

Exogenous Phospholipids Specifically Affect Transmembrane Potential of Brain Mitochondria and Cytochrome *c* Release*

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Lucia Piccotti‡, Cristina Marchetti§, Graziella Migliorati§, Rita Roberti‡,
and Lanfranco Corazzi†¶

From the ‡Department of Internal Medicine, Laboratory of Biochemistry and §Department of Clinical Medicine, Pathology and Pharmacology, University of Perugia, Perugia 06122, Italy

Release of cytochrome *c*, a decrease of membrane potential ($\Delta\psi_m$), and a reduction of cardiolipin (CL) of rat brain mitochondria occurred upon incubation in the absence of respiratory substrates. Since CL is critical for mitochondrial functioning, CL enrichment of mitochondria was achieved by fusion with CL liposomes. Fusion was triggered by potassium phosphate at concentrations producing mitochondrial permeability transition pore opening but not cytochrome *c* release, which was observed only at >10 mM. Cyclosporin A inhibited phosphate-induced CL fusion, whereas Pronase pretreatment of mitochondria abolished it, suggesting that mitochondrial permeability transition pore and protein(s) are involved in the fusion process. Phosphate-dependent fusion was enhanced in respiratory state 3 and influenced by phospholipid classes in the order CL > phosphatidylglycerol (PG) > phosphatidylserine. The probe 10-nonylacridine orange indicated that fused CL had migrated to the inner mitochondrial membrane. In state 3, CL enrichment of mitochondria resulted in a pH decrease in the intermembrane space. Cytofluorimetric analysis of mitochondria stained with 3,3'-diethyloxycarbocyanine iodide and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide showed $\Delta\psi_m$ increase upon fusion with CL or PG. In contrast, phosphatidylserine fusion required $\Delta\psi_m$ consumption, suggesting that $\Delta\psi_m$ is the driving force in mitochondrial phospholipid importation. Moreover, enrichment with CL and PG brought the low energy mitochondrial population to high $\Delta\psi_m$ values and prevented phosphate-dependent cytochrome *c* release.

The role of mitochondria in neurodegenerative disease has been increasingly supported (1). Mitochondrial dysfunction can lead to deleterious cell injury due to free radical generation, intracellular calcium concentration imbalance, and activation of mitochondrial permeability transition pore (mPTP)¹ (2). In

apoptosis, a caspase cascade often mediates cell death (3). Despite the complexity of the mechanisms involved, mitochondria appear to provide a link between the initiator caspases and the downstream effector caspases. Mitochondria release apoptosis-inducing factors that trigger DNA fragmentation of isolated nuclei (4, 5). In addition, Bax acts on mPTP, causing the release of cytochrome *c* into the cytosol (3, 6). Released cytochrome *c* binds to Apaf-1, thus initiating activation of caspase-1 and of downstream processes, leading to nuclear DNA fragmentation. Cytochrome *c* release is also stimulated by Bid (7, 8), whereas Bcl2 prevents it (9).

The mechanism responsible for signaling molecule release from mitochondria is under extensive study. It is known that these molecules are bound to the inner mitochondrial membrane, whose integrity appears to be essential for mitochondrial functions. Cardiolipin (CL) is present only within the inner mitochondrial membrane, strictly associated to mitochondrial electron transport complexes and transporters (10). The specific requirement of heart cytochrome *c* oxidase for CL has been demonstrated (11). The small basic protein cytochrome *c* specifically binds to CL (12, 13). Since functional cytochrome *c* binds to cytochrome *c* oxidase, via its surface positive charge (14), both proteins must be sensitive to CL lipid matrix. Studies on the release of cytochrome *c* from the inner mitochondrial membrane indicate that peroxidation of CL causes disruption of the molecular interaction between CL and the protein (12). Degradation of mitochondrial CL occurs during p53-induced apoptosis (15) or in mitochondrial damage induced by nitric oxide in human leukemia cells (16). A significant decrease in CL and other phospholipids was observed in brain mitochondria during ischemia (17). A genetic model system to study the role of anionic phospholipids in yeast mitochondria indicates that the complete lack of CL causes a decrease of mitochondrial membrane potential (18) and inhibition of translation of protein components of the electron transport chain (19). In aged rat heart mitochondria, cytochrome *c* oxidase activity is restored to the level of the young control by exogenous CL (20).

In this study, the ability of mitochondria to acquire CL, phosphatidylglycerol (PG), and phosphatidylserine (PS) from exogenous sources *in vitro* through a fusion process was demonstrated. The fusion rate was dependent both on the nature of lipids and on the respiratory state of mitochondria. The acquired phospholipids specifically influenced both the transmembrane potential of mitochondria and the release of cytochrome *c*.

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¶ To whom correspondence should be addressed: Dept. of Internal Medicine, Laboratory of Biochemistry, University of Perugia, Via del Giochetto, 06122 Perugia, Italy. Tel.: 39-075-585-7423; Fax: 39-075-585-7428; E-mail: corazzi@unipg.it.

¹ The abbreviations used are: mPTP, mitochondrial permeability transition pore; S/H, sucrose/HEPES; R₁₈, octadecyl rhodamine B chloride; AO, 3,6-bis(dimethylamino) acridine; NAO, 10-N-nonyl-3,6-bis(dimethylamino) acridine; DiOC₆(3), 3,3'-dihexyloxycarbocyanine iodide; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; CL, car-

diolipin; PG, phosphatidylglycerol; PS, phosphatidylserine; $\Delta\psi_m$, mitochondrial membrane potential; IGFL, integral of fluorescence.

FIG. 1. Flow cytometry analysis of mitochondrial CL. Mitochondria (0.3 mg of protein) were loaded with NAO (0.3 μ M) and incubated for 20 min at 37 °C in different respiratory conditions. Frequency histograms of NAO-stained mitochondria in the resting state (A) and in state 3 plus 5 mM potassium phosphate (B) are shown. A representative experiment of three is shown.

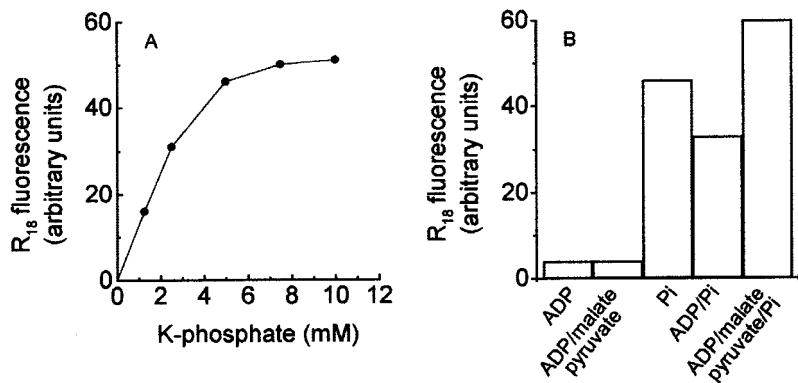
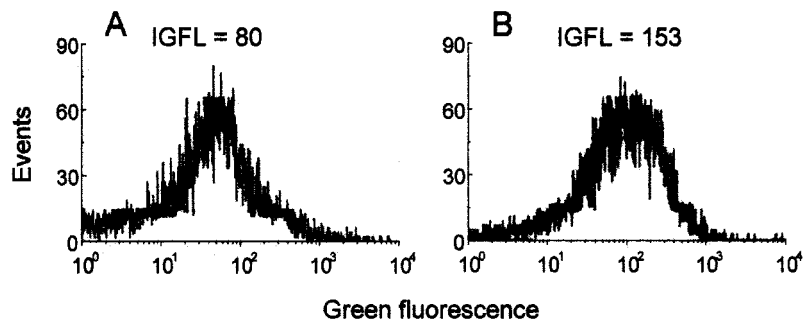


FIG. 2. Fusion of CL liposomes to R₁₈-loaded mitochondria. Liposomes (50 nmol of lipid) and R₁₈-loaded mitochondria (0.1 mg of protein) were added to 2 ml of S/H buffer. The elicited fluorescence was measured 2 min after the addition of potassium phosphate (*K*-phosphate; A), or respiratory substrates, in the presence or absence of 5 mM potassium phosphate (*Pi*; B). A representative experiment of three is shown.

EXPERIMENTAL PROCEDURES

Materials—Octadecyl rhodamine B chloride (R₁₈), 3,3'-diethyloxycarbocyanine iodide (DiOC₆(3)), and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) were from Molecular Probes Europe BV. Pronase, digitonin, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), 3,6-bis(dimethylamino) acridine (acridine orange; AO), and 10-*N*-nonyl-3,6-bis(dimethylamino) acridine (NAO) were from Fluka Chemie AG (Italy). Hepes, cytochrome *c*, complete protease inhibitor mixture, ADP (K⁺ salt), pyruvic acid, and malic acid were purchased from Roche Molecular Biochemicals. Pyruvic acid and malic acid solutions were adjusted to pH 7.0 with KOH. Cyclosporin A, CL, PG, and bovine brain PS were obtained from Sigma. Prior to use, phospholipids were purified by column chromatography (21). Rabbit anti-cytochrome *c* polyclonal IgG and donkey anti-rabbit horseradish peroxidase-conjugated IgG were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Mitochondria Preparation—Mitochondria were prepared from rat brain cortex as previously described (22). The mitochondrial pellet was resuspended in 0.32 M sucrose, 2 mM Hepes (pH 7.4) (S/H buffer), and biochemical characterization was performed as described (22). In some experiments, Pronase treatment of mitochondria was performed by incubating mitochondria (1 mg of protein in 1 ml of S/H buffer) with Pronase (25 μ g) at room temperature for 10 min. The reaction was stopped by adding protease inhibitor mixture (0.5 μ g) and pelleting by centrifuging 10 min at 9,000 \times *g*. Respiratory activity of mitochondria was modulated with ADP (0.8 mM), with pyruvate and malate (1.5 and 3.0 mM, respectively), or with ADP, pyruvate, and malate (0.8, 1.5, and 3.0 mM, respectively, state 3). When specified, potassium phosphate was added. In the resting state, substrates and phosphate were omitted.

Insertion of R₁₈ into the Outer Mitochondrial Membrane—An ethanolic solution of R₁₈ (10 μ l, 1 mg/ml) was added to 1 ml of mitochondrial suspension (1 mg of protein). The mixture was kept in the dark for 40 min at room temperature and chromatographed on a Sephadex G-50 column to remove the noninserted R₁₈. In some experiments, Pronase treatment of mitochondria was performed before the insertion of R₁₈. The amount of R₁₈ incorporated was calculated by comparing R₁₈ fluorescence of mitochondrial suspensions solubilized with 0.03% Thesit to that of standard R₁₈ solutions. For R₁₈ surface densities of \leq 10 mmol/mol of membrane lipid, the fluorescence quenching of the probe was linearly related to surface density, thus allowing the determination of fusion extent (23).

Preparation of Liposomes—CL, PG, or PS unilamellar vesicles were prepared in S/H buffer by reverse phase evaporation (24). Liposomes were sized by extrusion using polycarbonate Unipore membranes

(0.1- μ m pore size; Millipore Corp.). Phospholipid concentration was determined by phosphate assay (25).

Fusion Kinetics of Liposomes to Mitochondria—Fusion was assayed in a cuvette containing S/H buffer, R₁₈-loaded mitochondria (0.1 mg of protein), and liposomes (50 nmol lipid) in a final volume of 2 ml. After the addition of potassium phosphate and respiratory substrates, fusion was monitored by following fluorescence increments at 580 nm (λ_{exc} , 560 nm) using a Shimadzu RF-5000 spectrofluorometer. Minimal initial fluorescence values of R₁₈-mitochondria before starting fusion were set at 0%. Fluorescence in the presence of 0.03% Thesit was taken as 100%.

Localization in Mitochondria of Fused CL with the NAO Probe—Mitochondria (0.3 mg of protein) were incubated at 37 °C for 10 min in the presence of respiratory substrates and CL liposomes (20 nmol of lipid), in S/H buffer (total volume 0.45 ml). To remove nonfused CL, samples were layered on 0.5 ml of 0.6 M sucrose, centrifuged for 10 min at 11,000 \times *g*, and the pellets were resuspended with 0.4 ml of S/H buffer. NAO probe was added to a final concentration of 50 μ M, and samples were incubated for 5 min at room temperature. Each sample was then divided into two identical aliquots; one was treated with digitonin (0.4 mg/mg mitochondrial protein) to remove the outer mitochondrial membrane. After incubation in ice for 30 min with stirring, all samples were centrifuged at 9,000 \times *g* for 20 min, and NAO fluorescence (λ_{exc} , 494 nm; λ_{em} , 530 nm) was measured in mitoplasts and in the supernatant.

AO Accumulation in Mitochondria—The pH-sensitive dye AO was used to visualize the acidification of the intermembrane space of mitochondria. Mitochondria (0.6 ml, 0.8 mg of protein) were mixed with CL liposomes (15 nmol of lipid) in the presence of 5 mM potassium phosphate (pH 7.4), ADP, pyruvate, and malate. Potassium phosphate and respiratory substrates were omitted in controls (resting state). The mixtures were incubated for 10 min at 37 °C and centrifuged on 0.5 ml of 0.6 M sucrose for 10 min at 11,000 \times *g*. Pellets were resuspended in 0.6 ml of S/H buffer, and aliquots (0.1 mg of protein) were placed in a cuvette containing S/H buffer (total volume, 1.5 ml). After the addition of AO (4 μ M final concentration), the suspension was stirred for 2 min, and AO fluorescence (λ_{exc} , 494 nm; λ_{em} , 530 nm) was measured.

Swelling of Isolated Mitochondria—Mitochondrial swelling was determined by measuring the decrease of light scattering at 520 nm of a mitochondrial suspension (75 μ g of protein in 1.5 ml of S/H buffer) in the presence of potassium phosphate and respiratory substrates, with or without 15 μ M CCCP. In some experiments, mitochondria were pretreated for 5 min with cyclosporin A (5 μ M). The loss of scattering induced by CCCP within 5 min was considered the maximal swelling value.

Enrichment of Mitochondria with Selected Phospholipids—Mitochondria (0.4 mg of protein) were incubated with CL, PG, or PS lipo-

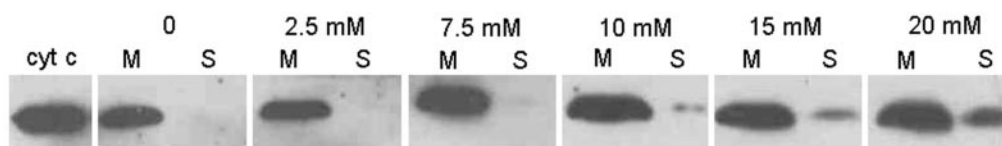


FIG. 3. **Potassium phosphate-dependent release of cytochrome *c* (*cyt c*).** Aliquots of mitochondria (0.2 mg of protein) were incubated for 20 min at 20 °C in the presence of increasing potassium phosphate concentrations and centrifuged for 10 min at $9,000 \times g$. Western blot analysis was performed in the mitochondrial pellet (*M*) and in the extramitochondrial medium (*S*). A representative experiment of three is shown.

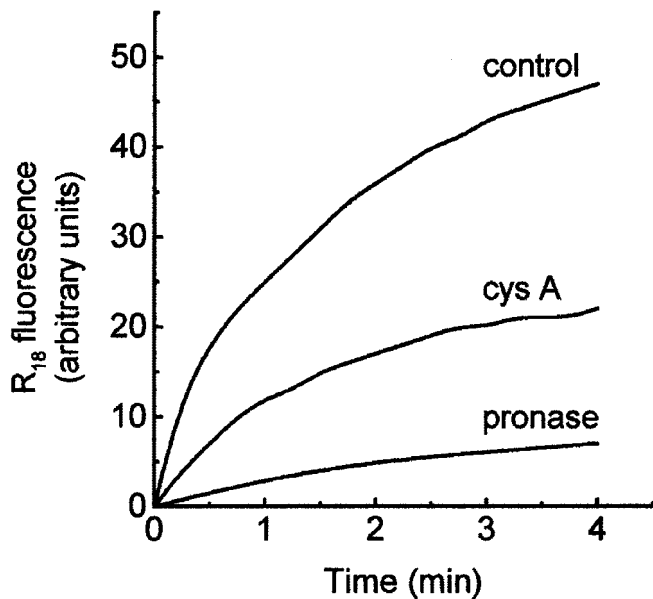


FIG. 4. **Fusion kinetics of CL liposomes to R_{18} -loaded mitochondria.** Liposomes (50 nmol of lipid) were added to 2 ml of S/H buffer containing 5 mM potassium phosphate and control or Pronase-treated R_{18} -mitochondria (0.1 mg of protein), and the elicited R_{18} fluorescence was monitored. The effect of cyclosporin A (*cys A*) (10 μM) on the fusion of CL with control mitochondria was evaluated. Pronase treatment of mitochondria was performed before the insertion of the fluorescent probe. Each sample presented the same maximal fluorescence intensity in the presence of 0.03% Thesit. A representative experiment of three is shown.

somes (20 nmol of lipid) in the presence of 5 mM potassium phosphate (pH 7.4). Respiratory activity of mitochondria was started by adding ADP, pyruvate, and malate in a total volume of 0.2 ml. The mixtures were incubated for 10 min at 37 °C, and lipid excess was eliminated by centrifugation on 0.5 ml of 0.6 M sucrose, as described. Lipids were extracted from aliquots of mitochondria (26), and phosphorus was determined in phospholipid classes after separation by two-dimensional thin layer chromatography (22). Mol % of CL was calculated taking into account 2 mol of phosphorus/mol of CL.

DiOC₆(3) Loading of Mitochondria and Release Kinetics—Mixtures containing mitochondria (0.8 mg of protein), 0.5 μM DiOC₆(3), 5 mM potassium phosphate (pH 7.4), and respiratory substrates (total volume, 0.5 ml) were preincubated for 10 min at 37 °C. Positive controls contained an additional 3 mM NaNO₃. After the addition of S/H buffer or CL liposomes (20 nmol of lipid), incubation was continued for 10 min. Samples were layered on 0.5 ml of 0.6 M sucrose and centrifuged for 10 min at $11,000 \times g$, and pellets were resuspended in 0.5 ml of S/H buffer. Aliquots of DiOC₆(3)-loaded mitochondria (0.1 mg of protein in 0.2 ml of S/H buffer) were incubated at 37 °C for various times. At the fixed time, mitochondrial suspensions were centrifuged for 10 min at $9,000 \times g$, the pellets were resuspended in 0.2 ml of S/H buffer, and DiOC₆(3) fluorescence was determined (λ_{exc} , 484 nm; λ_{em} , 501 nm).

Flow Cytometry Analysis—For mitochondrial membrane potential ($\Delta\psi_m$) determination, isolated mitochondria (0.3 mg of protein) were incubated for 5 min at room temperature in the dark, in the presence of 0.3 μM DiOC₆(3) or 1 μM JC-1, in different respiratory conditions. CL, PG, or PS liposomes (20 nmol of lipid) were added, and fusion was allowed for 5 min at room temperature. Suspensions were analyzed immediately by flow cytometry analysis, using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems) equipped with a focused argon laser. For complete depletion of $\Delta\psi_m$ (positive control), the

mitochondrial uncoupler CCCP (100 μM) was used. Data were analyzed and stored in a data management system (LYSYS software). DiOC₆(3) green fluorescence (FL1) was plotted on a logarithmic scale versus the frequency of events. The mean value of the integral of fluorescence (IGFL) was also evaluated (22). JC-1 fluorescence of mitochondria was detected as described (27), and red/green fluorescence emission (FL1/FL2) of particles was reported as a dot plot.

For the cytofluorimetric analysis of CL content, mitochondria (0.3 mg of protein) were incubated for 20 min at 37 °C with 0.3 μM NAO in different respiratory conditions. The green fluorescence (FL1) of the CL-specific dye was monitored as previously described.

Release of Cytochrome *c* from Isolated Mitochondria—First, mitochondria (0.2 mg of protein) were incubated for 20 min at 20 °C in the presence of increasing potassium phosphate concentrations (pH 7.4). In some experiments, incubations were performed in S/H buffer without phosphate for up to 60 min at 37 °C. The mitochondrial pellet and the extramitochondrial medium were recovered by centrifugation for 10 min at $9,000 \times g$.

Next, mitochondria (0.2 mg of protein) were incubated for 10 min at 37 °C in the presence of 5 mM potassium phosphate, respiratory substrates, and liposomes (15 nmol of lipid). Controls were without liposomes. Samples were centrifuged on 0.5 ml of 0.6 M sucrose as described. Recovered mitochondria were incubated for an additional 20 min at 37 °C in the presence of 20 mM potassium phosphate and centrifuged at $9,000 \times g$ for 10 min.

In both experiments, pellets and supernatants were subjected to Western blot analysis, using anti-(cytochrome *c*) IgG.

RESULTS

Flow Cytometry Analysis of Mitochondrial CL—The specific interaction of NAO probe with CL was exploited to evaluate CL level in mitochondria incubated in different metabolic conditions. Mitochondria were loaded with NAO in the presence or absence of respiratory substrates and analyzed by cytofluorimetry. In the resting state, the fluorescence histogram was shifted to the left, compared with metabolically active mitochondria (Fig. 1), suggesting a loss of CL during incubation at low energy.

Fusion of CL Liposomes with Mitochondria—Since CL decreased in the resting state, we examined the conditions required to supply CL from exogenous sources. Potassium phosphate, essential for mitochondrial respiration, was found to trigger the mixing of CL liposomes with the outer mitochondrial membrane. The fusion extent, measured as R_{18} fluorescence dequenching, increased up to 7 mM potassium phosphate and remained constant at higher concentrations (Fig. 2A). The same results were obtained when sodium phosphate replaced potassium phosphate. Respiratory substrates did not trigger fusion in the absence of phosphate but influenced the phosphate-dependent fusion. In fact, a mixture of ADP, malate, and pyruvate activated the process, whereas ADP was inhibitory (Fig. 2B).

Exposure of mitochondria to 5 mM potassium phosphate resulted in slow mitochondrial swelling and mPTP opening, as suggested by a 5% decrease of light scattering. Mitochondrial swelling did not increase at potassium phosphate concentrations higher than 5 mM but increased after the addition of 10 μM CCCP, producing a 12% decrease of light scattering. Opening of the mPTP was not accompanied by cytochrome *c* release, observed only for phosphate concentrations higher than 10 mM (Fig. 3). Cyclosporin A negatively affected the kinetics of phosphate-triggered CL fusion to mitochondria (Fig. 4), indicating that mPTP was involved in liposome fusion. Phosphate was

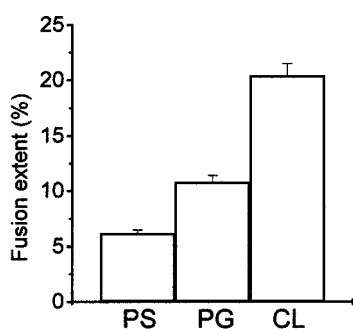


FIG. 5. **Fusion extent of different phospholipids with R₁₈-mitochondria.** Liposomes (50 nmol of lipid) and R₁₈-loaded mitochondria (0.1 mg of protein) were added to 2 ml of S/H buffer. Elicited fluorescence was measured 2 min after the addition of 5 mM potassium phosphate and respiratory substrates (state 3). The fusion extent was evaluated as a percentage of the maximal elicited fluorescence in the presence of 0.03% Thesit. Values are the mean \pm S.D. of four independent experiments.

ineffective in promoting CL fusion with Pronase-treated mitochondria (Fig. 4).

To test whether the ability of potassium phosphate to trigger fusion of liposomes to mitochondria depended on phospholipid classes, the fusion rate of CL was compared with that of PG and PS. The fusion extent of liposomes to R₁₈-mitochondria was CL > PG > PS (Fig. 5).

Enrichment of Mitochondria with Selected Phospholipids—Mitochondria were enriched with selected phospholipids by exploiting the ability of potassium phosphate to trigger the fusion of liposomes to mitochondria. In a standard experiment, metabolically active mitochondria were incubated with CL, PG, or PS liposomes in the presence of potassium phosphate and respiratory substrates. After fusion, the lipid excess was removed by centrifugation, and the phospholipid composition of fused mitochondria was determined. Total phospholipid content was within the value of 373 ± 18 nmol/mg of protein. Phosphatidylethanolamine and phosphatidylcholine were 32 ± 4 and $34 \pm 5\%$ of total, respectively. CL increased from 16 ± 2 to 23 ± 3 nmol/mg of protein (4.3–6.1%) in CL-fused mitochondria; PG increased from 14 ± 2 to 19.5 ± 3 nmol/mg of protein (3.8–5.2%) in PG-fused mitochondria; PS increased from 26 ± 3 to 32 ± 4 nmol/mg of protein (7–8.6%) in PS-fused mitochondria.

Localization of Fused CL in Mitochondria—NAO was used to evaluate the fate of fused CL in mitochondria, after incubation with CL liposomes, respiratory substrates, and/or potassium phosphate. After centrifugation on 0.6 M sucrose to remove nonfused CL, mitochondria were incubated with NAO, and aliquots were treated with digitonin to remove the outer mitochondrial membrane. NAO fluorescence was measured in mitoplasts and in intact mitochondria. In nonfused mitochondria, about 66% of NAO fluorescence was associated with mitoplasts, indicating that CL was localized mainly in the inner mitochondrial membrane. In CL-enriched mitochondria, NAO fluorescence in mitoplasts increased to 72 and 90% when fusion was performed in the presence of phosphate and phosphate plus respiratory substrates, respectively.

AO Fluorescence Measurement in CL-fused Mitochondria—Acidification of the intermembrane space of mitochondria that follows respiration causes trapping of the weak base AO in a protonated form, resulting in the formation of nonfluorescent dimer and multimer aggregates. Therefore, the decrease of pH in the intermembrane space of mitochondria can be measured as a decrease in AO fluorescence. Acidification was observed in the presence of respiratory substrates. Acidity was significantly enhanced in CL-enriched mitochondria during respira-

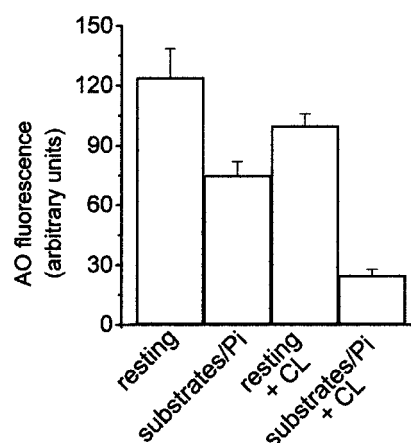


FIG. 6. **Probing of pH decrease in CL-fused mitochondria by AO fluorescence measurement.** Mitochondria (0.8 mg of protein) were incubated for 10 min at 37 °C in the resting state or in the presence of respiratory substrates and 5 mM potassium phosphate (Pi) (state 3), without or with CL liposomes (15 nmol of lipid). Nonfused CL was removed by centrifugation. Aliquots of mitochondria (0.1 mg of protein) were poured in a cuvette with 1.5 ml of S/H buffer, in the presence of 4 μ M AO, and fluorescence was measured after stirring for 2 min. Values are the mean \pm S.D. of four independent experiments.

tion but not in the resting state (Fig. 6). Acidification was also observed in PG-enriched but not in PS-enriched mitochondria (not shown).

$\Delta\psi_m$ Measurement in Phospholipid-enriched Mitochondria—DiOC₆(3)-loaded mitochondria were incubated in different respiratory conditions in the presence of CL, PG, or PS liposomes. After fusion, flow cytometry analysis was performed and IGFL values were calculated from single-parameter frequency histograms. IGFL value of 56 was measured in the resting state (Fig. 7). However, the basal value decreased during incubation of mitochondria at 37 °C (not shown). Complete depletion of $\Delta\psi_m$ in the presence of the mitochondrial uncoupler CCCP (positive control) and increase of $\Delta\psi_m$ in the presence of potassium phosphate and respiratory substrates were observed (Fig. 7). ADP, malate, and pyruvate had no effect on $\Delta\psi_m$ in the absence of potassium phosphate (not shown). An approximate 3.8- and 2.5-fold increase of IGFL was measured in CL- and PG-enriched mitochondria, respectively. In contrast, about 33% IGFL decrease was detected in PS-enriched mitochondria (Fig. 7). The observed increases of $\Delta\psi_m$ in CL- and PG-fused mitochondria were not measured when mitochondria were incubated with CL and PG in the absence of potassium phosphate (not shown). $\Delta\psi_m$ of Pronase-treated mitochondria was comparable with control in the resting state. However, Pronase treatment of mitochondria abolished the $\Delta\psi_m$ increase during incubation with respiratory substrates and CL liposomes (Fig. 7).

The fluorescent probe JC-1 was used to evaluate mitochondrial heterogeneity with respect to $\Delta\psi_m$. JC-1 selectively enters into mitochondria and changes color from green to orange after $\Delta\psi_m$ increase. $\Delta\psi_m$ distribution of mitochondrial populations is reported in Fig. 8. Each dot represents a single organelle analyzed for its green (*abscissa*) and orange (*ordinate*) associated fluorescence. In the resting state, mitochondria appeared as scattered, heterogeneous particles, most of them (80%) with green fluorescence. In the energized state, a shift toward orange fluorescence was observed, although about 20% of particles still exhibited a low energy state. Enrichment of mitochondria with CL produced a homogeneous mitochondrial population, 97% of particles with high fluorescence values.

Kinetics of DiOC₆(3) Release from Mitochondria—Mitochondria were loaded with DiOC₆(3) and incubated with respiratory substrates. CL liposomes were added, and fusion was allowed.

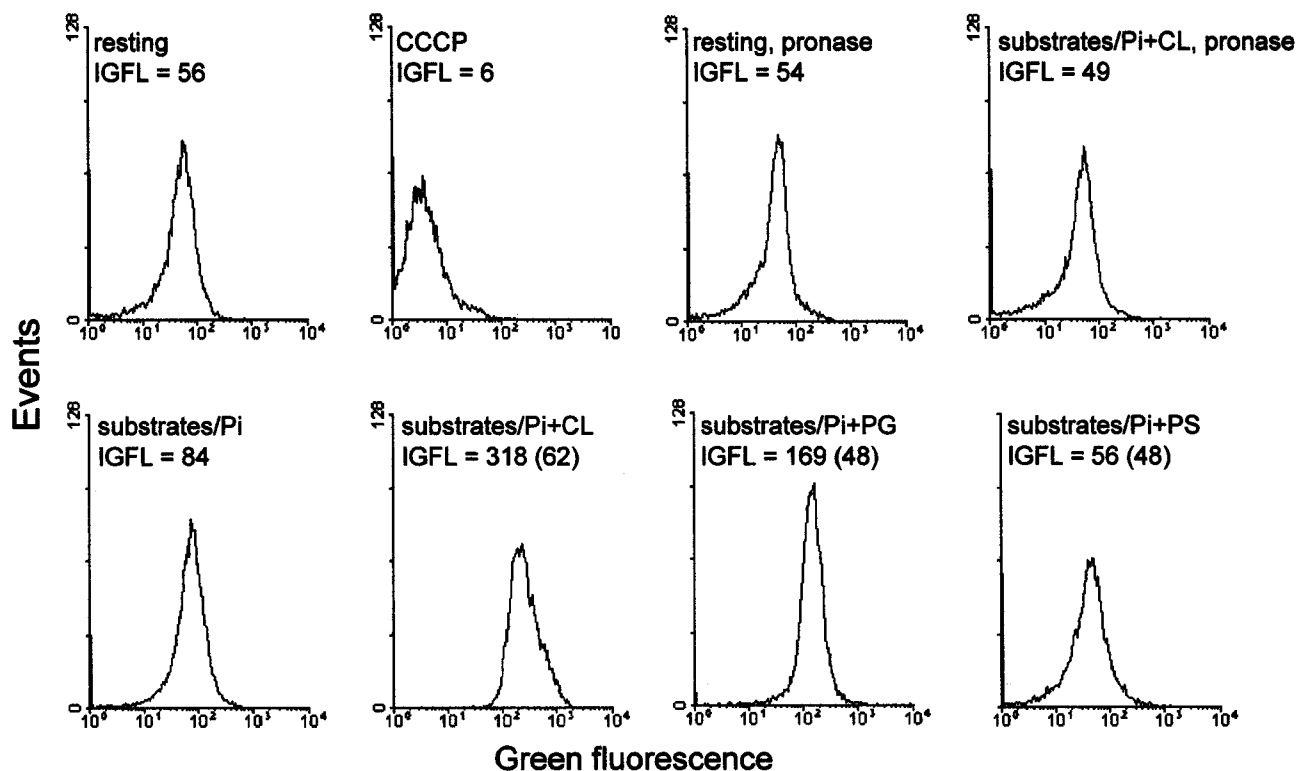


FIG. 7. Flow cytometry analysis of $\Delta\psi_m$ in DiOC₆(3)-stained mitochondria. Control or Pronase-treated mitochondria (0.3 mg of protein) were loaded with DiOC₆(3) (0.3 μ M) and incubated for 5 min at room temperature in the resting state or in state 3 plus 5 mM potassium phosphate (Pi). CL, PG, or PS liposomes (20 nmol of lipid) were added, and fusion was allowed for 5 min at room temperature before flow cytometry analysis. Numbers in parentheses indicate the IGFL value in the presence of CCCP. A representative experiment of three is shown.

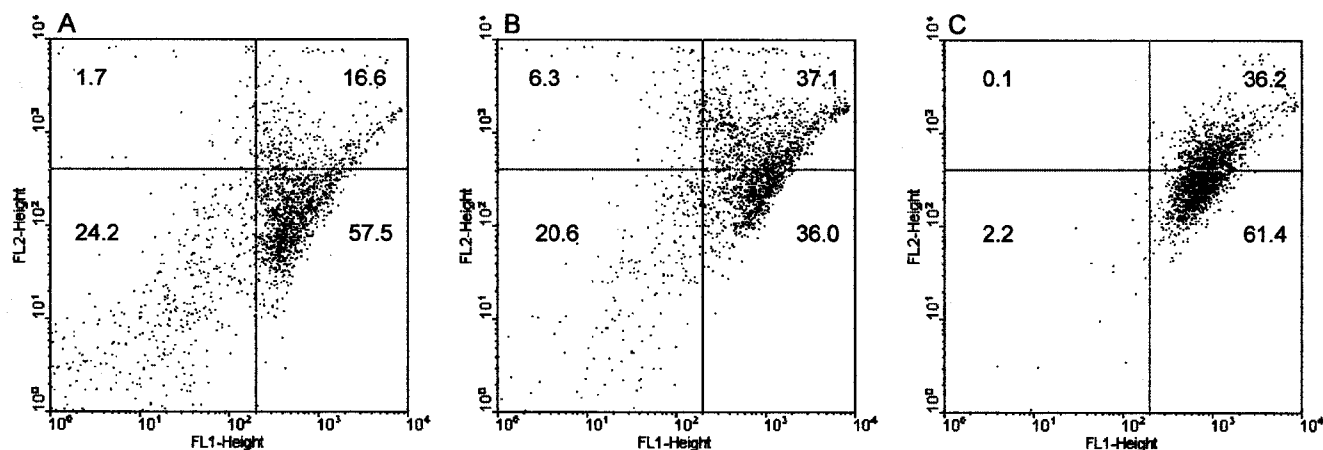


FIG. 8. Flow cytometry analysis of $\Delta\psi_m$ of single mitochondria stained with JC-1. Mitochondria (0.3 mg of protein) were loaded with JC-1 (1 μ M) in the resting state (A), in the presence of 5 mM potassium phosphate and respiratory substrates (B), and in the presence of 5 mM potassium phosphate, respiratory substrates, and CL liposomes (20 nmol of lipid) (C). The percentage values of mitochondria within prefixed energy sectors are reported. A representative experiment of three is shown.

After the removal of phospholipid excess by centrifugation on a sucrose gradient, the ability of DiOC₆(3)-loaded mitochondria to retain the fluorescent probe was tested. A time-dependent decrease of retained fluorescence was observed in control but not in CL-fused mitochondria (Fig. 9). A basal value of DiOC₆(3) fluorescence was retained in NaN₃-treated mitochondria. These data indicate that CL not only increases the uptake of DiOC₆(3) but also stabilizes the probe in the mitochondrial membrane.

Cytochrome *c* Release from Phospholipid-enriched Mitochondria—Mitochondria retained cytochrome *c*, when incubated for 20 min at 37 °C in S/H buffer; loss of cytochrome *c* was observed when incubation was prolonged to 60 min (not shown). A mas-

sive release of cytochrome *c* in the extramitochondrial medium was detected in the presence of 20 mM potassium phosphate (Fig. 10). However, in CL- and PG-enriched mitochondria, potassium phosphate did not elicit the same effect, and cytochrome *c* was retained in the inner membrane. PS enrichment of mitochondria did not prevent the loss of cytochrome *c*.

DISCUSSION

The present study provides evidence that some functional parameters of brain mitochondria are influenced by exogenous selected phospholipids acquired *in vitro* through a fusion process.

We found that mitochondria, incubated in the absence of

respiratory substrates, were subjected to $\Delta\psi_m$ decrease, cytochrome *c* release, and CL content reduction. CL is an essential phospholipid for many mitochondrial functions, which renders mitochondria completely autonomous for the synthesis of this lipid (28). Also, PG is synthesized in mitochondria, whereas other lipids, such as PS, are imported from mitochondria-associated membranes through a mechanism regulated by the respiratory state of mitochondria (22). Mitochondria can be enriched in CL through the fusion of CL liposomes. Fusion was triggered by phosphate (Fig. 2) in a concentration range that influenced mPTP but not cytochrome *c* release (Fig. 3). Mild proteolytic treatment of the outer mitochondrial membrane inhibited the phosphate-dependent fusion process (Fig. 4), suggesting that phosphate interacts with protein factors that, in mPTP opening conditions, allow mitochondrial acquisition of phospholipids. Hence, phosphate is not fusogenic *per se*, unlike divalent cations, such as Ca^{2+} (29).

The extent of liposome fusion depended on phospholipid classes (Fig. 5). Fused mitochondria were enriched in selected lipids. Compared with control, individual enrichment of CL, PG, or PS was about 44, 39, and 23%, respectively. Since in a standard fusion experiment, 20 nmol of lipids were mixed with 0.4 mg of mitochondrial protein, it can be calculated that 11–14% of added phospholipids were fused with mitochondria. Therefore, the enrichment was 5.5–7 nmol/mg of protein, less than 2% of total mitochondrial phospholipids.

The endogenous pool of CL is localized in the inner membrane of mitochondria (30). Experiments performed with NAO demonstrated that fused CL enriched the inner membrane. Consequently, the fusion process is consistent with a model in

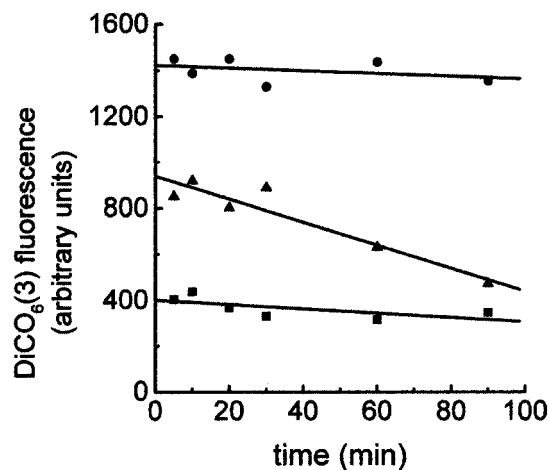


FIG. 9. Kinetics of DiOC₆(3) release from preloaded mitochondria. Mitochondria (0.8 mg of protein) were incubated with 0.5 μM DiOC₆(3) for 10 min at 37 °C, in the presence of 5 mM potassium phosphate and respiratory substrates, without (\blacktriangle – \blacktriangle) or with NaN_3 (\blacksquare – \blacksquare), or in the presence of 5 mM potassium phosphate, respiratory substrates, and CL liposomes (20 nmol of lipid) (\bullet – \bullet). After removal of nonfused CL, aliquots of DiOC₆(3)-loaded mitochondria were incubated for various times at 37 °C, and the fluorescence retained in mitochondria was measured. A representative experiment of three is shown.

which CL fuses with the outer mitochondrial membrane and flows inside through the contact points. The inhibitory effect of cyclosporin A on CL liposome fusion (Fig. 4), the localization of mPTP on the contact points (31), and the effect of potassium phosphate on mPTP suggest that mPTP opening is necessary for CL molecules to migrate into the inner mitochondrial membrane. This route might be operative also for PG, the biosynthetic CL precursor (32), and for PS, the substrate of PS decarboxylase, localized on the outer surface of the inner mitochondrial membrane (22).

AO is known to concentrate in acidic compartments. The enrichment of the inner mitochondrial membrane with CL favored the accumulation of AO. This result suggests that localization of the probe in the intermembrane compartment of mitochondria is a consequence of pH decrease that follows increased metabolic activity (Fig. 6). Evaluation of the functional heterogeneity of mitochondria populations under various metabolic conditions was performed by cytofluorimetry with DiOC₆(3) and JC-1 probes. Frequency determinations, using single-parameter fluorescence histograms, monitored the dynamic behavior of mitochondria. $\Delta\psi_m$ was modulated *in vitro* by respiratory substrates and strongly increased after CL or PG supply (Fig. 7), indicating that not only CL but also PG is possibly involved in mitochondrial respiration. Moreover, JC-1 indicated that the enrichment of mitochondria with CL brought the low energy population to high $\Delta\psi_m$ values (Fig. 8). In contrast, fusion of PS to mitochondria required $\Delta\psi_m$ consumption (Fig. 7), thus confirming that the driving force for importation of phospholipids resides in the mitochondrion (22). Despite the availability of substrates and the presence of CL, Pronase-treated mitochondria worked at basal level (Fig. 7). This result indicates that Pronase removed translocation factors without causing the rupture of the outer membrane, thus preventing substrates and CL from reaching the inner membrane of mitochondria.

DiOC₆(3) balanced between the aqueous medium and the inner mitochondrial membrane, depending on membrane potential (Fig. 9). Following the addition of CL, the equilibrium shifted to the right. DiOC₆(3) release kinetics indicated a time-dependent $\Delta\psi_m$ decline in control (Fig. 9). The release of DiOC₆(3) from CL-enriched mitochondria was very low, even after prolonged incubation, demonstrating that fused CL not only enhanced but also maintained the respiratory activity.

It has been hypothesized that the extent of cytochrome *c* association with the inner mitochondrial membrane is dependent on the interaction with CL and is regulated by the redox state (33). We found that phosphate, although acting on mPTP opening, did not induce cytochrome *c* release up to 10 mM, confirming that cytochrome *c* release is independent of the mPTP (34). This result suggests that phosphate-triggered cytochrome *c* release requires a phosphate concentration threshold, able to weaken CL-cytochrome *c* interactions. Enrichment of the inner membrane with CL or PG prevented cytochrome *c* release (Fig. 10), possibly by increasing the phosphate concentration threshold. The stabilization of cytochrome *c* in the membrane correlated directly with the high $\Delta\psi_m$ of brain mi-

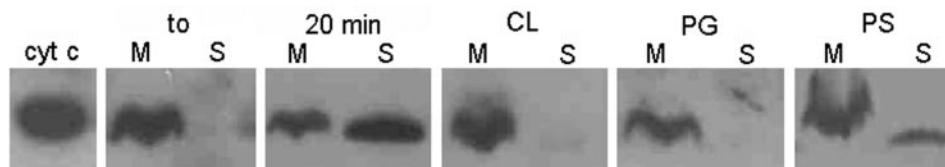


FIG. 10. Release of cytochrome *c* (cyt *c*) from mitochondria: Effect of fused phospholipids. Mitochondria (0.2 mg of protein) were incubated in the presence of 5 mM potassium phosphate and respiratory substrates in the presence or absence of liposomes (15 nmol of lipid). After recovery by centrifugation, mitochondria were incubated for 20 min at 37 °C in the presence of 20 mM potassium phosphate. Cytochrome *c* in the mitochondrial pellet (M) and in the supernatant (S) was detected by Western blotting. A representative experiment of three is shown.

tochondria after fusion with CL or PG (Fig. 7). In this respect, the ability of PG to partially substitute for CL is in agreement with the finding that cytochrome *c* presents binding sites for both CL and PG (35).

Detachment of cytochrome *c* from the inner membrane is an early event that leads to mitochondrial suffering and cell death. In cardiomyocytes, the decrease of mitochondrial membrane CL leads directly to decreased affinity of the membrane for cytochrome *c*, thus facilitating release for the initiation of programmed cell death (32). Mitochondrial defects occur in a wide variety of neurodegenerative disorders (36). Loss of CL has been documented in brain mitochondria during ischemia (17) and postischemic reperfusion (37). Cytochrome *c* release from mitochondria was monitored in cultured cerebrocortical neurons treated with staurosporine and *N*-methyl-*D*-aspartate (3) and in glutamate-induced neuron death (38). There are no data available on the correlation between CL and cytochrome *c* stability in the inner membrane of mitochondria in brain.

In this work, we exploited the possibility of transferring exogenous CL or PG to the inner membrane of brain mitochondria through a phosphate-mediated fusion process *in vitro*. The enrichment of the inner mitochondrial membrane with CL or PG may be relevant to the electrostatic interactions of cytochrome *c* with membrane lipids, resulting in the reinforcement of membrane-protein interactions. As a consequence, cytochrome *c* is retained in the mitochondrion, preventing the activation of downstream signals in apoptosis. In addition, a homogeneous mitochondrial population characterized by high $\Delta\psi_m$ is produced, thus improving mitochondrial functionality.

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Exogenous Phospholipids Specifically Affect Transmembrane Potential of Brain Mitochondria and Cytochrome *c* Release

Lucia Piccotti, Cristina Marchetti, Graziella Migliorati, Rita Roberti and Lanfranco Corazzi

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