane (Fig. 4). However, the biphasic behavior of fluorescence quenching indicates that the binding of additional cyt *c* occurs without interacting with the hydrophobic core of the membrane (Fig. 4). Bound cyt *c* is detached by phosphate via the so-called A site-dependent electrostatic interactions (Tuominen, Wallace & Kinnunen, 2002; Piccotti et al., 2004a) and without affecting $NBDC_{12}$-HPC fluorescence. Contrarily, fluorescence release accompanies cyt *c* removal by Ca^{2+}, indicating that phosphate and Ca^{2+} act on distinct cyt *c* pools (Fig. 5B). Furthermore, Ca^{2+} can remove the cyt *c* pool bound to the lipid phase even after the total removal of the loosely bound pool by 100 mM phosphate (Fig. 5A). Ca^{2+} affects CL liposomes, producing extensive leakage of fluorescent encapsulated ANTS/DPX because of the tendency of CL to form nonlamellar structures in the presence of Ca^{2+} (Epand et al., 2002), whereas phosphate does not affect the lamellar phase (Fig. 3). The use of models of CL liposomes may be open to question since the characteristics of membrane consisting exclusively of CL are different from those of mitochondrial membrane and the disruption of mitochondrial membrane by Ca^{2+}-dependent PTP activation is not mimicked in CL liposomes. Nevertheless, they have been advantageously employed in studies of molecular interactions with cyt *c* (Iverson et al., 2004). In favor of this simplified model is the finding that in mitochondria CL may be organized in patches (Piccotti et al., 2004b). Yet, it is well established that cyt *c* specifically and stoichiometrically binds to CL, providing a membrane attachment site for the protein and limiting the amount of the soluble pool (Tuominen, Wallace & Kinnunen, 2002; Iverson et al., 2004). Moreover, nonbilayer structures are shown in intact mitochondria (Van Venetie & Verkleij, 1982) and mitochondrial lipids convert from lamellar to hexagonal phases in the presence of Ca^{2+} (Cullis et al., 1980; Nicolay et al., 1985). Indeed, the propensity of the mitochondrial membrane to form hexagonal phases has been suggested to modulate the movement of Ca^{2+} through the membrane (Wolkowicz, 1988) as well as the activity of certain mitochondrial enzymes (Li, Zheng & Yang, 1995).

Structural and functional features of the interaction of cyt *c* with complex IV have been reported (Ferguson-Miller, Brautigan & Margoliash, 1976; Maneg et al., 2004). Complex IV activity is a marker of mitochondrial machinery efficiency in neurons (Wong-Riley et al., 1997). We assayed complex IV activity after incubation of mitochondria in different respiratory states. Compared to mitochondria incubated in the absence of exogenous substrates, mitochondria that had worked in state 3 presented about 25% reduction of activity (Fig. 6A). It is worth noting that complex IV is the only activity of the respiratory chain that is significantly decreased (about 25%) in the cortical regions of Alzheimer's disease *postmortem* brain tissue (Mutisya, Bowling & Beal, 1994; Kish et al., 1992). In our experiments, restoration of activity was obtained by supplementing mitochondria with exogenous CL (Fig. 6A), which fuses with the outer membrane and reaches the inner membrane of mitochondria (Piccotti et al., 2002). This result is in agreement with the finding that complex IV contains tightly bound CL, whose removal results in loss of structure and function (Sedlak & Robinson, 1999). Metabolically stressed mitochondria exhibited a more saturated fatty acid profile of CL than control (Piccotti et al., 2004a). This may account for the weakening of hydrophobic interactions and cyt *c* release, thus confirming the specific role played by CL in supporting the performance of respiratory complexes (Dale & Robinson, 1988; Zhang, Mileykovskaya & Dowhan, 2002). Phosphate-dependent cyt *c* removal did not affect complex IV activity, whereas activity was lost following Ca^{2+} pulse (Fig. 6B). Prevention of matrix Ca^{2+} accumulation by ruthenium red and CsA (Votyakova and Reynolds, 2005) avoided complex IV activity impairment. Ca^{2+} possesses a wide range of actions, including the removal

of tightly bound cyt *c* and modification of the layer structure of the inner mitochondrial membrane. Isolated mitochondria can accumulate Ca^{2+} in a process accompanied by the extrusion of protons and the decrease of $\Delta\psi_m$, whereas phosphate is transported across the inner membrane in exchange for OH^- (Nicholls & Budd, 2000). We found that phosphate loading of mitochondria and cyt *c* release did not alter $\Delta\psi_m$ (Fig. 7). This finding supports the idea that loosely bound cyt *c* is not involved in $\Delta\psi_m$ production. Adversely, impaired ability to produce potential is shown when mitochondria had previously accumulated Ca^{2+} (Fig. 7), suggesting that Ca^{2+} influences the components of the respiratory chain. It was found that exogenous CL exerts a stabilizing effect on the anchorage of cyt *c* with the inner membrane, preventing its release, and that the stabilization correlated directly with $\Delta\psi_m$ (Piccotti et al., 2002). Here, we confirm that CL enrichment of control, phosphate-, and Ca^{+2}-treated mitochondria produced a more homogeneous mitochondrial population characterized by higher $\Delta\psi_m$ (Fig. 7).

The coincubation of isolated nuclei and mitochondria in the presence of phosphate produced DNA fragmentation (Fig. 8). DNA fragmentation was prevented when mitochondria were previously enriched with CL or when incubation was performed in the presence of monoclonal anti-cyt *c* antibody. Hence, the enrichment of mitochondria with CL prevented phosphate-dependent cyt *c* release and downstream activated processes leading to DNA fragmentation.

In cerebellar neurons, cyt *c* is released from mitochondria in a reactive oxygen species (ROS)-dependent fashion and operates as a ROS scavenger (Atlante et al., 2000). The idea that the phosphate-dependent cyt *c* pool evidenced in this work could be the pool involved as ROS scavenger (Atlante et al., 2000) or as an electron shuttle between the outer and inner mitochondrial membrane (Bernardi & Azzone, 1981) may be a suggestive speculation.

Disturbance of intracellular Ca^{2+} homeostasis is associated with a wide range of neurodegenerative processes. Ischemia/reperfusion results in alteration in intracellular Ca^{2+} homeostasis and in inorganic phosphate accumulation to concentrations exceeding 20 mM (Crompton, 1999; Kowaltowski et al., 1996). Overstimulation of glutamate receptor in stroke or other chronic neurodegenerative diseases results in an increase of cytosolic Ca^{2+} (Lipton & Rosenberg, 1994). In these instances, mitochondria possess a high capacity to accumulate and retain Ca^{2+} in a process where phosphate is an accompanying ion (Nicholls & Chalmers, 2004). The separate effects exerted by phosphate and Ca^{2+}, otherwise not distinguishable when studying intact cells, have been evidenced in our study.

We report that in mitochondria cyt *c* bound to the inner membrane is organized in pools that are differently sensitive to factors such as phosphate and Ca^{2+}. Phosphate acts by detaching cyt *c* bound through electrostatic interactions, without affecting the geometrical order of the inner mitochondrial membrane. In contrast, Ca^{2+} may act by disturbing the geometry of the inner mitochondrial membrane, causing detachment of tightly bound cyt *c* and impairing membrane-bound activities. In particular, Ca^{2+} causes inhibition of complex IV activity, a key energy-metabolizing enzyme, leading to a reduction in energy stores, possibly contributing to neurodegenerative processes.

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