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Cardiolipin Regulates the Activity of the Reconstituted Mitochondrial Calcium Uniporter by Modifying the Structure of the Liposome Bilayer

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Abstract. Reconstitution of mitochondrial calcium transport activity requires the incorporation of membrane proteins into a lipidic ambient. Calcium uptake has been measured previously using Cytochrome oxidase vesicles. The enrichment of these vesicles with cardiolipin, an acidic phospholipid that is found only in the inner mitochondrial membrane of eukaryotic cells, strongly inhibits calcium transport, in remarkable contrast with the activation effect that cardiolipin exerts upon other mitochondrial transporters and enzymes. The relation of the inactivation of calcium transport to the physical state of the bilayer was studied by following the polarization changes of 1,6-diphenyl-1,3,5-hexatriene (DPH) and by flow cytometry in the cardiolipin-enriched liposomes with incorporated mitochondrial solubilized proteins. Non-bilayer molecular arrangements in the cardiolipin-supplemented liposomes, detected by flow cytometry, may produce the fluidity changes observed by fluorescence polarization of DPH. Fluidity changes correlate with the abolition of calcium uptake, but have no effect on the establishment of a membrane potential in the vesicles required for calcium transport activity. Changes in the membrane structure and uniporter function are observed in the combined presence of cardiolipin and calcium leading to a modified lipid configuration.

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Abbreviations: COV: Cytochrome oxidase vesicles from asolectin; COV + Cl: Cytochrome oxidase vesicles from asolectin enriched with cardiolipin; Cl: cardiolipin; PC: phosphatidylcholine; TEA: triethanolamine; TMPD: N,N,N′,N′-tetramethyl-phenylendiamine; DPH: 1,6-diphenyl-1,3,5-hexatriene; Dis-C_3(5): 3,3′-dipropylthiodicarbocyanine; PC: egg-yolk L-α-phosphatidylcholine; CCCP: carbonyl cyanide *m*-chlorophenyl-hydrazone; Cl: bovine heart cardiolipin.

Key words: Calcium uptake — Mitochondria — Cardiolipin — Flow cytometry — Fluorescence polarization

Introduction

One of the fundamental problems in membrane protein function is protein-phospholipid interaction. Although it is claimed that only the tight binding of phospholipids to membrane proteins represents a specific interaction that regulates membrane protein activities (Beyer & Klingenberg, 1985), it is clear that the lipid environment also influences protein activity, and thereby participates in the regulation of biochemical processes. Changes of the mitochondrial lipid pattern strongly influence respiration and energy production, two important functions of mitochondria (Robinson et al., 1980; Brown & Cunningham, 1982). Binding of lipids to mitochondrial proteins and activation of enzymes by mitochondrial lipids have been studied in detail. Cardiolipin is an anionic phospholipid found only in the inner mitochondrial membrane of eukaryotic cells or in the plasma membrane of some prokaryotic cells (Hoch, 1992). It plays an important role in the function of several mitochondrial inner membrane systems, e.g., cytochrome bc1 and cytochrome c oxidase of the electron transport chain (Sedlák & Robinson, 1999), FOF1 ATPase (Eble et al., 1990) and mitochondrial transporters, including ADP/ATP translocase, the tricarboxylate carrier, the α-ketoglutarate, aspartate/glutamate and palmitoyl carnitine carriers (Horváth et al., 1990; Hoch, 1992). In addition, cardiolipin is a hexagonal-phase (H_{II})-preferring lipid that can form non-lamellar molecular arrangements in lipid bilayers (Smaal et al., 1987; Aguilar et al., 1999). Mitochon-

drial proteins, almost all lipophilic and/or basic, bind most strongly to acidic phospholipids, particularly to cardiolipin among the inner membrane phospholipids (Ioannou & Golding, 1979). The identity of the mitochondrial calcium uniporter and therefore its primary structure remains obscure, but it is attractive to assume that it could also be strongly associated with this particular phospholipid constituent of the inner mitochondrial membrane, as are almost all studied mitochondrial proteins. We have reported the reconstitution of a partially purified mitochondrial protein fraction with calcium-uptake transport activity. This was achieved using liposomes capable of maintaining an inside-negative membrane potential, namely cytochrome oxidase vesicles (COV) (Zazueta et al., 1994). Searching for an activation effect on the calcium uptake of these reconstituted fractions, we used COV supplemented with cardiolipin. We found that the addition of this phospholipid inhibits the uniporter activity, in contrast with the activation that it exerts on other well-studied proteins and transporters of the mitochondrial inner membrane, such as cytochrome c oxidase (Sedlák & Robinson, 1999); NADH dehydrogenase (Fry & Green, 1981) and the ATP/ADP carrier (Beyer & Nuscher, 1996). The inactivation could be related to a change in the cardiolipin-liposome membrane lipid arrangement, as deduced from diphenyl hexatriene polarization fluorescence and from cytofluorometric analysis. The importance of lipids that don't form lamellae, such as cardiolipin, to the functioning of a number of peptides and proteins has been pointed out, but, indeed, the physical basis for this relationship is not yet firmly established and has been intensively studied only in a few cases. The present report supports the notion of the biological importance of this kind of modulation.

Materials and Methods

Liposome Preparation

Mitochondria from rat kidney cortex were prepared as described (Chávez et al., 1985) in 0.25 M sucrose, 10 mM TRIS and 1 mM EDTA, pH 7.3, as isolation medium. Mitochondrial proteins were solubilized with sodium cholate at a final concentration of 1.6% in 50 mM H_3PO_4/TEA, pH 7.0 buffer, and centrifuged at 12,000 × *g* for 10 min at 4°C. The supernatants were assayed for protein content using the biuret method (Gornall, Barda Will and David, 1949). Two mg of solubilized mitochondrial proteins were incorporated into preformed cytochrome oxidase vesicles (COV) as previously reported (Zazueta, Holguín & Ramíyez, 1991). Briefly, dried lipids were dispersed in a 50 mM H_3PO_4/TEA pH 7.4 buffer at a final phospholipid concentration of 30 mg/ml. Where indicated, the vesicles were formed with Type IV-s soybean asolectin (Sigma, St. Louis, MO; catalog number P3644) supplemented with 18% of dried commercial beef heart cardiolipin, containing $>80\%$ of linoleic acid (Sigma, C5646), to maintain the reported composition of that phospholipid in the inner mitochondrial membrane (Hoch, 1992). Mitochondrial solubilized proteins were reconstituted in COV ± commercial beef heart cardiolipin after a gentle sonication step for 10 sec, and exhaustive dialysis overnight at 4°C against 250 volumes of 50 mM KH_2PO_4, pH 7.4. The proteoliposomes formed were extruded through a 0.45-μm pore-size Millipore filter, to obtain a liposome population of homogeneous size. Finally, the liposomes were passed through a G-50 Sephadex column pre-equilibrated with the same buffer, to eliminate non-incorporated protein.

Ca^{2+} Uptake

Quantification of calcium uptake was carried out by a combination of the filtration technique using 250 μM $^{45}CaCl_2$ (specific activity 1000 cpm/nmol) and the protamine sulfate aggregation technique (Zazueta et al., 1994).

Cytochrome Oxidase Activity

Reconstituted cytochrome oxidase activity was measured polarographically with an oxygen electrode at 25°C. The medium used was 50 mM H_3PO_4/TEA pH 7.4, 7.5 mM ascorbate, 0.75 mM TMPD (N,N,N′,N′-tetramethyl-phenylendiamine) and 75 μg ferrocytochrome c/ml. The respiration in COV was stimulated with 10 μM CCCP (carbonyl cyanide *m*-chlorophenyl-hydrazone) and inhibited with 1mM NaCN.

Membrane Potential

The procedure used to quantify the membrane potential generated upon oxidation of ferrocytochrome c by oxidase vesicles was essentially like that described by Matsushita et al (1984). The concentration-dependent quenching of 3,3′-dipropylthiodicarbocyanine [Dis-C_3(5)] was followed fluorometrically as it accumulated within oxidase vesicles in response to the membrane potential. The assay medium consisted of 50 mM H_3PO_4, pH 7.4, 0.5 μM Dis-C_3(5), 75 μg/ml ferrocytochrome c and 7.5 mM ascorbate/0.75 mM TMPD. The reaction was initiated with cytochrome oxidase vesicles (600 μg of phospholipids) or ascorbate/TMPD. A calibration curve of known membrane potentials was prepared by assaying the fluorescent quenching of Dis-C_3(5) in protein-free vesicles containing 50 mM KH_2PO_4, pH 7.4. These vesicles were diluted with lower-concentration solutions of KH_2PO_4, pH 7.4 in the presence of 1 μM of valinomycin and 0.5 μM Dis-C_3(5). The potassium concentration gradient ratio (inside/outside) in the vesicles was used to calculate the membrane potential by using the Nernst equation. Data from Dis-C_3(5) quenching in COV, in the presence of ferrocytochrome c, were quantified by comparison with valinomycin-mediated diffusion potentials. Lipids were extracted from proteoliposomes with chloroform:methanol (2:1), and analyzed by thin-layer chromatography, using Whatman K5 Silica gel, 80 Å. Separation was achieved by using the solvent system: chloroform:hexane:methanol:acetic acid (50:30:10:5) v/v/v/v. Spots were visualized by iodine vapor treatment. Quantitative determination of phospholipids from chloroform-based solutions and from thin-layer chromatography spots, were made by using 10N-nonyl acridine orange for cardiolipin (Petit et al., 1992) or a modified Fiske & Subarrow's phosphate-content quantification for total phospholipids (Bartlett, 1958).

Fluorescence Polarization

Fluorescence polarization was measured in liposomes incubated in 2 ml of medium containing 50 mM H_3PO_4, pH 7.0, 1 μM 1,6-

Table 1. Calcium transport and cytochrome oxidase activity reconstituted in liposomes supplemented with cardiolipin

Liposomes	Reconstituted Calcium Transport (nmol $^{45}Ca^{2+}$/mg/5 min)	Cytochrome Oxidase Activity ($nAtO_2$/mg/min)
COV[1]	39.0 ± 2.3	423.6 ± 66.3
COV + Cl[2]	0.0	502.2 ± 37.2

[1]COV: Cytochrome oxidase vesicles formed with Type IV-s soybean asolectin.
[2]COV + Cl: Cytochrome oxidase vesicles formed with 18% commercial beef heart cardiolipin and 82% Type IV-s soybean asolectin. Measurements were made as described in Materials and Methods with 0.5 mM $CaCl_2$ at 30°C. Values represent the mean of three different experiments ± SD.

diphenyl-1,3,5-hexatriene (DPH) solubilized in dimethylformamide. The mixtures were incubated for 30 min and then each sample was subjected to polarization fluorometry: 340 nm (emission) and 417 nm (excitation) (Goldstein, 1984). Where indicated, the polarization was evaluated at different temperatures.

Flow Cytometry

For flow cytometry experiments, Type IV-s soybean asolectin liposomes (COV) and liposomes supplemented with commercial beef heart cardiolipin (COV + Cl) were incubated in the presence of 250 μM $CaCl_2$ for 5 rnin at 30°C. Flow cytometry was accomplished using a Beckton Dickinson FACSCalibur Flow Cytometer. Ten-thousand liposomes were analyzed using Cell Quest Software at FL1 compensation of 0.8% and a detector compensation threshold FSC-H of 52 V, with the detectors: FSC of E00, SCC of 401 V and FL1 of 748 V. Results are reported as relative forward (FSC) and side scatter (SCC) histograms and dot graphs in logarithmic mode (Lampariello, 2000). The diffraction of the laser beam (FSC) is proportional to the liposome surface area and/or liposomal aggregation, while refraction plus reflection of the beam (SSC) are proportional to the complexity of liposomal bilayers (Aguilar, et al., 1999). Liposomes prepared from PC/Cl (2:1 mole ratio) were incubated with 500 μM $CaCl_2$ and were used as positive control of the formation of nonlamellar arrangements in the liposomal bilayers (Aguilar et al., 1999).

Results

Cytochrome oxidase was incorporated into asolectin liposomes (COV) to provide an inside-negative membrane potential, able to drive calcium uptake when co-reconstituted with solubilized mitochondrial proteins. Table 1 shows mitochondrial calcium uptake in liposomes supplemented with cardiolipin (COV + Cl). Also shown is the effect of this anionic phospholipid on the activity of the reconstituted cytochrome oxidase. Calcium transport was abolished in cardiolipin-enriched liposomes, while the activity of cytochrome oxidase was not significantly modified. The obtained values were 423.6 ± 66.3 $nAtO_2$ in control liposomes *vs.* 502.2 ± 37.2 $nAtO_2$ in the cardiolipin-enriched liposomes. Addition of CCCP slightly increased cytochrome oxidase activity in control liposomes, i.e., 507 ± 29 $nAtO_2$ with no significance at $P < 0.05$, while respiration was not stimulated in the already activated cytochrome oxidase vesicles enriched with cardiolipin. These results

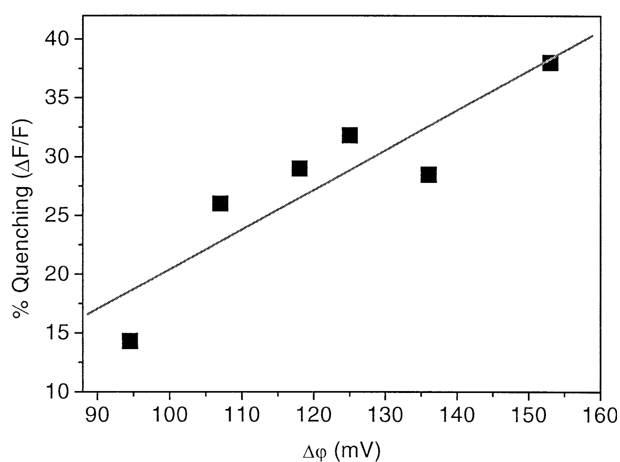

Fig. 1. Fluorescence quenching of Dis-C_3(5) induced by valinomycin-mediated diffusion potentials of defined magnitudes. Fluorescence quenching of Dis-C_3(5) was measured in protein-free liposomes, to generate a calibration curve. $\Delta\varphi$: Membrane potential (mV; inside negative).

are consistent with previous data from Paradies, Petrosillo & Ruggiero, (1997), who reported that the effect of cardiolipin on bovine cytochrome oxidase from normal rats was negligible when PC/CL liposomes were fused with mitochondria. In addition, we observed that the enrichment of asolectin vesicles with cardiolipin had no effect on the ability of complex IV to maintain a membrane potential that would support calcium uptake. A standard curve of DiS-C_3-(5) fluorescence quenching in response to protein-free vesicles exhibiting defined membrane potentials is shown in Fig. 1. This curve was similar both in presence or absence of cardiolipin. From this standard curve the membrane potential generated by oxidase vesicles reconstituted with solubilized mitochondrial proteins in the presence and absence of cardiolipin was determined (Table 2). Asolectin liposomes and cardiolipin-enriched asolectin liposomes maintained a membrane potential in the presence of TMPD and cytochrome c. The oxidation of ferrocytochrome c generated a membrane potential of about −124 mV and −99 mV, respectively. This is in good agreement with values reported by Singh & Nicholls (1986) of −100 mV, and Madden & Re-

Table 2. Quantitation of membrane potential ($\Delta\varphi$) in cytochrome oxidase vesicles upon ferrocytochrome c oxidation

Liposomes	Membrane potential (mV)			
	−Cardiolipin		+Cardiolipin	
	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$
COV	−124 ± 10	−112 ± 8.2	−99 ± 6.4	−97 ± 8.6
COV Plus mitochondrial kidney proteins	−101 ± 12.3	−102 ± 8.1	−96 ± 21.6	−117 ± 8.8

The reaction mixture contained 50 mM H_3PO_4, pH 7.4, 0.5 µM Dis-C_3(5), 75 µg/ml cytochrome c and 7.5 mM ascorbate/ 0.75 mM TMPD; the reaction was initiated with 600 µg of phospholipids. Values represent the mean of three different experiments ± SD. Where indicated, 250 µM $CaCl_2$ was added to the incubation medium. The means are not significantly different at $p < 0.05$ (One-way ANOVA test).

delmeier (1994) of −90 mV for proteoliposomes containing cytochrome oxidase. When mitochondrial proteins were incorporated into the vesicles, the observed potential was −101 mV in cytochrome oxidase liposomes and −96.5 mV in the liposomes supplemented with cardiolipin, showing that in the presence of cardiolipin the negative inside membrane potential was maintained in the liposomes. To discard the notion that the interaction of calcium with the cardiolipin head group promoted a permeability transition, we also measured the transmembrane potential in presence of high calcium concentrations, i.e., 250–500 µM. The values obtained were between −97 and −117 mV. There were no significant changes in the transmembrane potential in the vesicles assayed under the described conditions and they were similar to those determined in the absence of cardiolipin, −112 and −102 mV, respectively.

In Fig. 2, the temperature-dependent calcium accumulation in both liposome types is shown. COV maximum transport, e.g., 17 nmol Ca^{2+}/mg/5 min, was achieved at temperatures between 30°C and 55°C; however, at low temperatures, calcium was accumulated to moderate levels. In contrast, liposomes enriched with cardiolipin only accumulate calcium above 30°C. Arrhenius plots of COV calcium transport showed an inflection point around 30°C (Fig. 2*B*). At low temperatures, the energy of activation was calculated as 12.14 Kcal/mol/°K, while above 30° the energy of activation required for transport was diminished to 3.52 Kcal/mol/°K. The phase transition temperature in COV supplemented with cardiolipin was abolished and the energy of activation was 9.68 Kcal/mol/°K, close to the value determined for slow uptake in control liposomes (E_a = 12.14 Kcal/mol/°K).

The incorporation of cardiolipin into liposomes was verified by its isolation and separation on the second dimension thin layer chromatography. The first solvent system, chloroform:methanol:water (65:25:4), resolved cardiolipin as a spot with an Rf of 0.55. By using a second-dimension solvent system chloroform:hexane:methanol:acetic acid (50:30:10:5; v/v/v/v), we achieved separation of minor contaminants from cardiolipin. The *Rf* for cardiolipin was 0.75; a spot with comparable *Rf* was detected in liposomes supplemented with cardiolipin (*not shown*). Binding experiments using acridine orange were performed to determine the concentration of incorporated cardiolipin after reconstitution experiments in the vesicles. Cardiolipin values were calculated from the absorption spectrum ratio at 495 nm/474 nm of acridine orange. At the dye concentrations used (40 µM), the absorption spectrum of free acridine orange shifts its maximum from 495 nm to 474 nm, corresponding to the dimeric form of the dye. The decrease in absorption at 495 nm corresponds to the disappearance of acridine orange monomers, while the increase in the peak at 474 nm indicates the formation of dimers induced by cardiolipin binding. A plot of the ratio at 495 nm/474 nm of acridine orange in presence of increasing standard of reference commercial beef heart cardiolipin concentrations, results in a straight line. An almost 3-fold enrichment of cardiolipin in COV + Cl (45.2 ± 3 nmol Cl/mg phospholipid) was achieved with respect to liposomes formed with asolectin alone (15.9 ± 3 nmol Cl/mg phospholipid). The amount of total phospholipids in both kinds of liposomes quantified by their phosphate content (Bartlett, 1958), was 6.3 ± 0.3 and 3.4 ± 0.24 mg, respectively.

Asolectin is known to contain cardiolipin. Quantification of cardiolipin content in commercial Type IV-s soybean asolectin used in these experiments yields a value of 2%. It has also been noted that the incorporation of solubilized kidney mitochondrial proteins and cytochrome oxidase increased the content of cardiolipin in both kinds of liposomes (2.5 nmol Cl/mg phospholipid). Total cardiolipin fatty acyls were nearly the same, as cardiolipin from rat kidney contained 76.3% of linoleic acid (Clark et al., 1983), while cardiolipin from beef heart contains 78% of linoleic acid and cardiolipin from asolectin represented less than 2% of total phospholipids in the liposomes.

Fluorescence polarization of DPH embedded in a lipid bilayer is related to the membrane physical state: fluorescence polarization values are high in the gel-

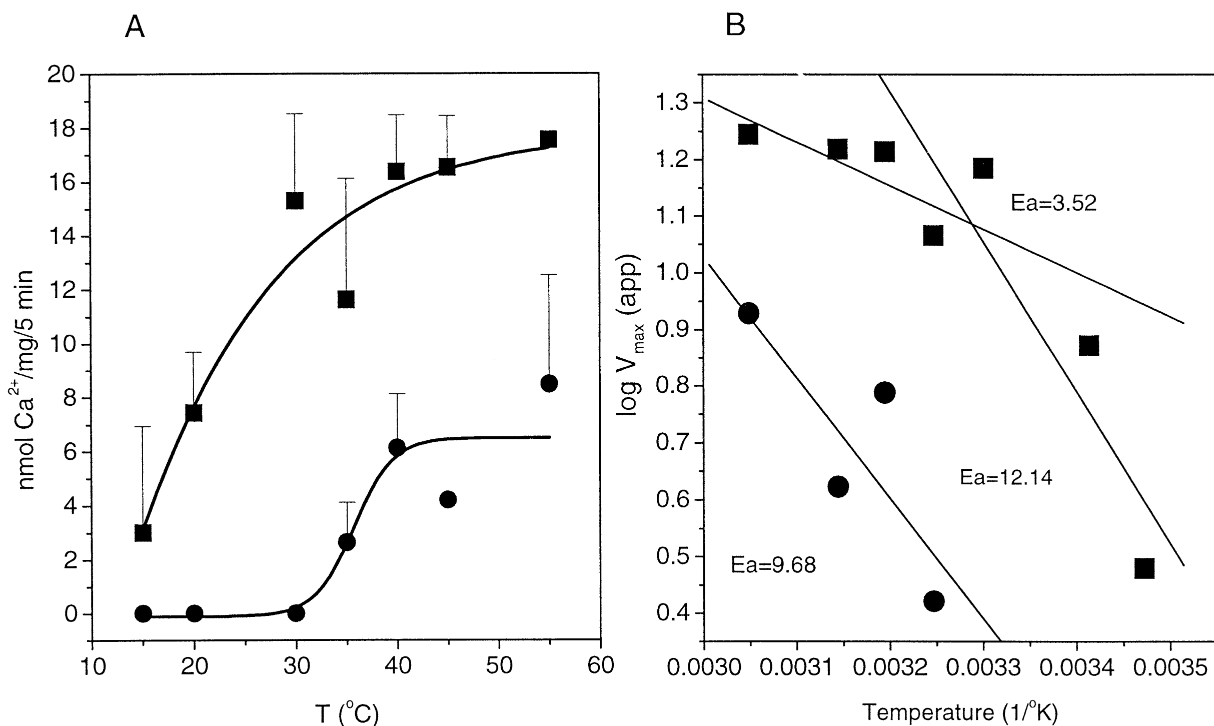

Fig. 2. Temperature-dependent calcium accumulation in cytochrome oxidase liposomes alone and supplemented with cardiolipin. (*A*) Calcium uptake as a function of temperature in cytochrome oxidase vesicles reconstituted with solubilized mitochondrial proteins, in presence (●) or absence (■) of commercial beef heart cardiolipin. (*B*) Arrhenius plot of calcium uptake in cytochrome oxidase vesicles reconstituted with solubilized mitochondrial proteins in presence (● $R = 0.82$) or absence of commercial beef heart cardiolipin (■; $R = 0.80$ and $R = 0.88$ for uptake at low and high temperatures, respectively). The assay medium consisted of 50 mM H_3PO_4, pH 7.4, 75 μg/ml cytochrome c, 7.5 mM ascorbate/0.75 mM TMPD and 250 μM $^{45}CaCl_2$ (specific activity 1000 cpm/nmol). After 5 min, the liposomes were aggregated by the addition of 0.8 mg of protamine sulfate. Immediately, an aliquot was withdrawn and filtered through a 0.25-μm pore size Millipore filter and washed with 10 mM $CaCl_2$. Specific activity values represent the difference between the energized (with cytochrome oxidase substrate) and nonenergized uptake rates (without substrate). Results are expressed as the mean of three different experiments ± SD. Experimental data were linearly fitted with the Microcal™ Origin™ Software, version 5.0. The standard deviation of the fits ranged between 0.05 and 0.1 with *p* values = 0.1.

crystalline phase and low in the fluid phase. Fluorescence polarization of DPH in vesicles supplemented with increasing cardiolipin concentrations was measured at 25°C. As the cardiolipin/phosphatidylcholine ratio increased, a decrease in polarization values was observed, indicating changes in fluidity of the liposomal bilayer (Fig. 3).

To evaluate the induction of changes on the physical state in cardiolipin-enriched liposomes in the presence of calcium, the fluorescence polarization of DPH was measured as a function of temperature under the transport-assay conditions. In the absence of calcium, similar polarization values of the probe DPH were observed in liposomes made mainly from asolectin and in those supplemented with cardiolipin. This pattern shows important changes when liposomes are incubated with calcium (Fig. 4). Polarization values increased in liposomes enriched with cardiolipin, indicating a shift to a gel-crystalline phase. It has been seen that calcium stabilizes Cl-liposomes, with its greatest effect being observed at high temperatures (>30°C) while Ca^{2+} fluidizes exogenous Cl-free liposomes, with its greatest effect being exerted at low temperatures (<30°C).

A change in the physical state of the liposomal membrane was also verified by flow cytometry experiments. In the absence of cardiolipin, cytochrome oxidase vesicles incubated with calcium under cation transport conditions, i.e., 250–500 μM Ca^{2+} for 5 min at 30°C, showed side scatter (SCC) laser beam values (Fig. 5, panel *a*), which increased 10-fold in liposomes supplemented with cardiolipin (Fig. 5, panel *b*). This increase in SCC, with differences (*D*) on a logarithmic scale, between calcium-treated liposomal populations $D = 0.70$ at $p < 0.001$, with a very high statistical significance, clearly showed a high bilayer complexity in liposomes supplemented with cardiolipin (Fig. 5, panel *c*). These SSC values revelead the presence of nonlamellar arrangements in the liposomes supplemented with cardiolipin. This effect was not detectable in liposomes made from asolectin ± cardiolipin, in absence of calcium (Fig. 5, panels *d*–e). Panels *c* and *f* clearly showed that the marked change in the bilayer organization ocurred only after the addition

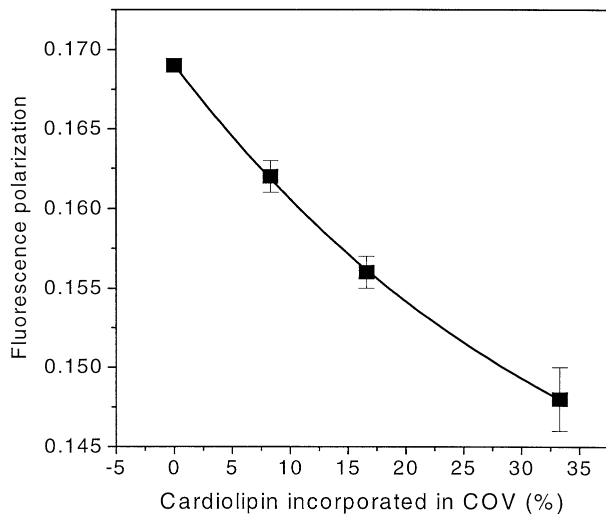

Fig. 3. Fluorescence polarization of DPH embedded in cytochrome oxidase vesicles (COV) reconstituted with mitochondrial proteins and supplemented with increasing cardiolipin concentrations. The assay medium was 50 mM KH_2PO_4, pH 7.4, at 25°C. Values represent the mean of three different experiments ± SD.

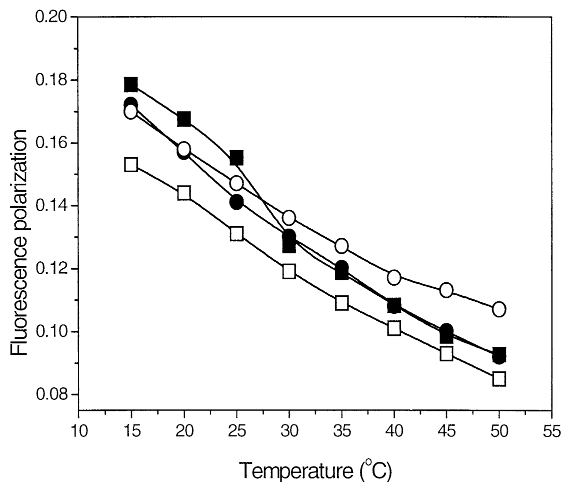

Fig. 4. Fluorescence polarization of DPH embedded in cytochrome oxidase vesicles enriched with 18% of cardiolipin and reconstituted with mitochondrial proteins. Fluorescence polarization of DPH was evaluated in presence (●) or absence (■) of cardiolipin without calcium and in presence (○) or absence (□) of cardiolipin with 250 μM $CaCl_2$. Results are representative of at least three different experiments.

of cardiolipin and calcium to the vesicles. The increment in SSC values was similar to that found in liposomes made from phosphatidylcholine/cardiolipin (Fig. 5 panels *g–i*), as previously shown (Aguilar et al., 1999). The small increase (3-fold) in FSC values observed in cytochrome oxidase vesicles supplemented with cardiolipin in presence of calcium (Fig. 5, panel *b*) compared with the vesicles without cardiolipin (Fig. 5, panel *a*) denotes a high liposomal surface area, probably due to the fusogenic properties of nonlamellar arrangements that produce larger liposomes (Smaal et al., 1987; Aguilar et al., 1999).

Discussion

The inner mitochondrial membrane contains, in addition to cardiolipin, phosphatidylcholine and phosphatidylethanolamine at nearly equimolar concentrations. However, compositions ranging from 2:3:1 to 4:3.5:1 (PC/PE/Cl) have also been reported (Hoch, 1992). One function that has been proposed for membrane lipids, particularly the acidic forms, is calcium transport. Kester and Sokolove (1989) have measured calcium translocation in liposome systems modeled on the mitochondrial inner membrane, without the participation of incorporated proteins. They found that Ca^{2+} uptake in this protein-free model is dependent on cardiolipin content. Calcium concentrations utilized (10 mM) greatly exceeded cytosolic levels, so their model could not account for a hypothesis that proposes that mitochondrial lipids mediate mitochondrial calcium processes, but still, this group proposed a putative regulatory role of cardiolipins in liposomal calcium uptake.

We have reported the reconstitution of calcium transport in cytochrome oxidase vesicles incorporated with solubilized mitochondrial proteins (Zazueta et al., 1994). Commercial asolectin used in those experiments contained 40% phosphatidylcholine and other phospholipids. In this work, the same source of asolectin was used and supplemented with cardiolipin to evaluate the effect of this phospholipid on the mitochondrial calcium uniporter function. The cardiolipin content in asolectin was quantified, yielding values around 2%, in contrast with reported values of 10% (Nalecz, et al., 1986), while mitochondrial protein contributes with 4.7%, probably as lipids tightly bound to incorporated proteins. Cardiolipin enrichment in liposomes supplemented with this anionic phospholipid was 2.85 times larger than that of liposomes without cardiolipin supplement. Cardiolipin-modeled liposome content correlates with that described for mitochondria (Hoch, 1992). The modified phospholipid composition in these liposomes correlates with a dramatically diminished activity of the reconstituted calcium uniporter. In this respect, Zara et al. (2000) reported a decrease in the transport activity of the mitochondrial tricarboxylate carrier from silver eel in liposomes enriched with cardiolipin. In our system, the inhibition was independent of the activity of cytochrome oxidase, since the respiratory rate of complex IV showed only a slight increase in cardiolipin-enriched liposomes. It has been reported that there are two classes of boundary-layer phospholipids that have different affinities for the cytochrome oxidase, the one with the lowest affinity is

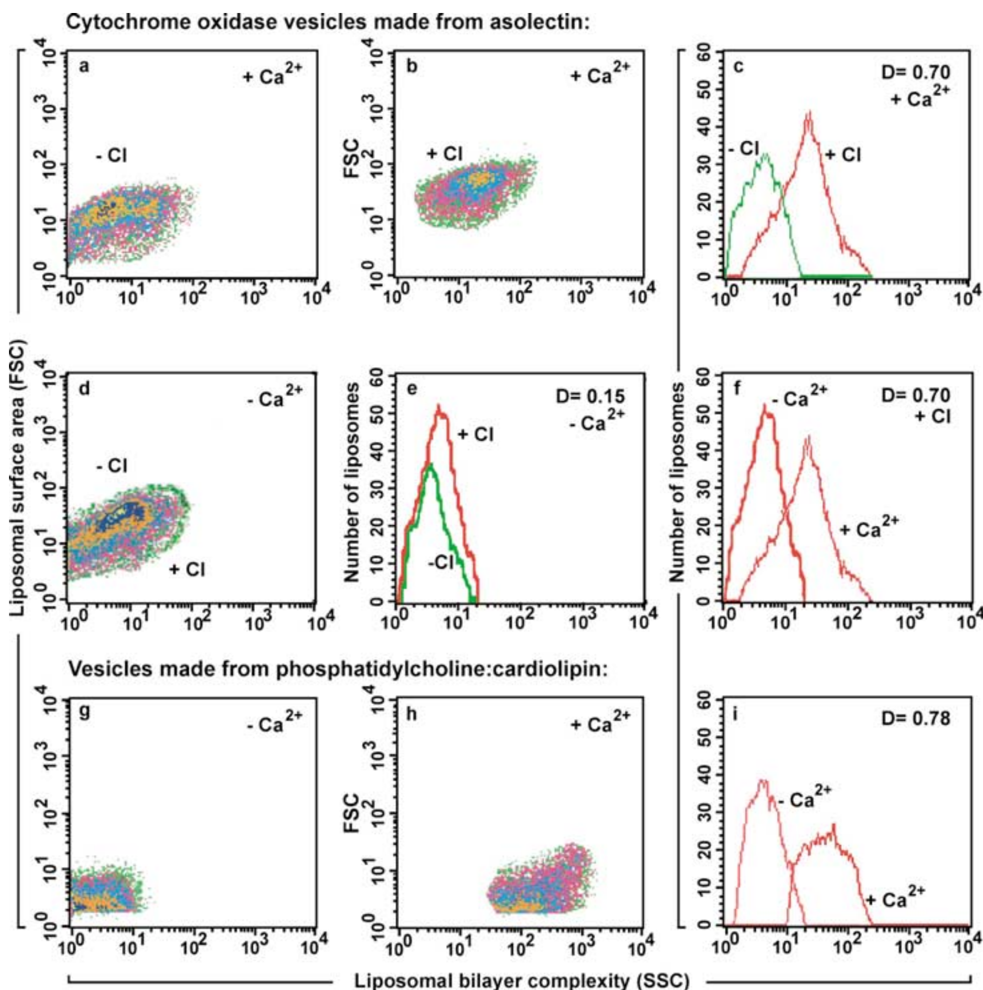

Fig 5. Flow cytometric analysis of liposomes. Cytochrome oxidase vesicles made from Type IV-s soybean asolectin were incubated with (panels *a–c*) or without (panels *d*, *e*) calcium 250–500 μM for 5 min at 30°C. Changes in bilayer complexity (SSC) and liposome surface area (FSC) due to the enrichment of asolectin liposomes with 18% commercial beef heart cardiolipin, were evaluated (panels *a–f*). Dot-plots from asolectin liposomes with and without cardiolipin are shown in panel *d*. Liposomes made from phosphatidylcholine/cardiolipin (2:1 mole ratio) were used as positive control of the presence of nonlamellar arrangements in liposomes (panels *g–i*). D refers to the difference (on a logarithmic scale) between liposomal populations. Values of $D \geq 0.5$ at $P < 0.001$ are highly significant and denote the presence of nonlamellar arrangements in liposomes. Red and green histograms denote liposomes with and without cardiolipin, respectively, while orange and dark blue colors in dot-plots represent zones with high liposomal density. One experiment representative of three is shown.

nonessential for activity and can be replaced by a variety of exogenous phospholipids and detergents. The second class is tightly bound to the enzyme and comprises two cardiolipins that are essential to maintain both quaternary structure and activity of bovine cytochrome oxidase (Sedlák & Robinson, 1999). In our experiments, we replaced the lipid environment of complex IV; in consequence, supplemented cardiolipin could be related to the first class of phospholipid binding. This cardiolipin-enriched environment did not affect the ability of complex IV to maintain a membrane potential, since the oxidation of ferrocytochrome generated similar membrane potentials in the presence or in the absence of cardiolipin.

It has been reported that the proton permeability of the bilayer is an important component of the proton permeability in intact mitochondria. An increased proton permeability has been observed in liposomes made from phospholipids extracted from mitochondria of hyperthyroid animals, in which the composition and saturation of fatty acid chains are modified (Brand et al., 1992). The phospholipid fraction that showed major changes is cardiolipin both in its quantity and its structure, but also other phospholipids, namely, phosphatidylcholine and phosphatidylethanolamine undergo similar changes (Guerrero et al., 1999). This fact probably accounts for the increased proton permeability already described. In contrast, the membrane potential is

maintained in our liposomes in the presence of bovine heart cardiolipin, but calcium accumulation was dramatically modified in cardiolipin-supplemented liposomes. The conservation of membrane potential in our liposomes discards the possibility that the addition of cardiolipin to asolectin liposomes sensitizes them to the opening of the permeability transition pore, in a similar way to that described in rat liver mitochondria (Bobyleva et al., 1997).

The effect of cardiolipin in reducing the activity of the calcium uniporter could be related to changes in the bilayer structure, as inferred from the higher energy-of-activation requirement of liposomes enriched with cardiolipin to support calcium uptake at high temperatures compared with control vesicles. In addition, the increased fluorescence polarization of DPH in liposomes enriched with cardiolipin in the presence of calcium strongly supports the notion of a decrease in the fluidity of the cardiolipin-supplemented liposomes. This diminished liposomal bilayer fluidity in the combined presence of cardiolipin and calcium can explain the strong inhibition of calcium transport, by exerting changes associated with lipid configuration. If the membrane fluidity diminished, the required energy configuration of the carrier would be modified and the calcium transport inhibited. In contrast, the activity of complex IV, which generates the membrane potential necessary for calcium transport, did not depend on liposomal fluidity. Furthermore, it has been reported that H_{II}-preferring phospholipids, such as cardiolipin (Van Dijck et al., 1978), form nonbilayer phospholipid arrangements that could be involved during the dynamic events of membrane function. This possibility was suggested in view of the ability of such structures to produce antiphospholipid antibodies (Baeza et al., 1995), which can participate in the development of the human antiphospholipid syndrome (Aguilar et al., 1999). Nonbilayer arrangements in the liposomal bilayers of cardiolipin-containing liposomes incubated with calcium under cation transport conditions (Fig. 5, panels *b*, *c*, and *f*), could modify liposomal fluidity and explain the increased fluorescent polarization values observed in these liposomes; this could be achieved by the interaction of the cardiolipin head group with calcium. In this respect it is known that cardiolipin head groups are important in determining the shape of the molecule and that their interactions with ions alter the lipid phase behavior (Schlame, Rua & Greenberg, 2000).

We cannot rule out that the mitochondrial uniporter requires for its proper function a specific lipid environment in which cardiolipin could be an important component. Indeed, our data support the conclusion that in a reconstituted system, exogenous cardiolipin inhibits the activity of the mitochondrial calcium uniporter. At the moment, a demonstration of a specific lipid-uniporter interaction is not possible due to the nature of the sample that we are handling. Tightly-bound lipids remained bound to mitochondrial proteins even after the solubilization process, as indicated by the finding that solubilized proteins increase the cardiolipin content in the reconstituted system.

A physiological implication of the diminished calcium accumulation observed in this model could be that it exists as an intrinsic regulatory system in some mitochondrial types that provides for protection from cell injury. In hyperthyroid mitochondria, cardiolipin content increases by 20–30% (Hoch, 1992). In this pathological condition, a variety of carriers and enzymatic complexes of the phosphorylation system are also incremented. Permeability transition, a well-known damaged state of mitochondria, is related to a massive calcium-overloading, regulated by the calcium cycling and mediated by the electrogenic uniporter, which catalyzes calcium uptake, and the Na^{+}-independent Ca^{2+}/nH transporter, which catalyzes calcium efflux. Furthermore, thyroid hormones may act synergistically with calcium influx, rapidly leading mitochondria into this damaged state (Hummerich & Soboll, 1989). Mitochondria from hyperthyroid rats have an increased leakiness to protons across the inner membrane (Hafner et al., 1988; Brand et al., 1992), in addition to undergoing permeability transition (Kalderon, Hermesh & Bar-Tana, 1995). An increased calcium-uptake transport has been observed in isolated hyperthyroid rat liver mitochondria (Chávez, personal communication), that could induce mitochondria into a hyperpermeable state. In vivo, the hyperthyroid cell could overcome this dangerous condition, regulating calcium uniporter activity by means of an increased cardiolipin content. In this context, it has been proposed that the low content of cardiolipin in hypothyroid mitochondria renders isolated liver mitochondria resistant to the opening of the membrane permeability transition pore (Chávez et al., 1998).

Cardiolipins, among the mitochondrial inner membrane phospholipids, are not only the least saturated and most acidic, the most rigidly fixed in an H-bond network that orients surface water molecules and cationic residues of proteins (Van Klompernburg & de Kruijff, 1998) and when neutralized are the most favorable for formation of nonbilayer, proton-conducing structures (Hoch, 1992), but in addition, they are also the most localized to one face of the inner membrane (the matrix face). By this token, the regulatory effect of cardiolipin on calcium uptake in liposomes could not only be explained in terms of a modified physical state of the membrane, but it could be related to the orientation interactions between cardiolipin and the reconstituted proteins. Neither can we discard the possibility that an excess of cardiolipin in our system affects the calcium uni-

porter reconstitution, blocking the activity of this porter.

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