Effect of carbonylcyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) on the interaction of 1-anilino-8-naphthalene sulfonate (ANS) with phosphatidylcholine liposomes

Andrea C. Cutró, Guillermo G. Montich & Oscar A. Roveri

Journal of Bioenergetics and Biomembranes

ISSN 0145-479X
Volume 46
Number 2

J Bioenerg Biomembr (2014) 46:119-125
DOI 10.1007/s10863-014-9545-0

Springer

J Bioenerg Biomembr (2014) 46:119–125
DOI 10.1007/s10863-014-9545-0

Effect of carbonylcyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) on the interaction of 1-anilino-8-naphthalene sulfonate (ANS) with phosphatidylcholine liposomes

Andrea C. Cutró · Guillermo G. Montich · Oscar A. Roveri

Received: 29 December 2013 / Accepted: 17 February 2014 / Published online: 26 February 2014

Abstract The weak hydrophobic acid carbonylcyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) is a protonophoric uncoupler of oxidative phosphorylation in mitochondria. It dissipates the electrochemical proton gradient ($\Delta\mu_H^+$) increasing the mitochondrial oxygen consumption. However, at concentrations higher than 1 μM it exhibits additional effects on mitochondrial energy metabolism, which were tentatively related to modifications of electrical properties of the membrane. Here we describe the effect of FCCP on the binding of 1-anilino-8-naphthalene sulfonate (ANS) to 1, 2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) unilamellar vesicles. FCCP inhibited the binding of ANS to liposomes either in the gel or in the liquid crystalline phase, by increasing the apparent dissociation constant of ANS. Smaller effect on the dissociation constant was observed at high ionic strength, suggesting that the effect of FCCP is through modification of the electrostatic properties of the membrane interface. In addition, FCCP also decreased (approximately 50 %) the quantum yield and increased the intrinsic dissociation constant of membrane-bound ANS, results that suggest that FCCP makes the environment of the ANS binding sites more polar. On those grounds we postulate that the binding of FCCP: i) increases the density of negative charges in the membrane surface; and ii) distorts the phospholipid bilayer, increasing the mobility of the polar headgroups making the ANS binding site more accessible to water.

Keywords ANS binding · FCCP · Surface potential · Liposomes · Binding site polarity

Electronic supplementary material The online version of this article (doi:10.1007/s10863-014-9545-0) contains supplementary material, which is available to authorized users.

A. C. Cutró · O. A. Roveri (✉)
Area Biofísica, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, S2002LRK Rosario, Argentina
e-mail: oroveri@fbioyf.unr.edu.ar

A. C. Cutró
e-mail: andreacutro@yahoo.com.ar

G. G. Montich
Centro de Investigaciones en Química Biológica de Córdoba (CIQUIBIC, UNC–CONICET), Departamento de Química Biológica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Córdoba, Argentina
e-mail: gmontich@fcq.unc.edu.ar

Present Address:
A. C. Cutró
Laboratorio de Biointerfases y Sistemas Biomiméticos, Laboratorios Centrales Centro de Investigación y Transferencia Santiago del Estero, CITSE UNSE CONICET, El Zanjón, RN 9, km1125, Santiago del Estero, Argentina

Introduction

The energy released in the electron transport from the mitochondrial respiratory chain substrates to oxygen is used to translocate protons across the inner mitochondrial membrane, thus generating an electrochemical potential gradient ($\Delta\mu_{H^+}$). This gradient is used by the mitochondrial ATPase complex to drive the synthesis of ATP from ADP and Pi (Nicholls and Ferguson 2002). A variety of compounds are known to uncouple oxidative phosphorylation. Most of them are weak acids capable of translocating protons across the membrane, dissipating the electrochemical proton gradient (Terada 1990). Among them, carbonylcyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) is a compound known for its action as protonophoric uncoupler of the oxidative phosphorylation (Benz and McLaughlin 1983). At concentration lower than 1 μM it stimulates the electron transport from NADH or succinate to oxygen (Chance et al. 1963). At higher

concentrations it exerts additional effects on mitochondrial energy metabolism that were tentatively related to modification of surface and/or dipole potential of the inner mitochondrial membrane (Reyes and Benos 1984). Furthermore, FCCP affected several biophysical properties of multilamellar liposomes such as thermotropic behavior and fluidity of multilamellar liposomes. In mixed lipid systems it also favors lateral phase separation and elastic curvature properties favoring lamellar/H_{II} transition (Monteiro et al. 2011).

To obtain additional evidences that FCCP at concentrations higher than 1 μM modifies electrical and/or structural properties of lipid bilayers, we decided to carry out a detailed study on the effect of FCCP on the binding of 1-anilino-8-naphthalene sulfonate (ANS) to large unilamellar vesicles (LUVs). It has been reported by Haynes and Staerk (Haynes and Staerk 1974) that ANS binds to pre-existing sites in the head group region of phospholipid membranes with a stoichiometry of 1 site per 4 phosphatidylcholine moieties. It has also been shown (Haynes 1974) that the apparent affinity of ANS for its binding site is influenced by the membrane surface potential. Here we show that FCCP completely inhibits the ANS fluorescence increase that accompanies the binding of ANS to phospholipid membranes, inhibition that is mainly due to an increase in the ANS dissociation constant, despite a small effect of FCCP on the fluorescence quantum yield of the bound ANS was also observed. Two factors contribute to the increase in the ANS dissociation constant: i) FCCP adds negative charges to the membrane surface with the consequent increase in the negative surface potential; and ii) FCCP increases the intrinsic dissociation constant of ANS by increasing the accessibility of the ANS binding sites to water.

Materials and Methods

Chemicals

FCCP and ANS were obtained from Sigma-Aldrich Co. 1,2-palmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, USA). Vesicles extrusion was carried out with an extruder from Avanti Polar Lipids, Inc. Solvents (pro-analysis quality) were obtained from Merck. All other compounds were of analytical reagent grade.

General

FCCP was dissolved in ethanol (spectrophotometric quality) and it was added to the LUVs aqueous suspensions. Controls with ethanol (less than 2 %, final proportion in the aqueous suspension) were performed for all determinations.

Preparation of large unilamellar liposomes (LUVs)

DOPC or DPPC from a stock solution in chloroform:methanol 2:1 V/V was placed in a glass tube. A thin lipid film was obtained by slow evaporation of the solvent using a stream of nitrogen. The residual mixture of organic solvents was removed by using a vacuum pump for 4 h. Once dried, the film was hydrated in a medium containing 250 mM sucrose and 30 mM Tris–HCl (pH 8.0). The multilamellar vesicle suspension thus obtained was disrupted by five freeze-thaw cycles. Finally, the lipid suspension was extruded ten times through a polycarbonate filter (pore diameter 100 nm) at 55 °C (http://www.avantilipids.com/index.php?option=com_content&view=article&id=533&Itemid=297. Accessed 20 December 2013).

Lipid quantification

Lipid concentration was determined by quantifying inorganic phosphorus. Samples were mineralized according to the method described by Hess and Derr (Hess and Derr 1975) and inorganic phosphate was colorimetrically quantified as phosphomolybdate essentially as described (Ames 1966; Chen et al. 1956).

ANS fluorescence measurements

Fluorescence measurements were carried out in a Cary Eclipse spectrofluorometer. Excitation was at 380 nm and fluorescence emission was measured at 480 nm. The FCCP absorbance at the excitation wavelength was used to correct the inner filter effect (Lakowicz 2006).

Lifetime fluorescence measurements

Fluorescence lifetimes were measured in a time correlation single photon counting (TCSPC) fluorometer. The samples were excited with a PLS340 LED driven by a PDL 800-B unit. The excitation wavelength was 340 nm and the frequency was 5 MHz. The emission was detected with a PMA182NM photomultiplier. Single photon counting was performed with a PicoHarp 300E TCSPC unit. The analysis of the fluorescence decay data was done with the Global FluoFit Fluorescence Decay Data Software. PDL 800-B, PicoHarp 300 and the software were from PicoQuant GmbH, Berlin.

Differential Scanning Calorimetry (DSC)

Thermograms were obtained using a scanning calorimeter DSC-VP from MicroCal (Northampton, MA). The reference cell was filled with a solution containing 250 mM sucrose, 30 mM Tris–HCl (pH 8.0). The sample cell (800 μL) was filled with liposomes (1 mg/mL) suspended in the same

medium. Both cells were pressurized to 26 psi. The scan rate was 30 °C/h.

Data analysis

Experimental data were analyzed by a non-linear regression procedure based on the Marquardt-Levenberg algorithm. Fitting parameters were expressed as the expectation value ± standard deviation. One-way ANOVA with the Student-Newman-Keuls as a post-test was used for the statistical analysis. A value of $p<0.05$ was considered statistically significant.

Results and discussion

Effect of FCCP on the thermotropic behavior of DPPC-LUVs

The effect of FCCP on the thermotropic behavior of unilamellar liposomes of DPPC was studied. LUVs of DPPC (1 mg/mL) exhibited a pre-transition around 34 °C and a main transition centered at 41.7 °C. The pre-transition practically disappeared in the presence of 30 μM FCCP and the main transition only slightly moved towards lower temperatures (the transition temperature was 41.3 °C in the presence of FCCP). A similar behavior has been previously reported for multilamellar DPPC liposomes (Monteiro et al. 2011).

Inhibition by FCCP of the binding of ANS to LUVs

The kinetic profile of the fluorescence increase observed when ANS binds to LUVs of DOPC showed two kinetic phases (Fig. 1):

$$\Delta F = \Delta F_1\left(1-e^{-k_1 t}\right) + \Delta F_2\left(1-e^{-k_2 t}\right) \quad (1)$$

ΔF_1 and ΔF_2 are the amplitudes of the fast and slow phase, respectively; k_1 and k_2 are the kinetic constants of those phases. The fast phase has been previously attributed to the binding of ANS to the surface of the liposomes and the slower phase to the migration of ANS into the inner hemilayer (Gains and Dawson 1975). The time constant of the fast phase was not determined since it was faster than the instrumental response. However, its amplitude -that accounts for approximately half of the total fluorescence change– was accurately measured by fitting Eq. 1 to the kinetic profiles. FCCP strongly decreased the amplitude of both kinetic phases (Fig. 2) without significantly modifying the kinetic constant of the slow phase (data not shown). The following equation (see Supplementary material) was fitted to the experimental data:

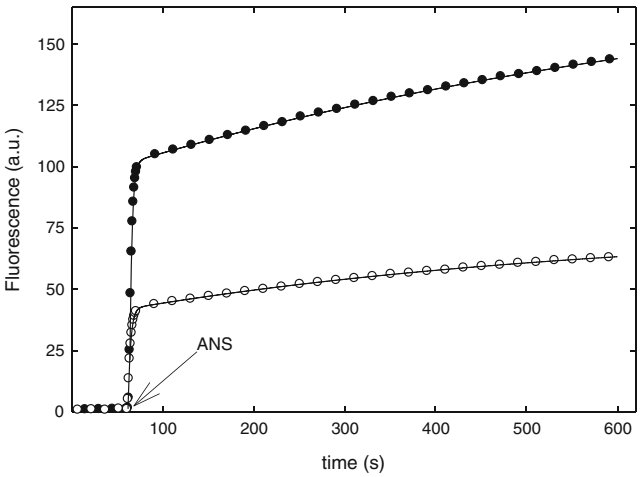

Fig. 1 Effect of FCCP on the ANS fluorescence increase upon binding to DOPC liposomes. The time course of ANS fluorescence change upon binding to DOPC liposomes was followed at 25 °C as described in Materials and Methods in the presence of 6.25 μM FCCP (*white circle*) and in its absence (*filled circle*). [DOPC]=0.1 mg/mL; [ANS]=62.5 μM. The lines indicate the best fit of Eq. 1 to the experimental data

$$\frac{\Delta F_1}{\Delta F_1^0} = \frac{(\Delta F_1)_\infty}{\Delta F_1^0} + \frac{\left[1-\frac{(\Delta F_1)_\infty}{\Delta F_1^0}\right] IC_{50}{}^n}{IC_{50}{}^n + [FCCP]^n} \quad (2)$$

ΔF_1^0, ΔF_1 and $(\Delta F_1)_\infty$ are the amplitudes of the fast phase in the absence, in the presence of FCCP at a given concentration and at infinite [FCCP]; IC_{50} is the [FCCP] that reduces 50 % the amplitude of the fast phase and n is the heterogeneity

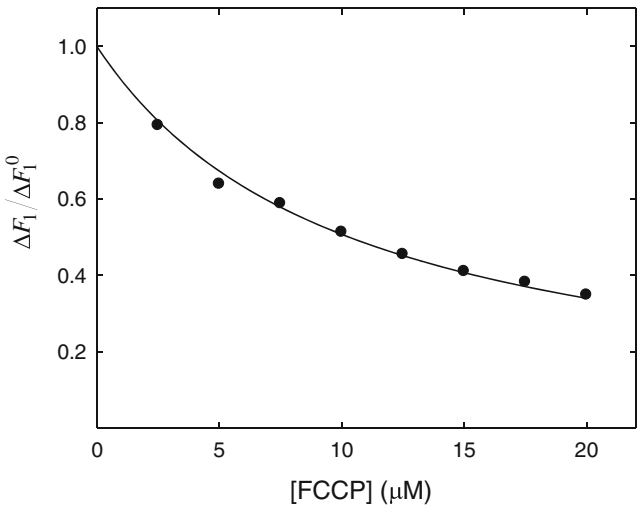

Fig. 2 Effect of FCCP on the fluorescence change observed upon binding of ANS to DOPC LUVs. The experimental conditions were described in Materials and Methods. [DOPC]=0.1 mg/mL and [ANS]=25 μM. The points represent the amplitude of the fast kinetic phase of fluorescence increase determined at different [FCCP] related to the amplitude estimated in the absence of FCCP (see Text). The line indicates the best fit of Eq. 2 to the experimental data

index (Disalvo and Bouchet 2014). No heterogeneity in the FCCP binding sites was detected since the statistical analysis showed that n was not significantly different than 1. The fitting procedure has also shown that $(\Delta F_1)_\infty$ was not significantly different from zero, indicating that at infinite [FCCP] the ANS fluorescence increase was completely inhibited (50 % inhibition was exerted by 10.3±0.2 μM FCCP). Similar behavior was observed with DPPC LUVs either in liquid crystalline or in gel phases. The IC_{50} value estimated for the gel phase (8±2 μM) was smaller than that estimated for the liquid crystalline phase (19±5 μM).

To determine if FCCP has a direct effect on the ANS fluorescence we made a Stern-Volmer plot for the quenching of ANS in ethanol. Fitting the fluorescence decay curve of pure ANS to a single exponential we obtained a fluorescence lifetime of 8.5 ns. Increasing concentrations of FCCP up to 7 μM did neither decrease the steady state fluorescence intensity nor the fluorescence decay time of ANS.

Experiments were carried out at different [ANS] in the absence and in the presence of a fixed [FCCP]. The amplitude of the fast phase, which is proportional to the amount of ANS bound to the outer hemilayer of the LUVs, exhibited a simple hyperbolic dependence on [ANS] either in the absence [7 and see Supplementary material] or in the presence of FCCP (Fig. 3):

$$\Delta F_1 = \frac{\Delta F_1^{\infty} \times [ANS]}{K_d^{'} + [ANS]} \qquad (3)$$

$K_d^{'}$ is the apparent dissociation constant of ANS from the external face of the bilayer (see Section 3.4) and ΔF_1^{∞} is the amplitude of the fast phase at saturating concentration of ANS. The latter depends on the number of ANS binding sites in the bilayer and on the fluorescence quantum yield of the bound ANS. FCCP produced an increase of $K_d^{'}$ and a decrease in ΔF_1^{∞} (Fig. 3). Qualitatively similar results were obtained with DPPC liposomes. In the gel phase (25 °C) FCCP 9.4 μM increased $K_d^{'}$ from 31±5 μM to 91±11 μM. Similarly, in the liquid-crystalline phase (50 °C) the ANS dissociation constant was increased from 20±3 μM to 60±10 μM by 18.7 μM FCCP.

Effect of FCCP on the maximal fluorescence change

Figure 4 shows K_d and ΔF_1^{∞} values measured from experiments similar to that shown in Fig. 3 in the presence of different [FCCP]. The effect of FCCP on ΔF_1^{∞} can be described by a hyperbolic decrease to a

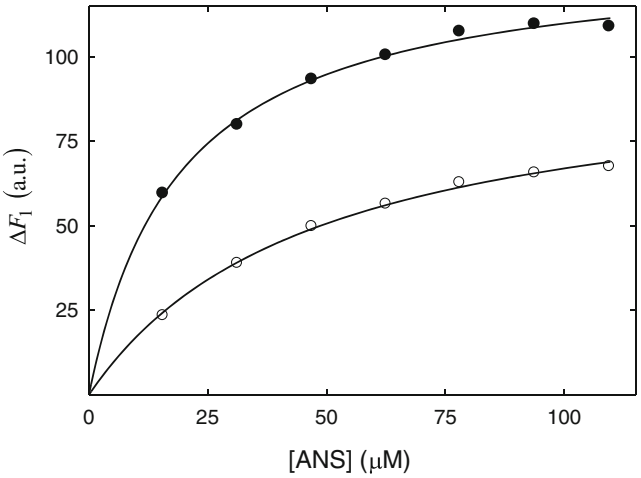

Fig. 3 Effect of FCCP on the binding of ANS to DOPC -LUVs. The amplitude of the fast phase of the ANS fluorescence increase was determined at different [ANS]. The other experimental conditions are similar to those described in the previous Legends. The points indicate the experimental values obtained in the absence (*filled circle*) and in the presence of 12.5 μM FCCP (*white circle*). The lines indicate the best fit of Eq. 3 to the experimentally obtained data

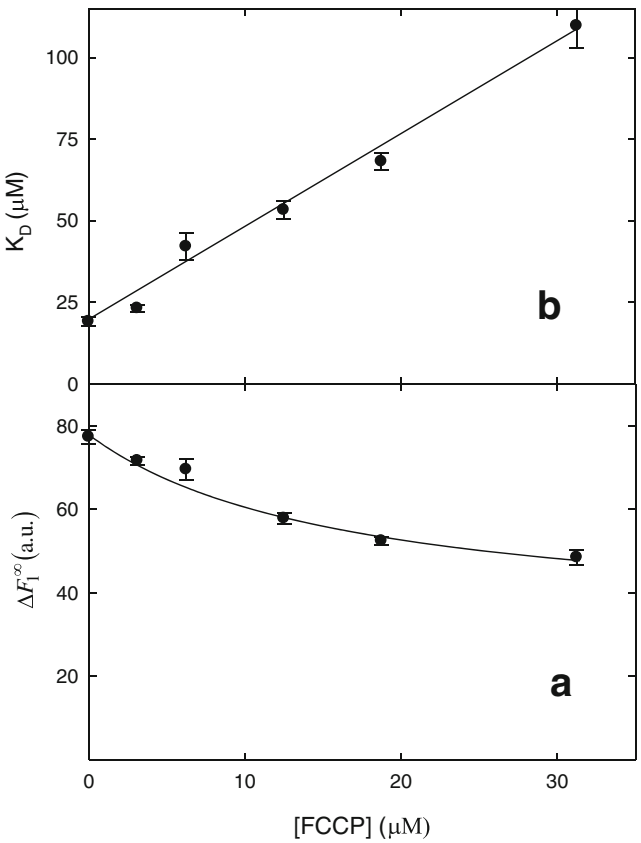

Fig. 4 Effect of FCCP on the binding parameters of ANS to DOPC liposomes. Experimental conditions were as described in the Legend to Fig. 3 except that titrations with ANS were performed at different [FCCP]. In the Figure the points represent ΔF_1^{∞} (a) and K_d (b) values estimated by nonlinear regression using Eq. 3, with their respective error bars. The lines are the weighted regression lines of Eq. 4 (a) or Eq. 5 (b)

value different than zero (see Supplementary material) according to (Fig. 4-a):

$$\Delta F_1^{\infty} = \left(\Delta F_1^{\infty}\right)_{[FCCP]=\infty} + \frac{\left(\left(\Delta F_1^{\infty}\right)_{[FCCP]=0} - \left(\Delta F_1^{\infty}\right)_{[FCCP]=\infty}\right) \times C_{0.5}}{C_{0.5} + 0.5[FCCP]} \quad (4)$$

$C_{0.5}$ (16.4±4.8 μM) is the [FCCP] that exerts half of the maximal effect; $(\Delta F_1^{\infty})_{[FCCP]=0}$ and $(\Delta F_1^{\infty})_{[FCCP]=\infty}$ are the ΔF_1^{∞} values in the absence and at infinite [FCCP], respectively. The latter is approximately 40 % of the value estimated in the absence of FCCP. Since ΔF_1^{∞} has a value significantly different from zero at infinite [FCCP], it can be concluded that the complete inhibition of the fluorescence increase cannot be explained by the effect of FCCP neither on the number of ANS binding sites nor on the fluorescence quantum yield of bound ANS.

To further characterize the effect of FCCP on ΔF_1^{∞}, the fluorescence lifetime of ANS was determined in the presence of variable [FCCP] and DOPC-LUVs. Either in the presence or in the absence of FCCP a bi-exponential decay of the fluorescence was observed. The shorter lifetime (τ_1) did not change with [FCCP] and it was assigned to ANS in solution. The longer lifetime (τ_2) was assigned to membrane-bound ANS. It was sensitive to the presence of FCCP and decreased with increasing [FCCP] (see Supplementary material) as follows (Fig. 5):

$$\tau_2 = \tau_2^{\infty} + \frac{\left(\tau_2^0 - \tau_2^{\infty}\right) C_{0.5}}{C_{0.5} + [FCCP]} \quad (5)$$

τ_2^0 and τ_2^{∞} are the lifetimes in the absence of FCCP and at infinite [FCCP]; $C_{0.5}$ is the [FCCP] at which half of the maximal effect was observed. By fitting the Eq. 5 to the experimental data we obtained values of τ_2^0=6.5±0.6 ns, τ_2^{∞}=3.3±0.3 ns and $C_{0.5}$=9.7±1.9 μM. Hence, FCCP decreased up to 50 % the fluorescence quantum yield calculated from the lifetimes estimated from the data shown in Fig. 5. Similar results were obtained with DPPC-LUVs either in liquid-crystalline or gel state (Table 1). Therefore, the decrease of ΔF_1^{∞} can be mainly attributed to the decrease in the fluorescence quantum yield of the bound ANS, despite a small effect on the number of ANS binding sites cannot be completely excluded. These results indicate that FCCP distorts the bilayer lattice increasing the accessibility of water to the ANS binding sites.

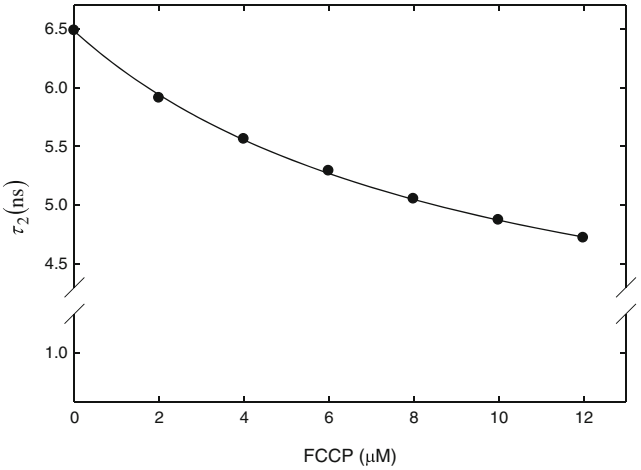

Fig. 5 Effect of FCCP on the fluorescence lifetime of ANS bound to DOPC-LUVs. The fluorescence lifetime of ANS bound to DOPC liposomes was determined at different [FCCP] at 25 °C. [DOPC]=0.1 mg/mL; [ANS]=25 μM. The line indicates the best fit of Eq. 5 to the experimentally determined data

Effect of FCCP on the ANS apparent dissociation constant (K_d')

On the other hand, the dissociation constant of ANS increased linearly (see Supplementary material) with [FCCP] (Fig. 4-b):

$$K_d' = K_d\left(1 + \frac{[FCCP]}{K_{FCCP}}\right) \quad (6)$$

K_d is the ANS dissociation constant in the absence of FCCP. K_{FCCP} is an apparent constant related to the intrinsic FCCP dissociation constant and to the effect of FCCP on the surface potential (see below). By fitting Eq. 6 to the experimental data (see Fig. 4-b), we obtained values of K_d=20±3 μM and of K_{FCCP}=7.1±1.4 μM. In summary, the linear increase in K_d' explains the complete inhibition of the fluorescence increase.

The apparent dissociation constant of an anion such as ANS from a charged surface depends on the intrinsic

Table 1 Effect of FCCP on the fluorescence quantum yield of ANS bound to liposomes

Temperature	Liposomes	τ_2^0 (ns)	τ_2^{∞} (ns)	$\Phi^{0\ a}$	$\Phi^{[FCCP]=\infty\ a}$
25 °C	DOPC	6.5±0.6	3.3±0.3	0.27±0.02	0.14±0.08
25 °C	DPPC	8.8±0.1	4.7±0.1	0.37±0.01	0.20±0.01
50 °C	DPPC	6.5±0.1	3.3±0.3	0.27±0.01	0.14±0.08

[a] Calculated from the fluorescence lifetimes estimated at zero and infinite [FCCP] using a natural lifetime equal to 24 ns (Fortes 1972)

dissociation constant K_d^0 and on the surface potential ψ_s, accordingly to (Robertson and Rottenberg 1983):

$$K_d = K_d^0 e^{-\frac{F\psi_s}{RT}} \quad (7)$$

The experiments described above were carried out at low ionic strength (in the absence of added NaCl; ionic strength–16.7 mM). To determine whether FCCP affects the membrane surface potential or the intrinsic dissociation constant, its effect on the binding of ANS in a high ionic strength medium (NaCl 200 mM) was studied. FCCP also inhibited the binding of ANS to DOPC LUVs at high ionic strength. Similarly to what happened at low ionic strength FCCP decreased ΔF_1^∞ and increased the ANS dissociation constant (see Table 2). Since this last result was obtained at high ionic strength at which a low surface potential value is expected, it can be concluded that FCCP increases the intrinsic dissociation constant of ANS from the bilayer.

$K_d^{'}$ values obtained at low and high ionic strengths are related to the surface potential under those conditions as follows (Robertson and Rottenberg 1983):

$$\Psi_{s(1)} - \Psi_{s(2)} = -\frac{RT}{F} \ln \frac{K_{d(1)}^{'}}{K_{d(2)}^{'}} \quad (8)$$

$\Psi_{s(1)}$ and $\Psi_{s(2)}$ are the surface potentials at high and low ionic strength, respectively and $K_{d(1)}^{'}$ and $K_{d(2)}^{'}$ the corresponding apparent ANS dissociation constants. When the dissociation constants determined in the absence of FCCP are used, Eq. 8 yielded a value equal to −3.05 mV. FCCP increased this negative value to −9.82 mV.

The surface potential is related to the surface charge density (σ) accordingly to the following equation (Robertson and Rottenberg 1983):

$$\sigma = 11.74\sqrt{[NaCl]}\, sinh \frac{\left(\psi_{s(1)} - \psi_{s(2)}\right)}{51.7} \quad (9)$$

Table 2 Effect of FCCP on the binding of ANS to DOPC-LUVs. Dependence on the ionic strength

Ionic strength (mM)	Additions	$K_d^{'}$ [a] (μM)	ΔF_1^∞ [a] (a.u)
16.7	None	18.9±1.3	130±2
216.7	None	16.8±2.1	122±4
16.7	FCCP	47.4±3.3	99±3
216.7	FCCP	32.3±3.7	103±4

[a] $K_d^{'}$ and ΔF_1^∞ were estimated by fitting Eq. 3 to experimental data obtained from experiments similar to those shown in Fig. 3 carried out at the stated ionic strength

Hence, the relation between the density of surface negative charges in the presence of FCCP (σ_{FCCP}) and that in its absence (σ_0) is:

$$\frac{\sigma_{FCCP}}{\sigma_0} = \frac{sinh\left(\psi_{s(1)} - \psi_{s(2)}\right)_{FCCP}}{sinh\left(\psi_{s(1)} - \psi_{s(2)}\right)_0} = \frac{sinh(-9.82)}{sinh(-3.05)} = 3.2 \quad (10)$$

Therefore, FCCP increases more than 3 times the density of surface negative charges.

General discussion

According to Monteiro et al. (Monteiro et al. 2011) FCCP is preferentially localized in the outer regions of lipids organized in lamellar phases. FCCP is a weak hydrophobic acid (pKa= 6.05, (Benz and McLaughlin 1983)). Since the experiments described here were carried out at pH 8.0, approximately 99 % of the compound is in its anionic form. The binding of an anion to the membrane surface would add negative charges to it but also would be sensitive to a negative electrostatic potential (Benz and McLaughlin 1983). As a matter of fact, a IC_{50} value three times smaller (3.6±0.2 μM) was estimated for the inhibition observed at high ionic strength (see previous section).

Haynes and Staerk (Haynes and Staerk 1974) suggested that the ANS binding site in phospholipid membranes is composed by four polar headgroups, whose mobility as well as the accessibility of water to the headgroups region would affect the ANS binding constant and the fluorescence quantum yield of the bound ANS. It has been reported by Monteiro et al. (Monteiro et al. 2011) that FCCP increases the fluidity of phospholipid membranes, hence disrupting the tight packing of the upper portions of the phospholipid acyl chains. Such disruption would increase the mobility of the polar headgroups and loosen the interaction between them. Therefore, the accessibility of water to the ANS binding sites would increase, consequently producing an increase of the ANS dissociation constant and a decrease in the fluorescence quantum yield of the bound ANS.

The above mentioned addition of negative charges to the membrane surface increases the negative surface potential, so inducing an additional increase in the apparent ANS dissociation constant. Reyes and Benos (Reyes and Benos 1984) have reported that FCCP induces changes in the interfacial potential of phospholipid monolayers that depend on the ionic strength in a way that they claim cannot simply be due to the induction of a double-layer potential at the membrane solution interface. However, their results were obtained with

[FCCP] lower than those used here. In addition, the effect on the intrinsic dissociation constant would mask partially the dependence on ionic strength of the increase in the apparent ANS dissociation constants.

Our results show that the affinity of gel phase for ANS and for FCCP (see Section 3.2) is higher than those of the liquid crystalline phase. The fact that FCCP inhibits the binding of ANS to liposomes in the gel phase is another evidence that FCCP binds preferentially at the lipid membrane interface.

Concluding remarks

FCCP binds as an anion to the membrane surface of liposomes. Upon binding to the bilayer, it exerts two effects: i) it increases the amount of surface negative charges, hence the surface potential and consequently the ANS apparent dissociation constant; and ii) it increases the accessibility of water to the ANS binding sites decreasing the fluorescence quantum yield of the bound ANS and increasing the ANS intrinsic dissociation constant, likely by an increased mobility of the polar headgroups.

Despite the increase in interfacial potential could reasonably explain the competition of succinate on the FCCP inhibition of the oxygen consumption in mitochondria (Reyes and Benos 1984), the effect of FCCP on the membrane fluidity and on the mobility of headgroups must be taken into account: i) to explain the additional effects on the mitochondrial metabolism observed at [FCCP] higher than 1 μM; and ii) for the pharmacological evaluation of uncouplers as FCCP.

Acknowledgments This work has been carried out with grants from CONICET (PIP 0307) and ANPCyT (PICT 2005/38056) to O.A.R. and also with support from the Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario. O.A.R. and G.M. are Members of the Carrera del Investigador from CONICET. A.C.C. was Fellow of ANPCyT (FONCyT) and of CONICET.

References

Ames BN (1966) Assay of inorganic phosphate, total phosphate and phosphatases. Methods Enzymol 8:115–118

Benz R, McLaughlin S (1983) The Molecular Mechanism of Action of the Proton Ionophore FCCP (Carbonylcyanide p-Trifluoromethoxyphenylhydrazone). Biophys J 41:381–398

Chance B, Williams GR, Hollinger G (1963) Inhibition of electron and energy transfer in mitochondria. III Spectroscopy and respiratory effects of uncoupling agents. J Biol Chem 278:439–444

Chen PS, Toribara TY, Warner H (1956) Microdetermination of phosphorus. Anal Chem 28:1756–1758

Disalvo EA, Bouchet AM (2014) Electrophoretic mobility and zeta potential of liposomes due to arginine and polyarginine adsorption. Colloids Surf A 440:170–174

Fortes, PAG (1972) Structural properties of the human red cell membrane and anion permeability studied with fluorescence probes. Ph. D. Dissertation, University of Pennsylvania, Philadelphia

Gains N, Dawson AP (1975) Transmembrane electrophoresis of 8-anilino-1-naphtalene sulfonate through egg lecithin liposome membranes. J Membr Biol 24:237–248

Haynes DH (1974) 1-anilino-8-naphtalenesulfonate: a fluorescent indicator of ion binding and electrostatic potential on the membrane surface. J Membr Biol 17:341–366

Haynes DH, Staerk H (1974) 1-Anilino-8-naphtalenesulfonate: a fluorescent probe of membrane surface structure, composition and mobility. J Membr Biol 16:313–340

Hess HH, Derr JE (1975) Assay of inorganic and organic phosphorus in the 0.1–5 nanomole range. Anal Biochem 63:607–613

http://www.avantilipids.com/index.php?option=com_content&view=article&id=533&Itemid=297. Accessed 20 December 2013.

Lakowicz, JR (2006). Instrumentation for fluorescence spectroscopy. In principles of fluorescence spectroscopy. New York, Springer, pp. 27–61

Monteiro JP, Martins AF, Lúcio M, Reis S, Geraldes CFGC, Oliveira PJ, Jurado AS (2011) Interaction of carbonyl-p-trifluoromethoxyphenylhydrazone (FCCP) with lipid membrane systems: a biophysical approach with relevance to mitochondrial uncoupling. J Bioenerg Biomembr 43:287–298

Nicholls DG, Ferguson SJ (2002) Chemiosmotic energy transduction. In: Bioenergetics3. Academic, London, pp 3–14

Reyes J, Benos DJ (1984) Changes in interfacial potentials induced by carbonylcyanide phenylhydrazone uncouplers: possible role in inhibition of mitochondrial oxygen consumption and other transport processes. Membr Biochem 5:243–268

Robertson DE, Rottenberg H (1983) Membrane potential and surface potential in mitochondria. fluorescence and binding of 1-anilinonaphtaleno-8-sulfonate. J Biol Chem 258:11039–11048

Terada H (1990) Uncouplers of oxidative phosphorylation. Environ Health Perspect 87:213–218