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Interaction of carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) with lipid membrane systems: a biophysical approach with relevance to mitochondrial uncoupling

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Abstract FCCP (carbonylcyanide *p*-trifluoromethoxyphenylhydrazone), a classical uncoupler of mitochondrial oxidative phosphorylation, is used in this study as a model to clarify how interactions of uncouplers with membrane lipid bilayers may influence membrane biophysics and their protonophoric activity itself. In order to disclose putative effects that may be important when considering using uncouplers for pharmacological purposes, an extensive characterization of FCCP membrane lipid interactions using accurate biophysical approaches and simple model lipid systems was carried out. Differential scanning calorimetry studies showed that FCCP molecules disturb lipid bilayers and favor lateral phase separation in mixed lipid systems. ^{31}P NMR assays indicated that FCCP alters the curvature elastic properties of membrane models containing non-bilayer lipids, favoring lamellar/H_{II} transition, probably by alleviation of hydrocarbon-packing constraints in the inverted hexagonal phase. Taking advantage of FCCP quenching effects on the fluorescent probes DPH (1,6-diphenyl-1,3,5-hexatriene) and DPH-PA (3-(p-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid), it is demonstrated that FCCP distributes across the bilayer thickness in both a single and a ternary lipid system mimicking the inner mitochondrial membrane. This behavior is consistent with the ability of the compound to migrate through the thickness of the inner mitochondrial membrane, an event required for its protonophoric activity. Finally, the study of the membrane fluidity in different lipid systems, as reported by the rotational correlation time (θ) of DPH or DPH-PA, showed that the extension at which FCCP disturbs membrane properties associated with the dynamics and the order of lipid molecules depends on the lipid composition of the model lipid system assayed.

Keywords FCCP · Lipid dynamics · Membrane fluidity · Lateral phase separation · Lamellar and inverted hexagonal phases · Differential scanning calorimetry · Fluorescence anisotropy · ^{31}P NMR

Abbreviations

DOPC	Dioleoylphosphatidylcholine
DOPE	Dioleoylphosphatidyl ethanolamine
DPH	1,6-diphenyl-1,3,5-hexatriene
DPH-PA	3-(p-(6-phenyl)-1,3,5-hexatrienyl) phenylpropionic acid
DPPC	Dipalmitoylphosphatidylcholine
DPPE	Dipalmitoylphosphatidylethanolamine
DSC	Differential scanning calorimetry
FCCP	Carbonylcyanide *p*-trifluoromethoxyphenylhydrazone
i.m.m	Inner mitochondrial membrane
^{31}P NMR	Phosphorous nuclear magnetic resonance
r_{ss}	Steady-state fluorescence anisotropy
TOCL	Tetraoleoyl cardiolipin

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Introduction

As a consequence of the central role of mitochondria in cellular energy production, interference with any of the pivotal steps in the mechanisms of energy production network results ultimately in cell damage (Boelsterli 2003). A well-known mitochondrial target for xenobiotics is the inner mitochondrial membrane (i.m.m.). An increased permeability of the i.m.m. to protons results into uncoupling of oxidative phosphorylation. The protonophoric activity of several agents leads to the dissipation of the proton gradient across the i.m.m. and, hence, to the collapse of the transmembranar potential, impairing the driving force for ATP synthesis. Electron transport and the pumping of protons into the intermembrane space become functionally disconnected (uncoupled) from ATP synthesis (Boelsterli 2003). Although the uncoupling phenomenon has been normally associated to mitochondria dysfunction and cell damage (Han et al. 2009), recent evidences show that a partial and transient uncoupling is beneficial in specific cases such as during excitotoxicity (Maragos and Korde 2004) or for post-ischaemic functional recovery (Brennan et al. 2006). The prevention of excessive mitochondrial calcium accumulation or a decreased rate of ROS production by the respiratory chain have been proposed as potential mechanisms for neuroprotection (Maragos and Korde 2004) derived from a "mild" uncoupling effect (Skulachev 1996). The same effect has been related to FCCP-induced ROS-dependent cardioprotection (Brennan et al. 2006).

FCCP (carbonylcyanide *p*-trifluoromethoxyphenylhydrazone, Fig. 1) is a classical uncoupler of oxidative phosphorylation. The mechanism of action of FCCP at the inner mitochondrial membrane level has been previously described (Terada 1990). FCCP is a weak acid with notorious lipophilic characteristics that allow it to migrate through the thickness of the bilayer in an electrically neutral form (protonated), as well as in the anionic form due to the charge delocalization provided by their conjugated ring structures (Skulachev et al. 1967). Classically, it has been described that the lipid composition and dielectric constant of the bilayer might play a role in FCCP-induced proton translocation across the i.m.m. (Benz and McLaughlin 1983). However, how FCCP-lipid interactions can modulate uncoupling and the extent to which the physical properties of the membrane lipid bilayer influence FCCP activity are aspects that remain to be clarified.

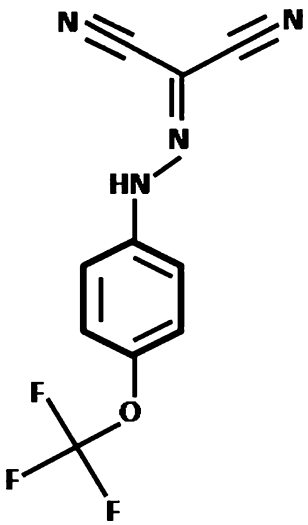

Fig. 1 Chemical structure of FCCP (carbonylcyanide *p*-trifluoromethoxyphenylhydrazone)

Previous work from our group has shown that many lipophilic compounds such as clinically-used drugs (Luxo et al. 1996; Monteiro et al. 2003; Sousa et al. 2008) and environmental pollutants, including insecticides (Donato et al. 1997; Martins et al. 2003; Monteiro et al. 2008) and organotins (Martins et al. 2005), promote alterations of membrane lipid physical properties, which may affect membrane-associated functions. Membrane protein activity related with active transport of protons and cations and several enzymatic activities have consistently shown dependence on the structure and dynamics of the membrane lipid bilayer component (Mouritsen and Jorgensen 1998; Andersen and Koeppe 2007; Lundbaek et al. 2010).

Fluidity has been the most evoked physical property of the lipid bilayer susceptible to be altered by the incorporation of membrane-active compounds, with impact on membrane physiology. However, other properties, namely concerning the lateral pressure profile, the curvature strain and the presence of different lipid-driven microdomains, have been shown to have a critical role in membrane function (Mouritsen and Jorgensen 1998; Andersen and Koeppe 2007; Lundbaek et al. 2010).

By using different biophysical approaches, the present work investigates alterations in the membrane physical properties induced by the incorporation of FCCP. Differential scanning calorimetry, quenching assays and fluorescence anisotropy studies allowed to assess FCCP-induced physical disturbances in different membrane systems and to predict its preferential localization across the lipid bilayer thickness. As a starting step, model membranes prepared from a single lipid species, dipalmitoylphosphatidylcholine (DPPC), were used. Later, by using more complex, binary and ternary lipid membrane models, including a mitochondrial membrane mimetic lipid system consisting of DOPC (dioleoylphosphatidylcholine), DOPE (dioleoylphosphatidylethanolamine) and TOCL (tetraoleoylcardiolipin), at 1:1:1 molar ratio, we evaluated FCCP-lipid interactions under more realistic conditions. The capacity of FCCP to induce non-lamellar phases was assessed by ^{31}P NMR, using the ternary lipid system in the presence of Ca^{2+} (at 1: 1 molar ratio to cardiolipin).

The results of the present work, contributing to identify FCCP-induced alterations on membrane lipid physical properties putatively correlated with its protonophoric activity or

side effects, may provide significant insights for predicting or modulating the impact of mitochondrial uncouplers, potentially used for pharmacological purposes, on target cells.

Experimental procedures

Chemicals

The lipids DOPC, DOPE, DPPC, DPPE (dipalmitoylphosphatidylethanolamine) and TOCL (at least 98% pure) were obtained from Avanti Polar Lipids, Inc. (Murcia, Spain). FCCP was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The probes 1,6-diphenyl-1,3,5-hexatriene (DPH) and 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid (DPH-PA) were purchased from Molecular Probes, Inc. (Eugene, OR, USA). All the other chemicals were of the highest commercially available purity.

Preparation of liposomes

Adequate portions of DPPC or mixtures of DPPE:TOCL (7:3) and DOPC:DOPE:TOCL (1:1:1) were dissolved in chloroform, and subsequently evaporated to dryness in a rotary evaporator. The dry residues were hydrated under N_2 atmosphere by gentle shaking with an adequate volume of buffer (10 mM Tris-maleate plus 50 mM KCl, pH 7.0, for DPPC and DPPE-TOCL liposomes and 50 mM HEPES plus 0.2 mM NaCl, pH 7.5, for DOPC-DOPE-TOCL liposomes) in a water bath at a temperature above the transition phase of the respective lipid preparations (room temperature for the ternary unsaturated lipid mixture, 55 °C for DPPC and 65 °C for DPPE-TOCL mixture). DPPC and DPPE-TOCL preparations (150 mM in phospholipids) used in DSC (differential scanning calorimetry) assays and DPPC and DOPC-DOPE-TOCL preparations (345 μM) used in spectrofluorimetric assays were vortexed three times during 1 min to disperse aggregates. For spectrofluorimetric purposes, lipid preparations were also briefly sonicated (3×30 s) in a low energy water sonicator to decrease the amount of scattered light. FCCP was then added to the liposome suspensions from a concentrated ethanol solution, and the preparations were allowed to equilibrate overnight at the respective temperatures at which liposomes were prepared (i.e. at temperatures above T_m, see above), since these conditions favor the incorporation of most compounds (Antunes-Madeira and Madeira 1989). Control samples were prepared with equivalent volumes of ethanol.

Differential scanning calorimetry

Multilamellar vesicles (150 mM in phospholipid) prepared as described above, containing different amounts of FCCP in order to obtain uncoupler:lipid molar ratios from 1:6 to 1:48, or ethanol (control), were sealed into aluminium pans and heating scans were performed over an appropriate temperature range, on a Perkin-Elmer Pyris 1 differential scanning calorimeter at a scan rate of 5 °C/min. To check data reproducibility, three heating scans were recorded for each sample. To compensate the effect of high heat capacity of the aqueous medium on the baseline, an estimated amount of buffer in the sample was used in the reference pan. Data acquisition and analysis were performed using the software provided by Perkin Elmer. Distinct temperatures are automatically defined for each endotherm: the onset temperature (T_0) and the temperature at the endotherm peak (T_m). To define the range of the phase transition or lateral phase separation (T_f-T_o), a third temperature was determined (T_f) by extrapolating to the baseline a tangent to the descendent slope of the endothermic peak. These critical transition temperatures were estimated as the mean value of three heating scans in at least three different samples from the same preparation. To determine the total amount of phospholipid contained in each pan, the pans were carefully opened at the end of the experiment and the content was dissolved in chloroform plus methanol. The phospholipid content was determined by measuring the amount of inorganic phosphate (Bartlett 1959), after hydrolysis of the extracts in 70% $HClO_4$, at 180 °C, for 60 min (Bottcher et al. 1961). Finally, peak profiles and enthalpy (ΔH) values were normalized to the exact phospholipid content in each pan.

^{31}P NMR

For ^{31}P NMR studies, preparations containing 5 mg of a mixture of DOPE:DOPC:TOCL (1:1:1) were obtained as described above. FCCP was added to the liposome suspensions from a concentrated ethanolic solution and the preparations were allowed to stabilize overnight. The samples were loaded into a 5-mm diameter NMR tube and a small volume of a $CaCl_2$ solution was added to reach 1:1 Ca^{2+}:cardiolipin molar ratio. Controls were prepared with a few μl of ethanol, corresponding to the volume of the FCCP solution assayed. Spectra were obtained using a Varian Unity 500 NMR spectrometer operating at 202.33 MHz in a 5-mm broadband probe over a 30-kHz sweep width in 60×1,024 data points. A 90° pulse width of 14.09 μs was used. Composite pulse decoupling allowed to removing any proton coupling. Generally, 800 free induction decays were processed using an exponential line broadening of 100 Hz prior to Fourier transformation. Probe temperature was maintained to ±0.2 °C by the variable temperature unit of the NMR spectrometer.

Incorporation of probes into liposomes

A small volume of the fluorescent probes DPH or DPH-PA in dimethylformamide were injected into liposome suspensions (345 μM in phospholipid) to give a lipid:probe molar ratio of 300. Liposome suspensions were then incubated overnight in the dark, prior to the incorporation of FCCP as described above. Control samples were prepared with equivalent volumes of dimethylformamide.

Fluorescence quenching and anisotropy

Fluorescence steady-state excitation and emission spectra of DPH and DPH-PA incorporated in DPPC or DOPC-DOPE-TOCL preparations, at which FCCP or ethanol (control) were added, were obtained in a Perkin-Elmer LS 55 Luminescence Spectrometer equipped with a thermostated cell holder. Excitation and emission wavelengths were set to 361 and 432 nm for emission and excitation spectra acquisition and also for the fluorescence anisotropy measurements. Fluorescence values were corrected for light scattering contributions by subtracting the intensities from unlabelled samples at the same conditions. These contributions were always negligible (less than 0.5%). Fluorescence intensity data were also corrected for absorbance of the quencher (FCCP) at the excitation wavelength of the probes. Fluorescence lifetime measurements were made with a Fluorolog Tau-3 Lifetime system. Excitation and emission wavelengths were set to the same values as in the case of steady-state measurements but excitation of the probes was achieved with a 450-W ozone-free xenon lamp. Modulation frequencies were acquired between 2 and 110 MHz. Integration time was 8 s. Manual slits used were 0.5 mm, slits for excitation monochromator were 7.000 (side entrance) and 0.8 mm (side exit) and for emission monochromator 7.000 (side entrance) and 7.000 (side exit). All measurements were made using Ludox as a reference standard (τ=0.00 ns) and recorded at a controlled temperature (50 °C for DPPC liposomes; 25 and 70 °C for the mixture DOPC:DOPE:TOCL). The probes were excited with vertically polarized light and resulting fluorescence intensities were recorded with the analyzing polarizer oriented parallel ($I_{\parallel}$) and perpendicular ($L_{\perp}$) to the excitation polarizer allowing the determination of steady-state fluorescence anisotropy for each labelled sample, as shown by Eq. (1) (Lentz 1993):

$$r_{ss} = \frac{I_{\parallel} - G.I_{\perp}}{I_{\parallel} + 2G.I_{\perp}} \quad (1)$$

where G is the grating correction factor given by the ratio of vertically to horizontally polarized emission components, when the excitation light is polarized in the horizontal direction.

Statistical analysis of data

DSC and fluorescence anisotropy data, namely transition temperature midpoints (T_m), transition temperature ranges (T_f-T_o) and enthalpy changes (ΔH), quenching percentages and anisotropy values were expressed as means ± standard deviation of 3–4 independent experiments. Multiple comparisons were performed using one-way ANOVA with the Student-Newman-Keuls as a post-test. A value of $p<0.05$ was considered statistically significant.

Results

FCCP interferes with the thermotropic behavior of model lipid membranes

Differential scanning calorimetric studies were performed to investigate the effects of FCCP on the biophysical properties of two membrane models: DPPC liposomes, as a classical model system for biophysical studies, and a mixed lipid system containing DPPE plus TOCL (representing two important lipid classes of the i.m.m.) at a 7:3 molar ratio. As expected, DSC thermograms of DPPC bilayers showed two endotherms, corresponding to a pre-transition at 34.6 °C, with a low enthalpy change (data not shown), and a main transition with a sharp peak centered at 41.9 °C, with an enthalpy change of 39.5 J/g (Table 1). The addition of FCCP to DPPC liposomes abolished the pre-transition peak (data not shown), even at the lowest concentration used (FCCP:DPPC molar ratio of 1:48). Increasing concentrations of FCCP, from 1:48 to 1:6 FCCP:phospholipid molar ratio, promoted a shift of the temperature range at

Table 1 Characterization of the phase transitions detected by DSC (temperature of the endothermic peak, T_m, transition temperature range, T_f-T_o, and enthalpy change, ΔH) in DPPC liposomes. Liposomes were incubated with FCCP to obtain FCCP:DPPC molar ratios from 1:48 to 1:6, or with a volume of ethanol corresponding to the maximal volume of the FCCP solution assayed for the control (FCCP:DPPC molar ratio of zero in the Table)

FCCP:DPPC	T_m (°C)	$T_f - T_0$ (°C)	ΔH (J/g)
0 (control)	41.92±0.30	3.36±0.36	39.48±0.32
1:48	41.15±0.52*	4.04±0.60*	39.93±0.51^{ns}
1:24	40.35±0.35**	4.62±0.40**	40.02±0.59^{ns}
1:12	39.40±0.22**	4.96±0.39**	40.05±0.53^{ns}
1:6	38.20±0.39**	5.96±0.33**	39.58±0.46^{ns}

a) Values presented are means ± standard deviation of four DSC experiments. Comparisons were performed using one-way ANOVA, with the Student-Newman-Keuls as a post-test for the following paired observations: liposomes with FCCP:DPPC molar ratios of 1:48, 1:24, 1:12 or 1:6 vs. control liposomes (without FCCP). **, $P<0.01$; * $P<0.05$; n.s., not significant

which lipid dispersions undergo a gel to a liquid-crystalline phase transition towards progressively lower temperatures. The highest uncoupler concentration assayed promoted a decrease of T_m of about 3.7 °C (Table 1). Additionally, the temperature range at which the transition takes place displayed a progressive broadening in the presence of increasing concentrations of the uncoupler, reflecting a decrease of the cooperativity of the DPPC phase transition. At the highest FCCP concentration, T_f-T_o increased 2.6 °C with regard to the control. However, FCCP did not significantly affect the enthalpy change of the DPPC main transition (Table 1).

Liposomes containing DPPE and TOCL prepared at a molar ratio of 7:3 form an ideal mixture showing a single, although broad (T_f-$T_o \approx 11$ °C), endotherm centered at about 48.7 °C (Fig. 2 and Table 2), despite the fact that the lipid components of the mixture display, when isolated, a large gap between their transition temperatures (64 °C for DPPE and below 0 °C for TOCL), as previously reported (Epand et al. 2006). The addition of FCCP (1:12 and 1:6 FCCP: phospholipid molar ratio) induced an alteration of the phase behavior of the lipid mixture, promoting the appearance of a new endothermic component with a higher transition temperature than the control single peak, but an enthalpy change significantly lower than that regarding the main endotherm (about one tenth for the highest FCCP concentration, Table 2). The appearance of this new endothermic component in the presence of FCCP, at temperatures closer, but still below, the phase transition of pure DPPE systems, suggests the formation of DPPE-enriched lipid domains. Regarding the enthalpy (ΔH) of the transitions, it was noticed that the value found in control conditions (20.2 J/g of phospholipids) is approximately equivalent to the sum of the transition enthalpies of the two endotherms obtained in FCCP-containing lipid preparations (Table 2). This supports the occurrence of a lateral phase separation, excluding that any other endothermic process was taking place.

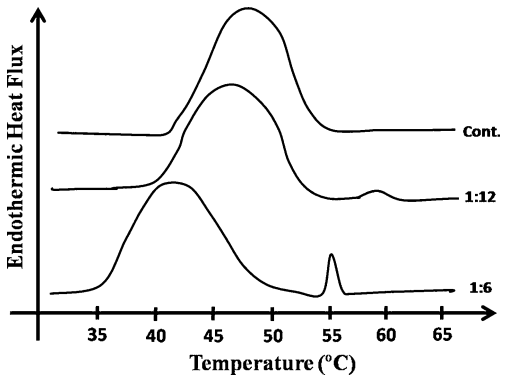

Fig. 2 DSC thermograms of liposomes prepared with a mixture of DPPE and TOCL (7:3, molar ratio) in the absence (Cont.) or the presence of FCCP at the FCCP:lipid molar ratios indicated on the scans. The DSC profiles were originated from heating scans. The thermograms represent typical traces of at least three independent experiments

FCCP favors hexagonal II phase formation in mixed non-bilayer and bilayer lipid systems

The influence of FCCP on the propensity of a heterogeneous lipid system containing bilayer (DOPC) and non-bilayer forming phospholipids (DOPE and TOCL plus calcium) for hexagonal II phase formation was assessed by ^{31}P NMR. In the absence of FCCP, the mixture of DOPC: DOPE:cardiolipin:Ca^{2+} (1:1:1:1, molar ratio) at 25 °C was shown to be organized in extended bilayers, evidenced by the characteristic asymmetrical ^{31}P NMR lineshape with a low-field shoulder and a high-field peak (Cullis and de Kruijff 1979) separated by approximately 10 ppm (Fig. 3). However, a small peak downfield of phosphoric acid (which was set at 0 ppm) is also noticed in this spectrum, evidencing the coexistence of lipids organized in an inverted hexagonal phase. As the temperature was increased, the hexagonal (H_{II}) component in the spectrum became more evident and, at 70 °C, it predominated relatively to the bilayer component. Simultaneously, a progressive reduction of spectrum width was observed, which is compatible with a bilayer to hexagonal (H_{II}) transition, affecting a significant part of the lipid vesicle population.

The incorporation of FCCP at the molar ratio of 1: 6 (FCCP to lipid), had a remarkable effect on lipid phase behavior. Thus, for the FCCP-containing lipid system, the lineshapes at and above 25 °C are significantly different from those of the control preparation (Fig. 3). At 40 °C, the hexagonal (H_{II}) component prevailed over the bilayer one and, at 70 °C, a lineshape typical of lipids in the inverted hexagonal phase, with a reversed asymmetry compared to the bilayer spectra and reduced width, was observed.

FCCP distributes along the hydrocarbon moiety of lipids in the lamellar phase, but closer to the methyl terminal groups of hydrocarbon chains in the H_{II} lattice

In order to investigate the preferential location of FCCP upon incorporation into lamellar/H_{II} lipid structures, steady-state fluorescence intensities and lifetimes were measured in liposomes labelled with two probes: DPH, buried in the hydrocarbon core (Andrich and Vanderkooi 1976), and DPH-PA located closer to the lipid/water interface due to its charged group (Trotter and Storch 1989). As a first approach, the effects of FCCP on the spectroscopic properties of both probes were studied in vesicles prepared with a single lipid species (DPPC). FCCP revealed to act as a potent quencher of both DPH and DPH-PA incorporated in DPPC liposomes, as illustrated for DPH in Fig. 4a. The

Table 2 Derived parameters of the phase transition peaks detected by DSC (temperature of the endothermic peaks, T_m, transition temperature range, T_f-T_o, and enthalpy change, ΔH) in DPPE:TOCL (7:3) liposomes. Liposomes were incubated with FCCP to obtain FCCP:lipid molar ratios from 1:12 to 1:6, or with a volume of ethanol corresponding to the maximal volume of the FCCP solution assayed for the control (FCCP:lipid molar ratio of zero in the Table)

	Peak 1			Peak 2	
FCCP:lipid	T_m (°C)	T_f–T_0 (°C)	ΔH (J/g)	T_m 2 (°C)	ΔH 2 (J/g)
0 (control)	48.66±1.52	11.12±2.12	20.20±0.61		
1:12	45.60±2.58*	11.45±1.33^{ns}	19.64±0.54^{ns}	57.32±2.66	0.34±0.07
1:6	41.95±0.55***	11.74±1.61^{ns}	18.38±0.57**	53.33±2.25^{ns}	1.54±0.62*

a) Values presented are means ± standard deviation of three DSC experiments. Comparisons were performed using one-way ANOVA, with the Student-Newman-Keuls as a post-test for the following paired observations: liposomes with a FCCP:lipid molar ratio of 1:12 or 1:6 vs. control liposomes (without FCCP), or liposomes with a FCCP:lipid molar ratio of 1:6 vs. liposomes with a FCCP:lipid molar ratio of 1:12 in the case of peak 2. ***, $P<0.001$; **, $P<0.01$; * $P<0.05$; n.s., not significant

same kind of profile was obtained for the excitation and emission spectra of DPH-PA-labelled liposomes (data not shown). Thus, for both probes, FCCP decreased the amount of absorbed light and induced a reduction of emitted light showing a strict dependence on FCCP concentration (Fig. 5). At the lower FCCP:phospholipid molar ratio assayed (1:96), FCCP decreased the fluorescence signal to approximately 50% and at the molar ratio of 1:24, a merely residual fluorescence was emitted by both probes.

In order to also appraise FCCP distribution in an inverted hexagonal lipid arrangement, quenching effects were evaluated when fluorescent probes were incorporated in liposomes composed of a mixture of DOPC:DOPE:TOCL (1:1:1) with Ca^{2+} 1:1 to cardiolipin. Quenching assays were performed at

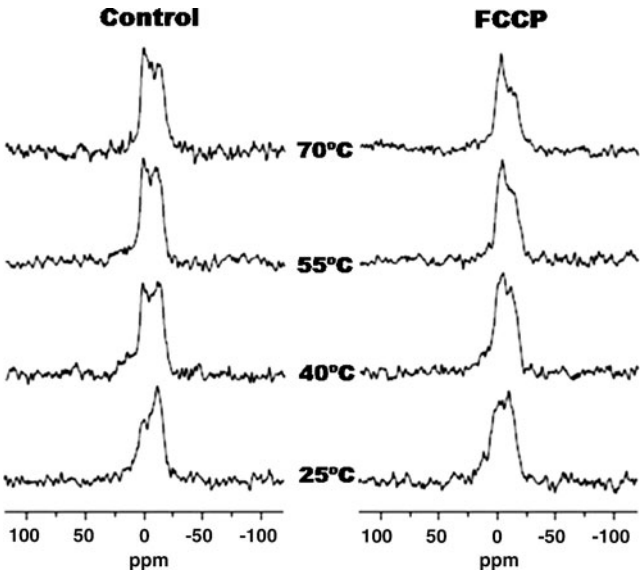

Fig. 3 ^{31}P NMR spectra of liposomes containing DOPC:DOPE:TOCL (1:1:1) with $CaCl_2$ (1:1 to cardiolipin) at different temperatures (indicated on the figure), in the absence (*Control*) or the presence of FCCP at a 1:6 FCCP to phospholipid molar ratio (*FCCP*). For each sample, the spectrum was first averaged at 25 °C over a period of about 20 min, and then the process was repeated over a range of temperatures with 15 °C increments. Each sample (600 μl) contained 5 mg of total lipid

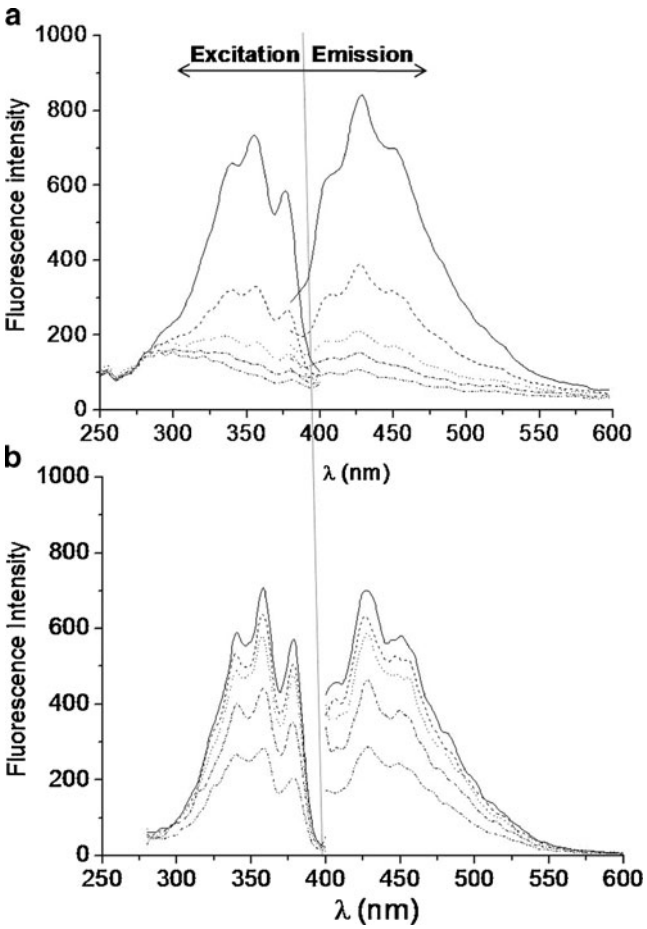

Fig. 4 Excitation and emission spectra of the fluorescent probe DPH incorporated in DPPC (A) or in DOPC:DOPE:TOCL (1:1:1) with $CaCl_2$ (1:1 to cardiolipin) liposomes (B) as affected by different ratios of FCCP:lipid (0, solid line; 1:96, dashes; 1:48, dots; 1:36, dash-dot; 1:24, dash-dot-dot). Curves were obtained after overnight incubation with FCCP, and the solid line curves represent the respective controls. Results are representative of at least 3 independent determinations

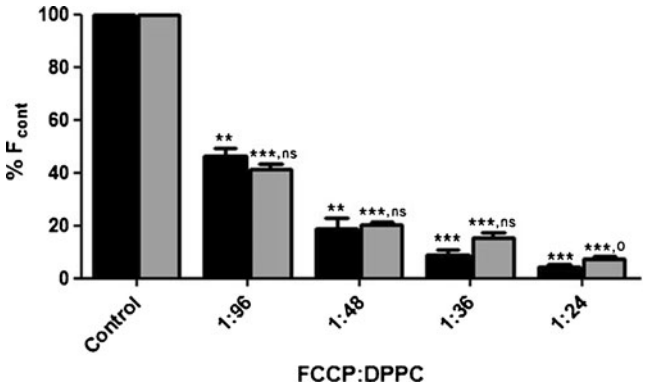

Fig. 5 Quenching effect of FCCP on DPH (black bars) and DPH-PA (grey bars) emission spectra, as a function of uncoupler concentration, in DPPC liposomes. The graph represents the data from at least three independent experiments for each condition. Comparisons were performed using one-way ANOVA, with the Student-Newman-Keuls as a post-test for the following paired observations: 1) liposomes with a FCCP:lipid ratio of 1:24, 1:36, 1:48 or 1:96 vs. control liposomes (without FCCP); ***, $P<0.001$; **, $P<0.01$; 2) liposomes labelled with DPH-PA vs. liposomes labelled with DPH, for the same FCCP concentration; °, $P<0.05$; ns, not significant

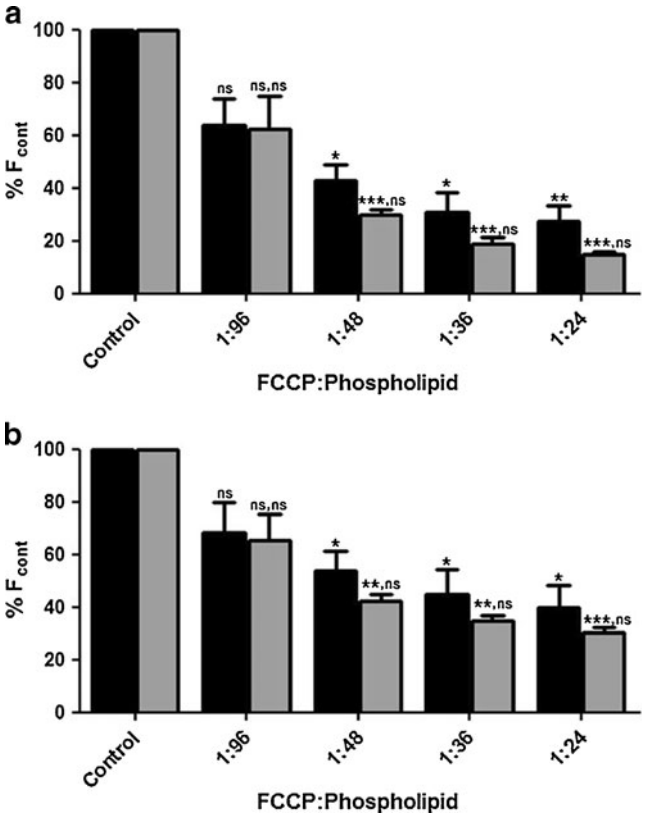

Fig. 6 Quenching effect of FCCP on DPH (black bars) and DPH-PA (*grey bars*) emission spectra, as a function of uncoupler concentration, in liposomes consisting of a mixture of DOPC:DOPE:TOCL (1:1:1) plus Ca^{2+} (at 1:1 Ca^{2+}:cardiolipin molar ratio) at 25 °C (**a**) or 70 °C (**b**). In the graph are presented the results from at least three independent experiments for each condition. Comparisons were performed using one-way ANOVA, with the Student-Newman-Keuls as a post-test for the following paired observations: 1) liposomes with a FCCP/lipid ratio of 1:24, 1:36, 1:48 or 1:96 vs. control liposomes (without FCCP); ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$; ns, not significant; 2) liposomes labelled with DPH-PA vs. liposomes labelled with DPH, for the same FCCP concentration (ns, not significant)

two different temperatures: 25 °C, at which the lamellar arrangement prevailed, and 70 °C, at which the H_{II} phase was the most relevant arrangement, according to ^{31}P NMR spectra (Fig. 3). Once more, typical excitation and emission spectra of fluorescence quenching in mixed lipid model membranes labelled with DPH, at different quencher (FCCP) concentrations, were observed (Fig. 4b). The same kind of profile was obtained when the same mixed lipid model systems were labelled with DPH-PA, regardless the temperature of the assay. Thus, at both temperatures assayed, FCCP was able to quench DPH and DPH-PA incorporated in this model system in a concentration dependent manner (Fig. 6), although the quenching effects were less drastic than in DPPC liposomes (Figs. 4 and 5).

The quenching efficiency of FCCP in each model system can be better compared after determining the Stern-Volmer constant (K_{sv}) from the Eq. (2) (Lentz 1993):

$$\frac{I_0}{I} = 1 + K_{SV}[FCCP] = \frac{\tau_0}{\tau} \quad (2)$$

where (I, τ) and (I_0, τ_0) are the fluorescence steady state intensity and lifetime with and without FCCP, respectively. According to this equation, it was possible to draw the Stern-Volmer plots of I_0/I and τ_0/τ against [FCCP]. Figure 7a illustrates the results obtained for quenching of DPH fluorescence by FCCP in DPPC liposomes, clearly showing that although a linear lifetime Stern-Volmer plot (τ_0/τ against the concentration of FCCP) has been obtained, a non-linear plot for the steady-state fluorescence intensity (I_0/I against FCCP concentration) with a positive deviation was observed. Furthermore, as the amount of collision quenching represented by the lifetime Stern-Volmer plot is smaller than the quenching that is observed by steady-state fluorescence measurements, an additional quenching process should be taking place, not influencing the observed excited state lifetime. Indeed, similar Stern-Volmer plots with the characteristic feature of an upward curvature, concave towards the y-axis, have been reported in the literature as related with a non-purely collisional quenching process, associated with a static quenching component (Lúcio et al. 2009). This type of positive deviation is usually interpreted in terms of a "sphere of action" static quenching model (Lentz 1993). According to this model, instantaneous or static quenching occurs if the quencher molecule (in this case FCCP) is very near to, or in contact with the probe, at the exact moment it happens to be excited. The probe molecules that are instantaneously quenched are therefore unobservable. The only observable probe molecules are those for which no adjacent molecules of FCCP exist. Hence, only a certain fraction of the excited

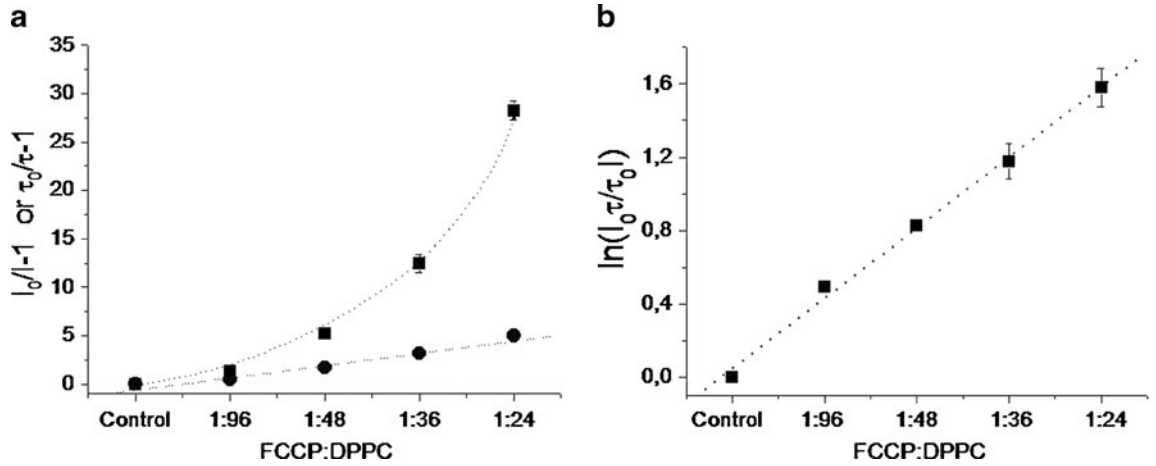

Fig. 7 **a** Stern-Volmer plots of the probe DPH incorporated in DPPC liposomes as a function of uncoupler concentration. Symbols (*black square*) represent Stern-Volmer plot obtained from steady state fluorescence measurements and (*black circle*) represent Stern-Volmer plot obtained from fluorescence lifetime measurements. **b** Fluorescence quenching of the probe DPH incorporated in DPPC liposomes as a function of uncoupler concentration. Line represents fit according to the sphere of action model

probe is quenched by the Stern-Volmer collisional mechanism (Lentz 1993). Moreover, this model assumes that, if the quencher (FCCP) is located inside a spherical volume (V) adjacent to the probe, the probability for the quencher to be inside this volume at the time of excitation depends on the volume itself and on the quencher concentration, according to Eq. (3) (Lentz 1993):

$$\ln\frac{I_0\tau}{I\tau_0} = V[FCCP] \tag{3}$$

Based on this equation it was possible to calculate the spherical volume where both FCCP and probe are located, which was given by the slope of the linear plot represented on Fig. 7b. Finally, we used the volume of the sphere of action (V) to assess the collision component of the observed quenching (Lentz 1993) by the introduction of an additional factor ($e^{V[FCCP]}$) in the fluorescence intensity ratio, which accounts for the fluorophore-quencher proximity effect and corrects the steady-state intensities, as shown by Eq. (4) (Lentz 1993):

$$\frac{I_0}{Ie^{V[FCCP]}} = 1 + K_{SV}[FCCP] = \frac{\tau_0}{\tau} \tag{4}$$

The slopes of these corrected steady-state fluorescence linear plots correspond to Stern-Volmer constants (K_{sv}) resultant from the collisional quenching between FCCP and the fluorophore DPH and DPH-PA in either DPPC liposomes or liposomes composed of the phospholipid mixture. The K_{sv} values calculated by the corrected steady-state Stern-Volmer plots (Table 3, column a) are equal (within the error) to the values of K_{sv} (Table III, column b) calculated from the slope of linear regressions of the experimental lifetime Stern-Volmer plots (Fig. 7a). For this reason and also because no specific interactions between probes and FCCP have been identified either in absorption or in fluorescence spectra, we assume that the applied sphere of action model is suitable to explain the observed quenching.

Although the K_{sv} values are adequate to evaluate the extension of the quenching for each probe individually, the comparison of quenching effects using two different probes (DPH and DPH-PA) requires the calculation of the bimolecular quenching rate constant ($K_q=K_{sv}/\tau_0$). This parameter reflects the efficiency of quenching or the accessibility of the fluorophores to the quencher (Lentz 1993), eliminating the specificity of the probe, which is related with the fluorescence lifetime of the probe (τ_0), experimentally measured in the absence of the quencher. Therefore, the analysis of the K_q values (Table 3, column c) indicates that: a) in DPPC liposomes, FCCP efficiently quenched the fluorescence of both probes (DPH and DPH-PA); b) in the ternary lipid system an higher K_q value was obtained with DPH-PA than with DPH at the lowest temperature (25 °C), but the opposite was observed at the highest temperature (70 °C). Thus, in conditions at which the mixture of lipids is preferentially organized in a lamellar structure (25 °C), the K_q value obtained with DPH was about 55% of that with DPH-PA, reflecting a preferential location of FCCP molecules closer to the outer regions of the lipid bilayer. In contrast, at 70 °C, temperature at which ^{31}P NMR spectra showed that the inverted hexagonal phase predominates (Fig. 3), FCCP seems to locate closer to DPH and hence farther from the aqueous lipid interface, as represented in Fig. 8. In fact K_q in those conditions was about six times higher for DPH than for DPH-PA (Table 3). These findings suggest a localization of FCCP molecules at the periphery of H_{II} tubes and at the interstices between H_{II} tubes, which is the most hydrophobic region of the hexagonal structure predictably occupied by DPH (Fig. 8).

Table 3 Values of the Stern–Volmer constant (K_{SV}) and the bimolecular quenching constant (K_q) obtained from measurements of fluorescence quenching of DPH and DPH-PA with different FCCP: lipid ratios (0; 1:96; 1:48; 1:36; 1:24). Lipid media consisted in liposomes of DPPC or a lipid mixture of DOPC:DOPE:TOCL (1:1:1) with Ca^{2+} 1:1 to cardiolipin at 25 °C or 70 °C

Lipid	Probe	$K_{SV}(M^{-1} \times 10^{-5})$ (a)	$K_{SV}(M^{-1} \times 10^{-5})$(b)	$K_q(M^{-1}s^{-1} \times 10^{-13})$(c)
DPPC	DPH	3.29±0.42	3.20±0.27	4.22±0.14
	DPH-PA	1.60±0.10*	1.28±0.36**	3.59±0.69***
Lipid Mixture 25 °C (L Phase)	DPH	0.30±0.42	0.29±0.40	0.31±0.05
	DPH-PA	0.32±0.02$^{n.s.}$	0.32±0.01$^{n.s.}$	0.56±0.01*
Lipid Mixture 70 °C (H Phase)	DPH	0.28±0.02	0.25±0.0	3.60±0.15
	DPH-PA	0.24±0.57$^{n.s.}$	0.24±0.12$^{n.s.}$	0.58±0.14*

(a), (b) and (c) Values presented are means ± standard deviation of three fluorescence quenching experiments. Comparisons were performed using one-way ANOVA, with the Student-Newman-Keuls as a post-test for the following paired observation: liposomes labelled with DPH-PA vs. liposomes labelled with DPH. ***, $P<0.001$; **, $P<0.01$; * $P<0.05$; n.s., not significant

(a) K_{sv} values calculated from the slope of the lifetime linear plots (τ_0/τ vs. [FCCP]) obtained from data fitting to Eq. 2

(b) K_{sv} values calculated from the slope of the steady-state fluorescence linear plots ($I_0/I_e^{V[FCCP]}$ vs. [FCCP]) obtained from data fitting to Eq. 4

(c) K_q values calculated using the K_{sv} values of column (a) and experimental τ_0 of the probe

FCCP affects the lipid order in lamellar and hexagonal II lipid structures

The effects of FCCP on lipid dynamics and phospholipid acyl chain packing were studied by fluorescence anisotropy, using the rotational correlation time (θ) of the probe to which FCCP localises closest in each preparation, i.e. DPH in DPPC liposomes, DPH-PA at 25 °C and DPH at 70 °C in preparations containing a mixture of DOPC:DOPE:TOCL (1:1:1) with Ca^{2+} 1:1 to cardiolipin. The rotational correlation time was calculated from measured r_{ss} values and τ variation, considering that the rotational motion of the probe was not hindered ($r_\infty=0$), as shown by Eq. (5):

$$\theta = \tau\left(\frac{r_{ss} - r_\infty}{r_0 - r_{ss}}\right) \quad (5)$$

Since θ gives information about the probe motion, it will also report the fluidity of the lipid environment surrounding it. Results (Fig. 9) show that FCCP promoted a significant increase of fluidity of the lipid mixture at 25 °C (lamellar phase), which was evident even for the lowest FCCP:lipid molar ratio (1:96). However, when FCCP molecules predominantly reside in the most disordered regions of the lamellar or inverted hexagonal lattices (the central region of DPPC bilayers and the interstices between H_{II} cylinders formed by the mixed lipid system at 70 °C, respectively), its effects on hydrocarbon chain packing, monitored by DPH, were negligible as expected (Fig. 9). Consistently, DSC also showed that the transition enthalpy (reporting the extension of hydrocarbon chain interactions) was negligibly affected by FCCP in DPPC liposomes.

Discussion

FCCP is a weak acid mostly known for its uncoupler properties on mitochondria. Since the outer mitochondrial membrane is very permeable, FCCP can easily reach the intermembrane

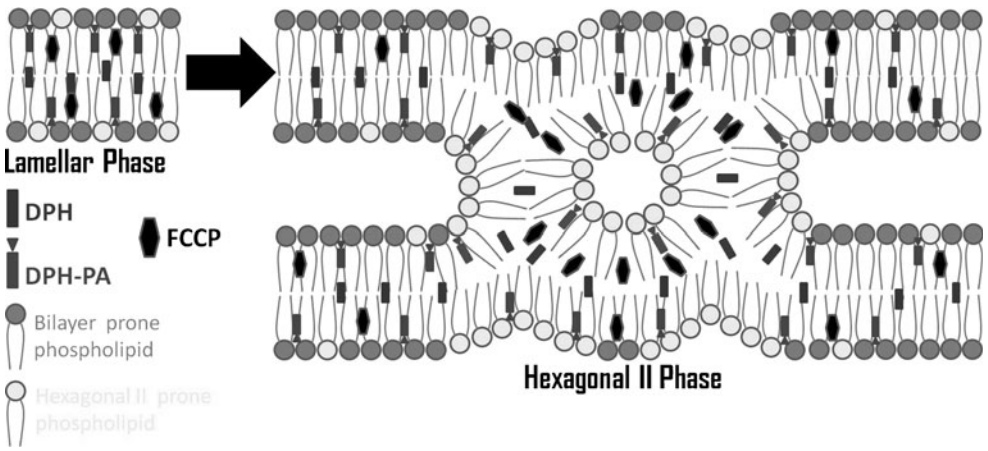

Fig. 8 Representative diagram showing the proposed distribution of FCCP and fluorescent probe (DPH and DPH-PA) molecules in lamellar and non-lamellar lipid phases

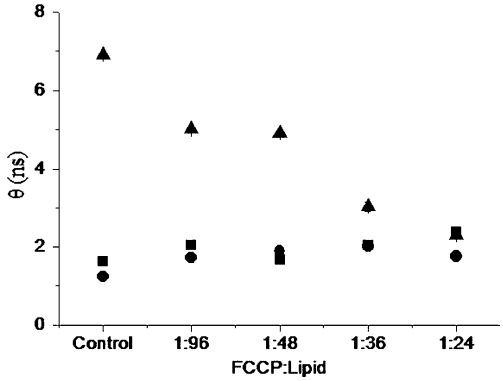

Fig. 9 Rotational correlation time (θ) of DPH in DPPC liposomes (*black square*) and DPH or DPH-PA in DOPC:DOPE:TOCL:Ca^{2+} (at 1:1:1:1 molar ratio) liposomes at 70 °C (*black circle*) and 25 °C (*black triangle*), respectively, as a function of the uncoupler concentration. Results are representative of at least three independent determinations

space of mitochondria. In this acidic compartment, due to the extrusion of protons by the mitochondrial proton pumps, FCCP is protonated and, in its uncharged form, moves across the mitochondrial inner membrane, losing its acquired proton in the matrix, because of the inside-out difference of approximately 0.6 pH units (Boelsterli 2003). In its negative form, FCCP is driven back out across the membrane by the inside-out negative membrane potential. The net result of this process is the shuttling of protons into the mitochondrial matrix, short-circuiting both the transmembrane proton concentration gradient and the membrane electric potential, compromising mitochondrial ATP synthesis (Terada 1990).

Since the mechanism of action of FCCP involves the diffusion of both the uncharged and anionic forms of the molecule across membranes, it is predictable that the composition and physical characteristics of the lipid environment are susceptible to interfere with the uncoupling process. In fact, it was demonstrated that the presence of negatively charged lipids in the membrane decreases the concentration of the anionic form of FCCP at the membrane-solution interface and the FCCP ability to act as a protonophore (McLaughlin and Dilger 1980).

Due to its lipophilic characteristics, assured by charge delocalization provided by the conjugated ring structures (Skulachev et al. 1967), FCCP is assumed to easily incorporate into membranes. Therefore, as demonstrated for many other lipophilic compounds (Luxo et al. 1996; Donato et al. 1997; Rosa et al. 2000; Martins et al. 2003, 2005; Monteiro et al. 2003, 2008; Sousa et al. 2008), FCCP should be able to disturb membrane physico-chemical properties, the nature and the severity of the disturbance being predictably dependent on the composition of the lipid bilayer. In the present study, we investigated FCCP/lipid interactions using different membrane systems and different biophysical approaches.

For the present studies, FCCP concentrations expressed in terms of FCCP:lipid molar ratios from 1:96 to 1:6 were used. Considering that the most commonly used concentration of FCCP to promote proton shuttling across the i.m.m. is 1 μM, corresponding to 1 nmol FCCP per mg of liver mitochondrial protein (Oliveira et al. 2000) and in view of the value of Fleischer et al. (1967) of 0.18 mg of phospholipid/mg liver mitochondrial protein and the phospholipid average molecular weight of 775 (Jurado et al. 1991), we can calculate that a FCCP:lipid molar ratio of 1:96 corresponds to roughly 1 nmol FCCP per 0.4 mg of mitochondrial protein, which is a concentration of the same order of magnitude of the one used to promote uncoupling of isolated mitochondria. Taking into account that several studies with cell cultures use FCCP concentrations from 2 to 10 μM (Leyssens et al. 1996; Aronis et al. 2002; Caputo and Bolaños 2007; Serafim et al. 2008), we predict that our data could be reliably extrapolated for the mitochondrial membrane. Moreover, we should also consider the occurrence of FCCP localized effects on the membrane, namely in lipid microdomains whose composition may assure a higher affinity for the compound and, hence, where it could attain higher concentrations (for instance in cardiolipin-enriched domains, as suggested for our DSC studies).

The steady-state fluorescence anisotropy proved to be a very sensitive technique to monitor alterations of fluidity in artificial as well as in native membranes (Luxo et al. 1996; Videira et al. 1996; Donato et al. 1997; Rosa et al. 2000; Martins et al. 2003, 2005; Monteiro et al. 2003, 2008; Sousa et al. 2008). This technique is particularly reliable when fluorescence decay experiments are conducted in parallel, since that way it is possible to confirm whether anisotropy changes are due to alterations of the lipid order rather than to changes in the fluorescence lifetime of the fluidity probe (Heyn 1979). Data presented in this work shows that a FCCP concentration as low as 1:96 FCCP to lipid molar ratio was enough to promote a significant increase of fluidity at 25 °C in a membrane prepared from the main classes of i.m.m. phospholipids, i.e. PC, PE and cardiolipin (Fig. 9). At this temperature, lipids are organized in a L_α lamellar phase, as reflected by ^{31}P NMR spectra (Fig. 3), and FCCP molecules are preferentially localized in the outer regions of the bilayer monitored by DPH-PA, as suggested by the K_q values (Table 3). As denoted by the high value of θ in the control preparation (without addition of FCCP), this system shows a high level of lipid packing in the upper regions of the hydrocarbon chains, probably due to the interaction of the phospholipid headgroups, particularly those of the anionic phospholipid (cardiolipin), with calcium ions. The ability of Ca^{2+} to increase lipid order has been previously detected in bacterial PE and cardiolipin-containing liposomes (Jurado et al. 1991). On the other hand, the fluidizing drug tamoxifen, which shares with FCCP the ability to decrease the T_m of saturated PC species (Custódio et al. 1993), also promotes an increase of fluidity in the outer

regions of bilayers formed by cardiolipin plus Ca^{2+}-containing lipid systems (Luxo et al. 2000). Therefore, FCCP should promote the disruption of the tight packing of the upper portions of phospholipid acyl chains, which in turn impairs Ca^{2+} coordination by the respective phospholipid headgroups. Considering the significant local concentrations that calcium may predictably attain at the interface of the anionic i.m.m. (Epand et al. 2002), FCCP may promote relevant alterations of the dynamic equilibrium between different membrane lipid microdomains, particularly when involving cardiolipin-enriched domains, hence influencing lipid-protein and protein-protein interactions.

The DSC studies performed in the present study also showed that FCCP is able to induce lateral phase separation (Fig. 2, Table 2), efficiently segregating cardiolipin from the zwitterionic lipid PE in a mixture of DPPE:TOCL used as a model of an heterogeneous membrane. FCCP is likely excluded from the saturated lipid-enriched domains, preferentially partitioning into the most disordered cardiolipin-enriched domains. This behavior is consistent with that observed in DPPC liposomes, where the profile skewed towards low temperatures (data not shown) suggesting a non-homogeneous FCCP distribution in the plane of the membrane, with a preference for the liquid-disordered regions. FCCP-induced formation of PE-rich domains and lateral phase segregation should have significant implications for the functionality of mitochondrial membranes. Firstly, due to the tendency of PE to form structures with negative curvature, an eventual destabilization of the bilayer with impact on membrane permeability is predictable. Secondly, the destabilization of the dynamic heterogeneous membrane structure and the appearance of leaky interfacial regions between coexisting differentiated lipid domains should have disastrous consequences for the normal mitochondrial membrane functioning.

FCCP also disturbed the phase behavior of the i.m.m. model containing DOPC:DOPE:cardiolipin at 1:1:1 molar ratio, including calcium at a ratio with cardiolipin of 1:1. By using ^{31}P NMR, we show for the first time that FCCP promotes the formation of a highly curved non-lamellar phase, by increasing the negative curvature strain in membrane systems composed by bilayer and non-bilayer preferring lipids (Fig. 3). As indicated by the quenching studies (Table 3), FCCP shows a distribution similar to that reported for other hydrophobic molecules such as alkanes (Sjolund et al. 1985; Siegel et al. 1989), accumulating in the core of the bilayer phase and preferentially residing in the interstices of the inverted hexagonal phase, partitioning into more disordered regions in both phase lattices. This behavior explains FCCP ability to favor the lamellar/inverted hexagonal (L_α/H_{II}) phase transition, by relaxing hydrocarbon-packing constraints, filling out the volumes between H_{II} cylinders (Sjolund et al. 1985).

The conjunction of a high propensity to induce lateral phase separation and to initiate the formation of non-lamellar phases may confer FCCP a high potential to disturb mitochondrial membranes, namely at the contact sites between the inner and the outer mitochondrial membranes, which are enriched in cardiolipin (Ardail et al. 1990), with which FCCP has a preferential interaction, as previously discussed. Cardiolipin, besides providing a high organization of the respiratory system components (Wittig and Schagger 2009), has also been proposed to mediate the targeting of proapoptotic proteins to mitochondria, actively participating in the release of proteins relevant for apoptosis execution (Gonzalvez and Gottlieb 2007). Peroxidation, hydrolysis and re-distribution of CL seem to occur early in apoptosis (Kagan et al. 2006). Therefore, destabilization of cardiolipin-enriched bilayer domains caused by FCCP and eventually facilitated by the increased calcium concentration in mitochondria (Epand et al. 2002), should have implications in mitochondrial apoptotic signaling.

Additionally, quenching results suggest that FCCP incorporated in DPPC or DOPC:DOPE:TOCL:Ca^{2+} (1:1:1:1, molar ratio) lamellar structures displays a broad distribution, from the hydrophobic core to the outer regions of the lipid bilayers. This distribution across the bilayer thickness is consistent with the ability of FCCP to migrate through the i.m.m. when performing its uncoupler activity (Skulachev et al. 1967; Benz and McLaughlin 1983; Terada 1990). Parallel studies performed in our laboratory with other weak acid-drug harboring protonophoric activity, the nonsteroidal anti-inflammatory drug nimesulide, showed also a wide distribution of the uncoupler molecules across the bilayer, although distinct effects on membrane physical properties were observed (unpublished data). From this information we can infer that: a) as expected, different molecular mechanisms may lead to perturbations at the membrane level promoting protonophoresis, eventually with different efficiency; b) the secondary effects resulting from membrane physical disturbance induced by different drugs used for a same pharmacological purpose (e.g. mild uncoupling) may be modulated by structural design.

We are aware that model lipid systems and their ionic environment are not as complex as those present in biological systems. Nevertheless, there are several lines of evidence that show that structural and material properties of lipid bilayers, which are relevant for the functionality of native membranes, may be adequately modeled in artificial lipid systems, showing a tight correlation with lipid composition (Andersen and Koeppe 2007; Lundbaek et al. 2010).

In summary, the present study provides evidence that FCCP promotes distinct biophysical effects in different lipid systems and in disparate regions of lipid mixtures

prepared to resemble the heterogeneous composition of native membranes. Moreover, some of the effects here described for FCCP may actually interfere with its uncoupling activity. Although FCCP protonation/deprotonation cycles, which occur under physiological conditions, are extremely complex to mimic in model lipid systems, data reported in the present work may provide useful insights into the role of mitochondrial lipid membrane composition in the action of modulators of the mitochondrial function, at physiologically relevant concentrations.

Therefore, our studies point to two distinct aspects of toxicological significance: a toxicokinetic one, indicating that alterations of mitochondrial lipid composition, as those occurring in malignant cells (Garcea et al. 1980) or as a consequence of the diet (Innis and Clandinin 1979, 1981), may interfere with FCCP partition and localization into the membrane, modifying its impact on mitochondrial activity; and a toxicodynamic aspect, suggesting that the uncoupler may modulate the physical properties of the membrane, with predictable repercussions in its uncoupling activity and secondary pharmacological effects. These aspects may contribute for a better toxicology understanding of uncoupler-membrane interactions, aiding in the future design of more efficient uncouplers for pharmacological purposes.

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