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Dodecyl and octyl esters of fluorescein as protonophores and uncouplers of oxidative phosphorylation in mitochondria at submicromolar concentrations

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ABSTRACT

In our search for fluorescent uncouplers of oxidative phosphorylation, three esters of fluorescein, *n*-butyl-, *n*-octyl-, and *n*-dodecyl-oxycarbonyl-fluorescein (C_4-FL, C_8-FL, C_{12}-FL) were synthesized and characterized. With increasing liposomal lipid content, the long-chain alkyl derivatives of fluorescein (C_8-FL, C_{12}-FL and commercially available C_{18}-FL), but not C_4-FL and unsubstituted fluorescein, exhibited an increase in fluorescence polarization reflecting the dye binding to liposomes. C_{12}-FL induced proton permeability in lipid membranes, while C_4-FL was inactive. In contrast to C_4-FL and C_{18}-FL, C_{12}-FL and C_8-FL increased the respiration rate and decreased the membrane potential of isolated rat liver mitochondria with half-maximal effective concentrations of 700 nM and 300 nM, respectively. The effect of C_n-FL on the respiration correlated with that on proton permeability of the inner mitochondrial membrane, as measured by induction of mitochondria swelling in the potassium acetate medium. Binding of C_8-FL to mitochondria depended on their energization, which was apparently associated with pH gradient generation across the inner mitochondrial membrane in the presence of a respiratory substrate. In wild-type yeast cells, C_{12}-FL localized predominantly in plasma membrane, whereas in AD1-8 mutants lacking MDR pumps, it stained cytoplasmic organelles with some preference for mitochondria. Fluorescent uncouplers can be useful as a tool for determining their localization in a cell or distribution between different tissues in a living animal by fluorescent microscopy.

1. Introduction

Uncouplers are low molecular weight compounds which are capable of carrying protons across inner mitochondrial membrane (IMM) and uncoupling respiration and ATP synthesis in mitochondria [1–5]. Current interest in this class of compounds is associated with their ability to protect cells from damage in a number of physiological models leading to the idea of their use in therapy [6–10].

It is generally accepted that uncoupling of respiration and ATP synthesis in mitochondria is due to IMM depolarization resulting from flow of protons down their electrochemical gradient across IMM facilitated by protonophores (Fig. 1A). The findings, showing that a small reduction in the electrochemical proton gradient (proton motive force) attenuates ROS production dramatically [11–14], have validated application of uncouplers for treatment of diseases associated with oxidative stress [6–8,10]. In particular, the use of uncoupling agents was proposed as a novel therapeutic approach for the treatment of neuronal injury following acute CNS insults, spinal cord contusion and traumatic brain injury [15–18]. According to [19], mild uncouplers can also play the role of cardioprotectors. Earlier, mitochondrial uncoupling was widely discussed as a target for drug development for the treatment of obesity [6]. Besides, uncoupling agents can serve as inducers of protective compensatory mechanisms in neurons [20] and control hyperglycemia similar to antidiabetic drugs [9]. However, toxicity of known uncouplers limits their potential for therapeutic use and necessitates design of novel uncoupling agents.

Although having been studied for more than half a century, the mechanism of uncoupling is still not fully understood. There are several lines of evidence that are not compatible with proton-carrying activity

Abbreviations: C_{12}-FL, fluorescein *n*-dodecyl ester; C_8-FL, fluorescein *n*-octyl ester; C_4-FL, fluorescein *n*-butyl ester; C_{18}-FL, fluorescein *n*-octadecyl ester; FL, unsubstituted fluorescein; C_n-FL, fluorescein *n*-alkyl ester with n hydrocarbon units; DPhPC, diphytanoylphosphatidylcholine; EggPC, egg yolk phosphatidylcholine; DiS-C_3-(5), 3,3′-dipropylthiadicarbocyanine iodide; TPP^+, tetraphenylphosphonium cation; FCCP, carbonyl cyanide-*p*-(trifluoromethoxy)phenylhydrazone; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; TMRE, tetramethylrhodamine ethyl ester; DNP, 2,4-dinitrophenol; BLM, bilayer lipid membrane; IMM, inner mitochondrial membrane; RLM, rat liver mitochondria

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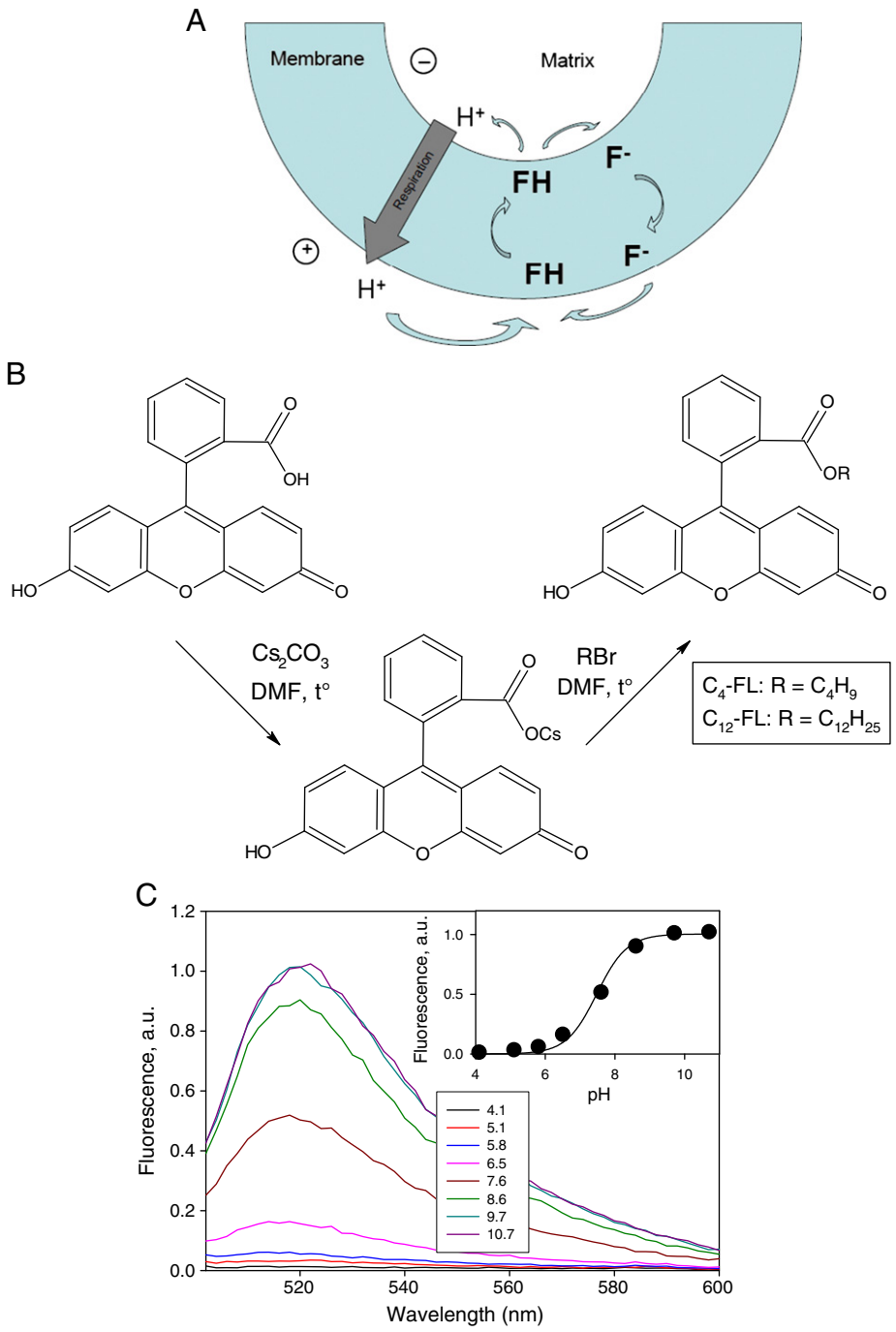

Fig. 1. A. Scheme of protonophore-mediated uncoupling on the inner mitochondrial membrane. B. Scheme of synthesis of alkyl-substituted fluoresceins. C. Emission spectra of C_{12}-FL at different pH. Inset: pH-dependence of C_{12}-FL fluorescence and a fitting curve according to Henderson–Hasselbalch equation with apparent $pK_a = 7.5$. C_{12}-FL concentration, 10 nM. The solution was 100 mM KCl, 10 mM Tris, 10 mM MES, 10 mM β-alanine. Excitation, 490 nm.

of known uncoupling agents as the sole basis of their uncoupling action. In many cases, strict correlation between the protonophoric activity on model lipid membranes and uncoupling of mitochondria was not found [1,21], albeit it was reported earlier for a series of uncouplers [22]. Moreover, Starkov and colleagues discovered that the dipole potential modulator 6-ketocholestanol [23] blocked the uncoupling action of FCCP on mitochondria, but stimulated the protonophoric activity of FCCP in planar lipid bilayers [24,25]. Besides, early works revealed tight binding of the protonophores 2,4-dinitrophenol (DNP) and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) to certain proteins of IMM [26,27]. To gain more insight into mechanistic details of mitochondria uncoupling, conjugation of a fluorescent probe to an uncoupler seems to be useful. Given a brightly fluorescent uncoupler, one gets an opportunity to examine its binding to membrane components including proteins and probably find specific interaction with a certain protein site. Besides, it could be helpful in studying distribution of uncouplers in tissues.

It has been shown recently that highly fluorescent dodecyl ester of rhodamine 19 can serve as an uncoupler in cells and isolated mitochondria [28]. However, its effective concentrations are rather large (tens of micromoles), and the mechanism of action differs considerably from that of conventional anionic uncouplers such as 2,4-dinitrophenol

(DNP) or carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP). Earlier uncoupling activity at high concentrations was also found for another fluorescent cationic dye, 3.3′-dihexyloxacarbocyanine (DiO-C_6-(3)) [29]. The question arises whether anionic fluorescein, having limited permeability through lipid membranes [30,31], can be modified so as to be used as an uncoupler. Fluorescein has two ionizable groups, one carboxyl group (pK_a close to 4) and one hydroxyl group in the aromatic core with pK_a close to 6.5 (Fig. 1B). Fluorescein does not uncouple mitochondria, but is capable of pH gradient-driven accumulation in mitochondria [32–34], which might be mediated by metabolite anion carriers, e.g., the α-ketoglutarate transporter, the tricarboxylate carrier, and the glutamate-aspartate exchanger [35]. In the present work we have synthesized three esters of fluorescein with *n*-butyl (2′-O-butyloxycarbonyl-fluorescein, C_4-FL), *n*-octyl (2′-O-octyloxycarbonyl-fluorescein, C_8-FL) and *n*-dodecyl groups (2′-O-dodecyloxycarbonyl-fluorescein, C_{12}-FL) as shown in Fig. 1B. A C_{12}-alkyl chain has been selected by analogy to our previous work with rhodamine derivatives, where an alkyl group of twelve carbon atoms provided maximal protonophoric activity [28]. It is shown here that C_{12}-FL and C_8-FL but not C_4-FL can uncouple rat liver mitochondria in the submicromolar range. Wild-type yeast cells exhibit efflux of C_{12}-FL, while MDR-deficient mutant cells can accumulate C_{12}-FL in cytoplasm where the dye partially colocalizes with tetramethylrhodamine.

2. Materials and methods

2.1. Materials

Fluorescein was from Reakhim (Moscow, Russia). Fluorescein octadecyl ester (C_{18}-FL), pyranine and tetramethylrhodamine ethyl ester (TMRE) were from Invitrogen. Diphytanoylphosphatidylcholine (DPhPC), egg yolk phosphatidylcholine (EggPC) and cholesterol were from Avanti Polar Lipids (Alabaster, Alabama), other chemicals were from Sigma.

2.2. Synthesis of C_{12}-FL, C_8-FL and C_4-FL

To increase lipophilicity of fluorescein, we used esterification of the carboxyl group with a C_{12}, C_8, or C_4 alkyl chain. C_{12}-FL and C_4-FL were synthesized by using an approach for preparation of alkyl rhodamine-19 derivatives developed in our previous work [36], namely: via the reaction between bromoalkanes and the previously obtained Cs salt of fluorescein, in dimethylformamide at 65 °C, the yields being rather low (about 10%) apparently due to formation of a bis-alkyl product. A rather similar procedure of synthesis was described earlier for *n*-octadecyl ester of fluorescein [37]. Briefly: the solution of cesium carbonate (0.49 g, 1.5 mmol) in water (0.5 ml) was added under mixing at 65 °C to the solution of fluorescein (1 g, 3 mmol) in DMF (7.5 ml). After 30 min, 1-bromododecane (0.75 g, 3 mmol, 300 μl) was added to the reaction mixture and heating (65–75 °C) continued for 20 h. Target substances were purified by column chromatography on silica gel in solvent mixtures containing chloroform and methanol at different ratios and characterized by HPLC, NMR and LC-MS (Supplementary Materials). C_8-FL was synthesized according to [38]. Briefly, fluorescein (1.026 g, 3 mmol) was dissolved in *n*-octanol (6 ml, 3.8 mmol), supplemented with concentrated sulfuric acid (0.5 ml) and incubated at 190 °C for 18 h. The resulting solution was diluted with ethyl acetate and concentrated by rotary evaporation. The precipitate (1.108 g) representing a mixture of unsubstituted fluorescein and the required ester was then separated by column chromatography on silica gel with ethyl acetate and an ethyl acetate/ethanol (3/1) mixture as eluents. Fractions containing pure C_8-FL were collected and concentrated by rotary evaporation. The yield of the product amounted to 54%. LC–MS was employed to estimate the purity.

2.3. Planar bilayers

Bilayer lipid membrane (BLM) was formed by the brush technique [39] from a 2% decane solution of diphytanoylphosphatidylcholine (DPhPC) on a 0.6-mm aperture in a Teflon septum separating the experimental chamber into two compartments of equal size (3-ml volumes). Electrical parameters were measured with two AgCl electrodes placed into the solutions on the two sides of the BLM via agar bridges, using a Keithley 428 amplifier (Cleveland, Ohio, USA).

2.4. Detection of proton transport in pyranine-loaded liposomes

The lumenal pH of the liposomes was assayed with pyranine by a slightly modified procedure of [40]. To prepare pyranine-loaded liposomes, lipid (5 mg diphytanoylphosphatidylcholine (DPhPC)) in a chloroform suspension was dried in a round-bottom flask under a stream of nitrogen. The lipid was then resuspended in buffer (100 mM KCl, 20 mM MES, 20 mM MOPS, 20 mM tricine titrated with KOH to pH 6.0) containing 0.5 mM pyranine. The suspension was vortexed and then freeze-thawed three times. Unilamellar liposomes were prepared by extrusion through 0.1-μm-pore size Nucleopore polycarbonate membranes using an Avanti Mini-Extruder. The unbound pyranine was then removed by passage through a Sephadex G-50 coarse column equilibrated with the same buffer solution. To measure the rate of pH dissipation in liposomes with lumenal pH 6.0, the liposomes were diluted in solution buffered to pH 8 and supplemented with 2 mM *p*-xylene-bis-pyridinium bromide to suppress the fluorescence of leaked pyranine. The pH was estimated from the ratio I_{455}/I_{410} of the intensities of fluorescence measured at 505 nm upon excitation at 455 nm (I_{455}) and 410 nm (I_{410}), respectively [41], as monitored with the Panorama Fluorat 02 spectrofluorometer. At the end of each recording, 1 μM nigericin was added to dissipate the remaining pH gradient. To prevent the formation of H^+-diffusion potential, the experiments were carried out in the presence of 10 nM valinomycin.

2.5. Isolation of rat liver mitochondria

Rat liver mitochondria (RLM) were isolated by differential centrifugation [42] in a medium containing 250 mM sucrose, 10 mM MOPS, 1 mM EGTA, pH 7.4. The final washing was performed in the medium additionally containing bovine serum albumin (0.1 mg/ml). Protein concentration was determined using the Biuret method. Handling of animals and experimental procedures were conducted in accordance with the international guidelines for animal care and use and were approved by the Institutional Ethics Committee of A.N. Belozersky Institute of Physico-Chemical Biology at the Moscow State University.

2.6. Mitochondria respiration and mitochondrial membrane potential measurements

Respiration of isolated mitochondria was measured using a standard polarographic technique with a Clark-type oxygen electrode ("Strathkelvin Instruments", UK) at 25 °C using 782 system software. The incubation medium contained 250 mM sucrose, 5 mM MOPS, 1 mM EGTA, pH 7.4. Mitochondrial protein concentration was 0.4 mg/ml. Membrane potential was measured with the help of a tetraphenylphosphonium (TPP^+)-sensitive electrode (NIKO-ANALIT, Moscow, Russia) in the medium described above plus 5 mM KH_2PO_4. TPP^+ measurements were accompanied by measurements of respiration by a Clark-type electrode in a home-made two-electrode setup similar to that described in [43].

2.7. Swelling of mitochondria

The protonophoric ability of fluorescein derivatives was tested by induction of swelling of non-respiring rat liver mitochondria incubated

in buffered isotonic potassium acetate in the presence of valinomycin. Under these conditions, mitochondria do not swell, because acetate can cross the membrane only as undissociated acetic acid, and the transmembrane passage of potassium in the form of the K^+-valinomycin complex generates a charge imbalance, preventing further permeation of K^+ [44]. Intramitochondrial accumulation of potassium acetate becomes possible only if H^+ can be exported from the inner compartment, thus enabling the influx of K^+ resulting in mitochondria swelling. This process can be mediated, for example, by synthetic protonophores [45]. Swelling of mitochondria was recorded as the decrease of absorbance at 600 nm of the mitochondrial suspension. In short, an aliquot of mitochondria (0.5 mg mitochondrial protein) was added to 1 ml of the 'swelling medium' containing 145 mM potassium acetate, 5 mM Tris, 0.5 mM EDTA, 3 μM valinomycin and 1 μM rotenone at pH 7.4.

2.8. C_n-FL binding to mitochondria measured by fluorescence correlation spectrometry (FCS)

As shown in our previous works, FCS can be used to measure binding of cationic rhodamines to mitochondria [46,47]. We used the same setup for the measurements of fluorescein derivatives although the use of 532-nm laser was not optimal for excitation. The fluorescence signal from a suspension of dye-doped mitochondria represented a sequence of peaks of different intensity reflecting their random walk through the confocal volume [46]. The experimental data were obtained under stirring conditions which increased the number of events by about three orders of magnitude thus substantially enhancing the resolution of the method. The setup of our own construction was described previously in [46]. Briefly, fluorescence excitation and detection were provided by a Nd:YAG solid state laser with a 532-nm beam attached to an Olympus IMT-2 epifluorescence inverted microscope equipped with a 40× NA 1.2 water immersion objective (Carl Zeiss, Jena, Germany). The fluorescence passed through an appropriate dichroic beam splitter and a long-pass filter and was imaged onto a 50-μm core fiber coupled to an avalanche photodiode (SPCM-AQR-13-FC, PerkinElmer Optoelectronics, Vaudreuil, Quebec, Canada). The output signal F(t) was sent to a personal computer using a fast interface card (Flex02-01D/C, Correlator.com, Bridgewater, NJ). The signal was measured in Hz meaning number of photons per second. The data acquisition time T was 30 s. The card generated the autocorrelation function of the signal G(τ) defined as

$$G(\tau) = \frac{\langle \delta F(t) \cdot \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2} \quad (1)$$

where $\langle F(t) \rangle$ is the mean fluorescence intensity and $\delta F(t) = F(t) - \langle F(t) \rangle$ is a deviation from the mean. Autocorrelation function measurements are usually employed to determine diffusion coefficients by $G(\tau)$ curve fitting to the theoretical equation of three-dimensional diffusion [48]. However, in the case of objects as large as mitochondria, statistically reliable measurements of $G(\tau)$ encounter certain difficulties due to the necessity of signal accumulation during a very long time interval. In this work $G(\tau)$ was measured under stirring, when the dependence of G on τ is defined by the rate of liquid flow generated by the stirrer. It should be pointed out that under the conditions of dye binding to mitochondria (like in the case of C_{12}-FL), the inflection point of the $G(\tau)$ function in the absence of stirring was found in the time range of 10–1 s, which was indicative of the presence of dye molecules on objects with a characteristic size of 1 μm. When the mitochondria suspension is stirred, the autocorrelation function shows both the average residence time of a fluorescent particle in the confocal volume and the average number of particles present in this volume. In our experiments, fluorescent particles are free dye molecules and mitochondria carrying bound dye molecules. The $G(\tau)$ value in the limit of low τ is used for quantitative estimation of an average number of fluorescent particles, namely, for suspension of identical particles, according to [49]:

$$G(\tau \to 0) = \frac{1}{N} \quad (2)$$

where N is the average number of fluorescent particles in the confocal volume. An increase in the parameter N in our system can be attributed to the release of bound dye molecules from mitochondria leading to an increase in the total number of fluorescence particles due to multiple binding of the dye to mitochondria.

2.9. Yeast strains, growth conditions and microscopy

In this study we used *W303-1A* (*ade2-101 his3-11 trp1-1 ura3-52 can1-100 leu2-3*) as a control strain and *AD1-8* (*W303-1A, yor1::hisG, snq2::hisG, pdr5::hisG, pdr10::hisG, pdr11::hisG, ycf1::hisG, pdr3::hisG, pdr15::hisG*) strain lacking major multidrug resistance genes [50]. Cells were grown on solid YPGly medium (2% glycerol, 2% bacto-peptone, 1% yeast extract, 2% agar) and then transferred into sterile 10 mM MES buffer (pH 7.1), the cell density was set as $4 \cdot 10^6$ cells/ml.

Cells were stained with C_{12}-FL, C_4-FL or TMRE and photographed using an Olympus BX2 microscope with two different filter sets: U-MNIABA3 (excitation 470–495 nm, 505 nm dichroic beamsplitter, emission 510–550 nm) for C_{12}-FL and C_4-FL and U-MNG2 (excitation 530–550 nm, 570 nm beamsplitter filter, emission >590 nm) for TMRE.

2.10. Octanol-water partition coefficient

The octanol–water partition coefficient (P_{ow}) of C_{12}-FL and other fluorescein esters was determined using a method based on correlation of this parameter with the retention time in a reverse-phase high performance liquid chromatography (HPLC) column [51]. It was previously shown for related compounds that this method estimates the coefficient with satisfactory accuracy [52]. The mobile phase consisted of 0.1% trifluoroacetic acid in water and a gradient of acetonitrile from 40 to 95% (11 min; a flow rate of 1.5 ml/min). The logarithm of the partition coefficient $logP_{ow}$ was calculated from the equation:

$$\log P_{ow} = 4.26 \times \log(t_R - t_0) - 7.95$$

where t_0 is the dead time, while t_R has the usual meaning of retention time for an analyte. All the chromatographic runs were performed on an Agilent 1100 chromatograph (Agilent, USA) at 25 °C. The HPLC columns used were Luna C18(2), 5 μm, 4.6 × 150 mm.

3. Results

3.1. pK_a of C_{12}-FL and its octanol–water partition coefficient

Fig. 1C shows the dependence of C_{12}-FL emission spectrum on pH of the buffer solution. As expected for a derivative of fluorescein, fluorescence intensity increased upon shifting pH to alkaline values while the position of the maximum (λ_{max}), 519 nm, was independent of pH. C_4-FL spectra exhibited similar pH dependence with $\lambda_{max} = 516$ nm (not shown). Insert to Fig. 1B shows the pH dependence of the fluorescence intensity at maximum of the C_{12}-FL spectra and a fitting curve obtained by the Henderson–Hasselbach equation with $pK_a = 7.5$. This value is close to the data presented in [53] for fluorescein *n*-octadecyl ester. The pH dependences of fluorescence intensity for C_4-FL and FL led to pK_a values of 6.2 and 6.6, respectively (data not shown). A shift in pK_a between C_4-FL and C_{12}-FL can be related to formation of aggregates of micelles in aqueous solution in the case of C_{12}-FL.

One of the important parameters of a chemical compound is its octanol–water partition coefficient (P_{ow}). It characterizes the compound's aqueous solubility and permeability through lipid and

cellular membranes. We estimated P_{ow} for fluorescein alkyl esters by reverse phase HPLC as described in Materials and methods. The following values of P_{ow} were determined: C_4-FL, 6.94; C_8-FL, 288; C_{12}-FL, 2700; C_{18}-FL, 13400. The values of P_{ow} for C_8-FL and C_{12}-FL are in the range characteristic for strong uncouplers [5].

3.2. Binding of C_n-FL to liposomes

Study of fluorescence anisotropy in lipid membranes reflects the effect of motional restriction imposed on a fluorophore by the viscous environment [54–57] and therefore can be used as a reporter of fluorophore interaction with lipid membranes. Fig. 2 shows the dependence of fluorescence polarization of fluorescein and its alkyl esters on the concentration of EggPC liposomes. With increasing lipid concentration, fluorescence polarization of C_{12}-FL and C_{18}-FL increased from that in aqueous buffered solution and finally reached a maximum value at the saturation level of probe–liposome interaction. The mole ratio of C_{12}-FL to lipid at saturating concentrations was about 1:1000. The increased polarization value compared to that in bulk aqueous solution indicates that the fluorophore experiences a motionally restricted environment inside the lipid membranes. The growth of polarization, which apparently reflected the dye binding to liposomes, was large with C_{12}-FL and C_{18}-FL, much less pronounced with C_8-FL, and negligible with C_4-FL and unsubstituted fluorescein. Based on these data, it can be concluded that C_{12}-FL and C_{18}-FL exhibited strong binding to liposomes, whereas the binding of C_8-FL appeared to be rather weak, although stronger than that of C_4-FL and FL which obviously remained in solution. These results corresponded well to the binding of alkyl-substituted nitrobenzoxadiazole (NBD) to liposomes differing by about 1800-fold for C_4-NBD and C_{12}-NBD [58].

3.3. C_{12}-FL induced proton current on BLM

To test the idea that C_{12}-FL induces proton permeability in BLM, the electrical current was measured under voltage-clamp conditions in this model system. A typical trace of an increase in the current upon several additions of C_{12}-FL in micromolar concentrations is shown in Fig. 3, Panel A. Slow kinetics of the increase in the current could be associated with the poor stirring of the solutions bathing the membrane. Unfortunately, an increase in the stirring rate led to instability of the BLM. The data were in line with the nonlinear dependence of the current on the concentration of C_{12}-FL, i.e. a two-fold increase in C_{12}-FL concentration (from 1 μM to 2 μM) led to about four-fold increase in the current (Fig. 3A). A similar increase was observed upon doubling the concentration from 2 μM to 4 μM (Fig. 3A). The concentration dependence was studied in detail in another model system, liposomes, where C_{12}-FL binding was fast (see below). Similar experiments on BLM with C_4-FL showed that it did not cause an increase in the current up to 8 μM while C_8-FL exhibited activity comparable to that of C_{12}-FL (data not shown).

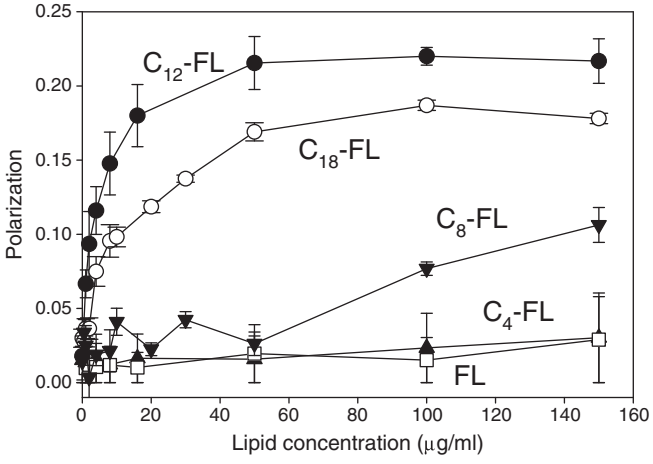

Fig. 2. Dependence of fluorescence polarization of C_{18}-FL, C_{12}-FL, C_8-FL, C_4-FL and FL on the concentration of EggPC liposomes. C_{18}-FL, C_{12}-FL, C_8-FL, C_4-FL and FL concentration, 10 nM. The solution was 10 mM Tris, 100 mM KCl, pH 8.5. Excitation, 490 nm; emission, 520 nm.

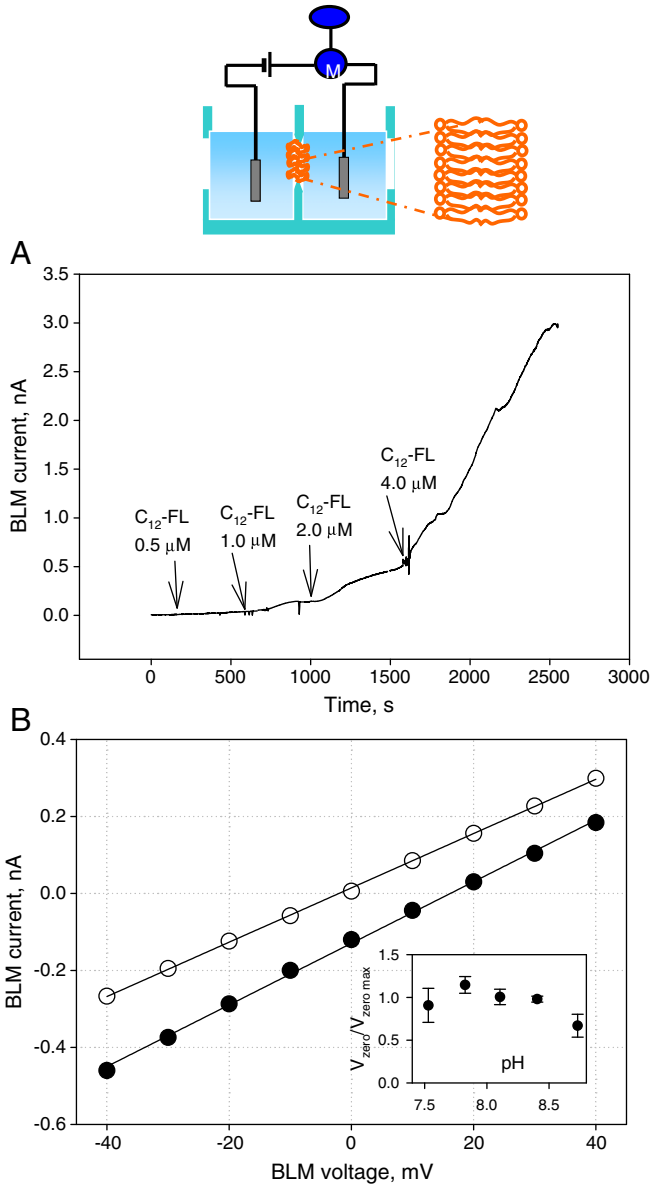

Fig. 3. A. C_{12}-FL induced electrical current through planar BLM made from DPhPC. The solution was 40 mM Tris, 10 mM KCl, pH 8.5. BLM voltage 50 mV. B. I–V curve for 3 μM C_{12}-FL under symmetrical (open circles, pH cis and trans were 8.56) and asymmetrical conditions (closed circles, pH cis was 8.56, pH trans was 8.25). Insert: pH dependence of the zero voltage current. The solution in panel B was 10 mM Tris, 10 mM MES, 10 mM β-alanine, 10 mM KCl.

To determine ion selectivity of C_{12}-FL-mediated permeability, I-V curves were measured under symmetrical (Fig. 3, Panel B, open circles) and asymmetrical conditions (Fig. 3, Panel B, closed circles) of different pH ($pH_1 = 8.56$, $pH_2 = 8.25$). The value of zero-voltage current under asymmetrical conditions ($V_{zero} = 17$ mV; with the "plus" sign at the side with lower pH) corresponded to nearly ideal proton selectivity of the conductance ($V_{max} = \frac{RT}{F} \cdot \Delta pH = 18.3$ mV at $\Delta pH = 0.31$). This analysis relied on the Nernst equation following [1]. High proton selectivity was observed in the pH range from 7.5 to 9 (inset in Fig. 3B), exhibiting maximum close to $pH = pK_a$ with a shoulder at the alkaline

side, which agreed with pH dependences of ionic selectivity of other uncouplers [1,59–63].

3.4. Liposomes loaded with pyranine

According to [40,64,65], liposomes loaded with the pH-sensitive probe pyranine can serve as a useful system to study the action of protonophores. Fig. 4 shows the kinetics of dissipation of a pre-formed pH gradient on membranes of liposomes after the addition of FCCP, C_{12}-FL or C_4-FL. The addition of C_{12}-FL led to significant changes in liposomal pH within minutes, suggesting specific protonophoric activity, close to that of FCCP (Fig. 4A). Remarkably, C_4-FL was completely inactive in this system up to concentrations of 30 nM (Fig. 4B). At higher concentrations, fluorescence of C_4-FL contributed to an increase in the fluorescence signal which complicated the measurements. The effective concentrations of C_{12}-FL in the liposome assay were by approximately two orders of magnitude lower than those in planar BLM (Fig. 3), which could be associated with differences in membrane lipid compositions and/or in C_{12}-FL/lipid ratios. Similar lowering of the efficacy in planar BLM compared to liposomes was found with CCCP, which

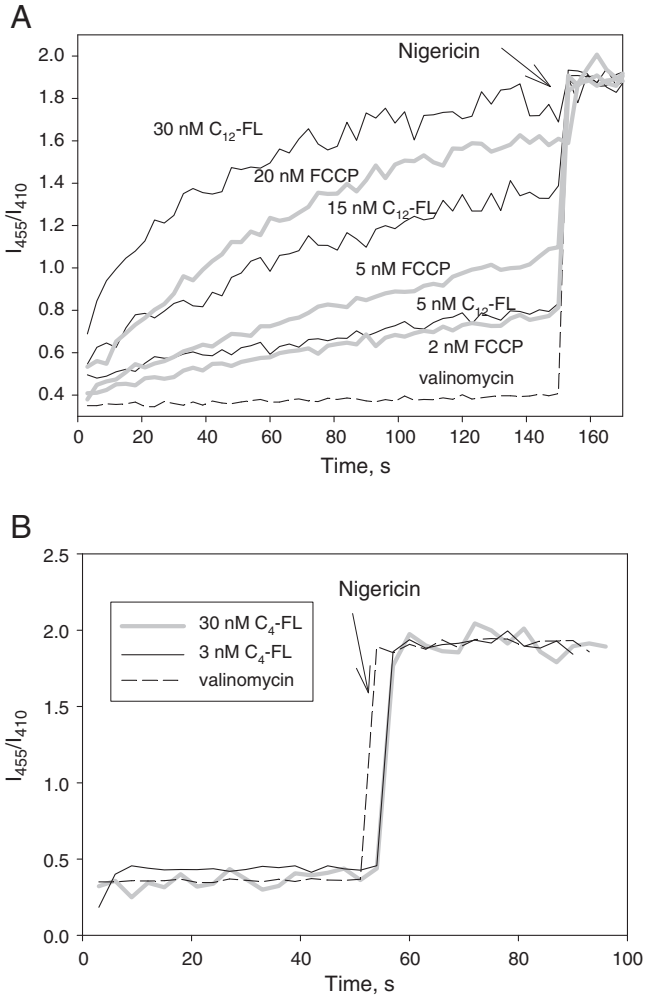

Fig. 4. Dissipation of pH gradient on membranes of pyranine-loaded liposomes by C_{12}-FL and FCCP (panel A) and C_4-FL (panel B) added at t = 0. Inner liposome pH was estimated from the ratio (I_{455}/I_{410}) of pyranine fluorescence intensities measured at 505 nm upon excitation at 455 nm and 410 nm, respectively. 1 μM nigericin was added at 150 s (A) and 50 s (B) to equilibrate the pH. Other conditions: see Materials and methods. Lipid concentration was 20 μg/ml.

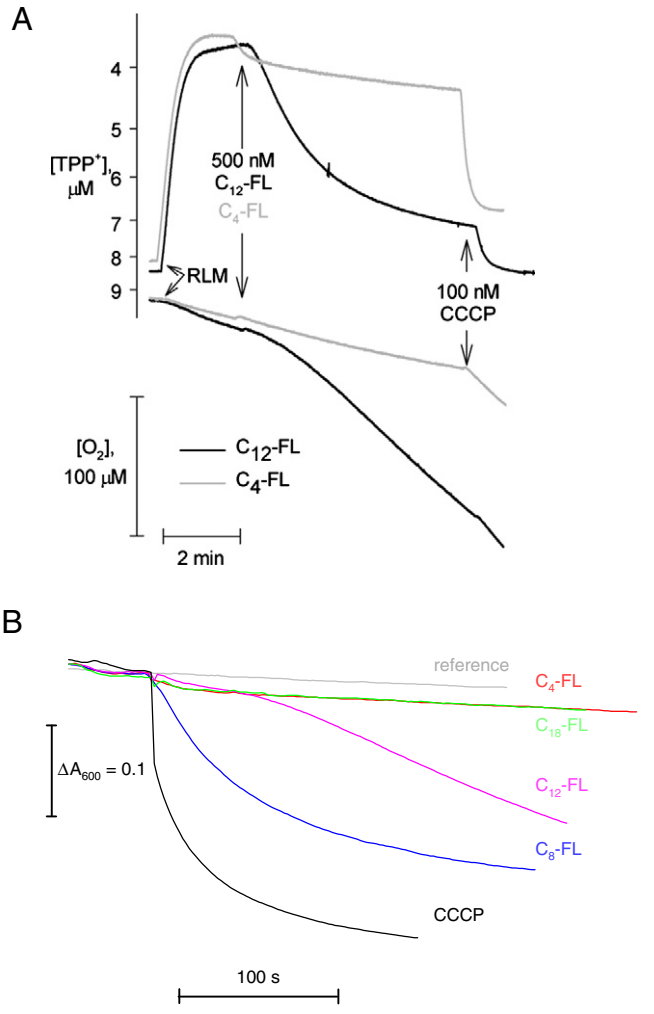

Fig. 5. A. Effect of C_{12}-FL and C_4-FL on the stimulation of rat liver mitochondria respiration and on the mitochondrial membrane potential. Shown are traces of the rate of respiration and TPP accumulation in the medium described in "Materials and methods". Additions: succinate 5 mM and 2 μM rotenone. B. Effect of fluorescein analogs on the swelling of rat liver mitochondria in potassium acetate medium. Incubation mixture: 145 mM potassium acetate, 5 mM Tris, 0.5 mM EDTA, pH 7.4, 3 μM valinomycin and 1 μM rotenone. Mitochondrial protein 0.2 mg/ml. Additions: FL, C_4-FL, C_8-FL, C_{12}-FL, and C_{18}-FL; 1.5 μM; FCCP 1 μM.

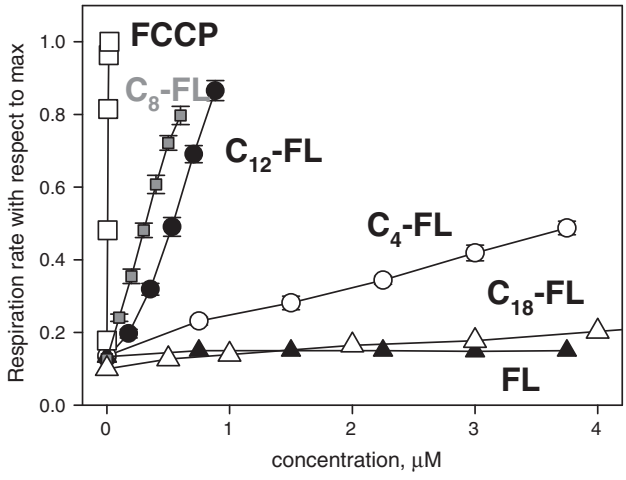

Fig. 6. Dose dependence of respiratory stimulation by C_8-Fl, C_{12}-Fl, C_{18}-Fl, C_4-FL and FL, and FCCP.

corresponded to the literature data [40,66]. Noteworthy, the experiments with pyranine-loaded liposomes were carried out in the presence of valinomycin, the powerful K^+-ionophore. No pH changes were detected in the absence of valinomycin. According to [40], this effect is associated with $\Delta\psi$ formation on liposome membranes, which blocks proton transport without valinomycin. Valinomycin per se did not induce pH changes under our experimental conditions (lower curve in Fig. 4A).

3.5. Mitochondrial respiration, membrane potential and proton permeability

The n-dodecyl ester of fluorescein (C_{12}-FL) stimulated respiration of rat liver mitochondria at submicromolar concentrations, while C_4-FL was substantially less active (Fig. 5A). Succinate was used as a substrate and rotenone was added to prevent an inhibition by oxaloacetate. The stimulation of respiration by C_{12}-FL was accompanied by a substantial decrease in membrane potential as measured by a TPP^+ electrode (Fig. 5A, top curves). Therefore, protonophoric activity of C_{12}-FL led to the uncoupling activity similar to other uncouplers. Interestingly, the C_{12}-FL-mediated decrease in potential developed with time as previously observed for the dodecyl derivative of rhodamine 19 [28].

Proton permeability of the inner mitochondrial membrane can be estimated by mitochondrial swelling in the potassium acetate medium in the presence of valinomycin [44,45,67,68]. Panel B of Fig. 5 shows the effect of derivatives of fluorescein on the proton permeability. In agreement with the data on the respiration of mitochondria, C_8-Fl was most effective in the swelling while all the derivatives comprise the series C_8-FL > C_{12}-FL > C_4-FL > C_{18}-FL.

Fig. 6 shows the concentration dependence of the stimulation of respiration. The C_{50} values for C_8-FL, C_{12}-FL, and C_4-FL were about 0.3 μM, 0.7 μM, and 5 μM, respectively. The unsubstituted fluorescein as well as C_{18}-FL was inactive at these concentrations (closed and open triangles in Fig. 6), while FCCP was effective at substantially lower concentrations compared to C_{12}-FL (open squares in Fig. 6).

Comparison of Figs. 4A and 6 reveals a noticeable difference between the relative efficacies of C_{12}-FL with respect to FCCP in liposomes and mitochondria. It can be assumed that this difference is associated with the interaction of the uncouplers with some mitochondrial proteins. Therefore, we performed experiments with agents known to modulate the action of uncouplers, 6-ketocholestanol [24,25] and carboxyatractylate [21,69,70]. As seen from Fig. 7, 6-ketocholestanol almost completely removed the effect of CCCP on mitochondrial membrane potential in line with the data of Starkov and colleagues [24,25,71], but practically did not alter the effect of C_{12}-FL, which points to a mechanistic difference in the action of these uncouplers. Atractylate also did not change the effect of C_{12}-FL (data not shown), although this inhibitor of the ATP/ADP-antiporter is known to modulate the effect of DNP on respiration and membrane potential of mitochondria [21]. It can be suggested that unlike FCCP which might exert its uncoupling effect with the help of some protein, C_{12}-FL uncouples mitochondria without involvement of proteins, which leads to its lower activity in mitochondria compared to liposomes.

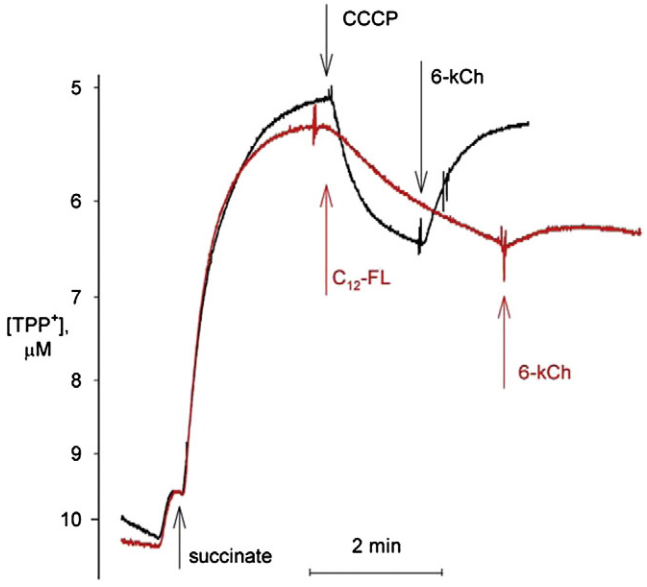

Fig. 7. Effect of 6-ketocholestanol on the mitochondrial membrane potential in the presence of FCCP and C_{12}-FL.

3.6. Binding of C_n-FL to mitochondria

Bright fluorescence of C_n-FL can be used for the study of their binding to isolated mitochondria. As it was described previously [46], the method of FCS has several advantages for this purpose. For example, it does not require centrifugation of mitochondria which by itself could affect the binding. Fig. 8 shows autocorrelation functions ($G(\tau)$) of

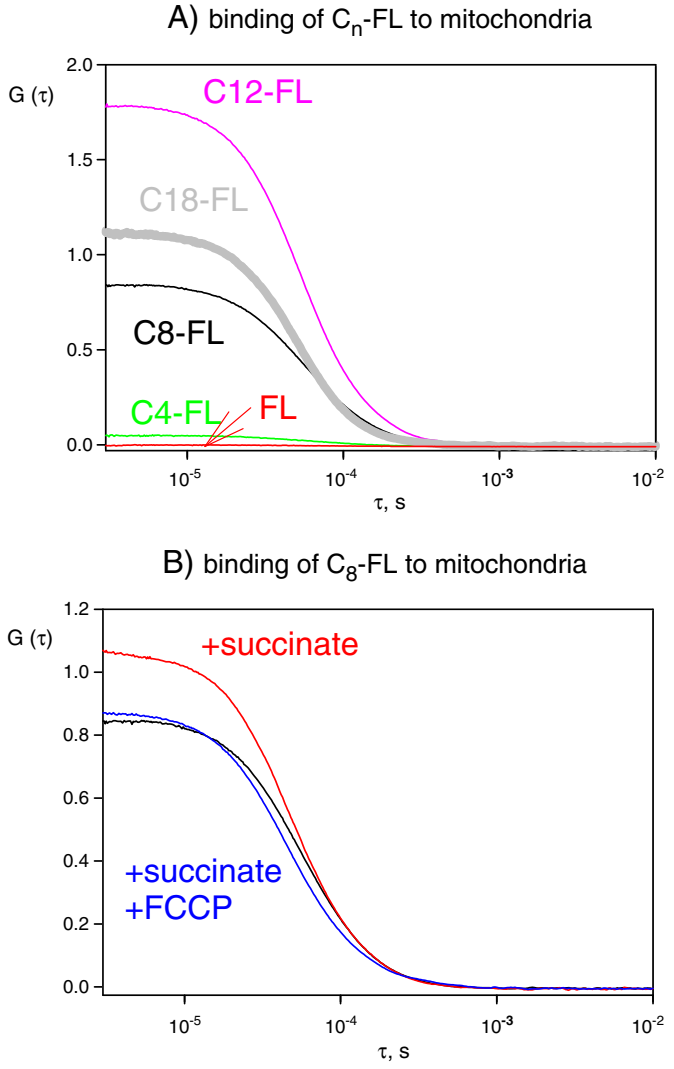

Fig. 8. A. FCS measurements of FL-, C_4-FL-, C_8-FL-, C_{12}-FL-, and C_{18}-FL-binding to mitochondria. Autocorrelation functions $G(\tau)$ of the corresponding compounds (concentration 100 nM) in a suspension of rat liver mitochondria in incubation buffer (0.1 mg protein/ml). Incubation mixture: 250 mM sucrose, 20 mM MOPS, 1 mM EGTA, 5 μM rotenone, pH 7.4. B. Autocorrelation functions $G(\tau)$ of C_8-FL (100 nM) in a suspension of rat liver mitochondria in incubation buffer in the absence (curve 1) and in the presence (curve 2) of 5 mM succinate, and after the addition of 1 μM FCCP (curve 3).

the recordings of FL, C_4-FL, C_8-FL, C_{12}-FL, and C_{18}-FL fluorescence measured in a suspension of rat liver mitochondria in the presence of rotenone, i.e. when mitochondria are deenergized. The measurements were carried out under stirring conditions which determined the time-resolved shape of the $G(\tau)$, while the amplitudes of $G(\tau)$ were related to the mean number of fluorescent particles in a small observation spot weighted by their brightness [49,72]. It has been previously shown using a series of rhodamine derivatives that the amplitude of $G(\tau)$ at low τ under these conditions correlates with the dye binding to mitochondria [47]. According to Fig. 8A, FL and C_4-FL had poor affinity to mitochondria, while the binding of C_8-FL, C_{12}-FL, and C_{18}-FL was close to saturation as the amplitude of $G(\tau)$ in the case of penetrating cationic dye TMRE was about 0.5 under the conditions of energized mitochondria (in the presence of 5 mM succinate).

It was interesting to study the effect of energization on the binding of fluorescein derivatives. Panel B of Fig. 8 shows $G(\tau)$ of the recordings of C_8-FL fluorescence measured in the suspension of mitochondria before (curve 1), after (curve 2) the addition of succinate and after the subsequent addition of FCCP (curve 3). The amplitudes of $G(\tau)$ in the presence of C_8-FL increased to some extent upon energization of mitochondria (Fig. 8A), while in the case of C_{12}-FL and C_{18}-FL the amplitude of $G(\tau)$ did not depend on energization (data not shown). In the case of C_4-FL the amplitude of $G(\tau)$ increased from 0.05 to 0.09 upon the addition of succinate and returned to the initial value after the addition of FCCP (data not shown). These data showed on a qualitative level that in contrast to C_8-FL and C_4-FL, C_{12}-FL accumulation in the mitochondrial matrix did not depend upon energization, and C_{12}-FL was tightly bound to mitochondria even in the absence of membrane potential. It should be pointed out that the concentrations of the majority of C_n-FL used in these experiments (200 nM) were far below the half-maximal uncoupling concentrations (Fig. 6) except for C_8-FL which produced a considerable effect on the respiration rate at this concentration. The decrease in the concentration resulted in a low fluorescence signal of C_n-FL and led to unreliable measurements of $G(\tau)$.

3.7. Intracellular distribution of C_{12}-FL in yeast cells

To study distribution of C_{12}-FL at a cellular level, we performed experiments on wild-type and mutant AD1-8 yeast cells (with inactivated multidrug resistance ABC pumps). C_{12}-FL did not accumulate in control *S. cerevisiae* cells, staining the cellular membranes only, while it penetrated into AD1-8 cells (Fig. 9, left). Comparison of distribution of C_{12}-FL with that of the mitochondrial marker TMRE in AD1-8 cells (right-side images in Fig. 9B) revealed that C_{12}-FL partially colocalized with TMRE. A noticeable portion of C_{12}-FL remained in the medium (uniform green fluorescence) in contrast to TMRE which accumulated in the cells almost completely. C_4-FL accumulated neither in wild type nor in the mutant cells (data not shown).

4. Discussion

With the aim to design a highly fluorescent uncoupler, i.e. a compound having both high fluorescence yield and the ability to carry protons across membranes, we decided to construct a lipophilic weak acid on the basis of fluorescein. Taking into account an increasing body of evidence that C_{12} chain length is optimal for membrane-translocating activity [28,73,74], a C_{12} alkyl group was chosen for conjugation to the carboxyl group of fluorescein, thereby leaving free only its hydroxyl group with the pK_a close to neutral. The present data on liposomes and planar bilayers have demonstrated the protonophoric activity of C_{12}-FL (Figs. 3, 4) causing uncoupling action on mitochondria (Fig. 5). Importantly, the ability of the fluorescein alkyl esters to bind to lipid membranes (Fig. 2) appears to be necessary for the protonophoric activity. Conventional protonophores are weak acids with pK_a in the physiological pH range, which can permeate through lipid membranes in neutral and anionic forms [1]. The proton translocation cycle includes protonation of the anionic form C_{12}-FL^- at one side of the membrane, movement of the neutral form to the other side C_{12}-FL-H, subsequent deprotonation and movement of C_{12}-FL^- back to resume the cycle (scheme in Fig. 1A). The pK_a of C_{12}-FL (7.5) favors functioning of the uncoupler under living cell conditions.

Our data revealed the non-monotonous pattern of the dependence of the uncoupling action of fluorescein derivatives on the hydrocarbon chain length, with C_{18} being completely inactive in mitochondria. Relevantly, nonlinear dependence of the activity on the alkyl chain length was also found in our laboratory for the ability of alkyl rhodamines to transport protons and organic anions across model membranes [28,74]. The increase in the activity with n at low n can be associated with an increase in binding of the compounds to membranes (Fig. 2). The decrease in the activity at high n might result from a concomitant decrease in the translocation rate constant. Actually, the rate of flip-flop of lipids was shown to decrease with increasing n in the range of n = 8-12 [75], which was attributed to increased restriction of transmembrane mobility at high n. Another reason for the reduced

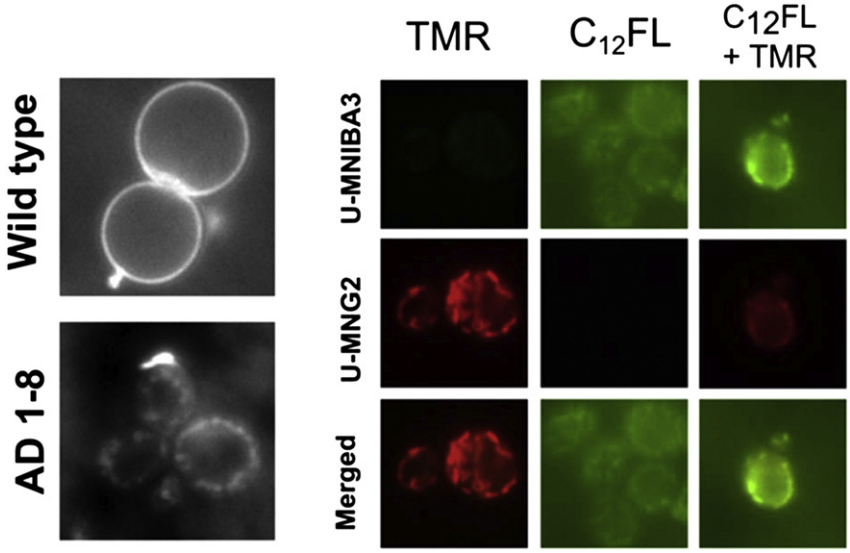

Fig. 9. Left: C_{12}-FL in *Saccharomyces cerevisiae* cells (wild type and AD1-8). Right: AD1-8 cells loaded with C_{12}-FL (green channel, 2.5 μM), TMRE (red channel, 0.5 μM) and overlap showing co-localization in yellow.

activity of long-chain fluoresceins could be a low rate of partitioning and redistribution of the carriers between different vesicles as was shown for long-chain rhodamines [74].

C_{12}-FL partially co-localized with mitochondria in yeast cells (Fig. 9). Owing to inside-negative potential in mitochondrial matrix, the anionic form of C_{12}-FL must be expelled from mitochondria. However, the expulsion may be ineffective due to the presence of the neutral form. Accumulation of C_{12}-FL in mitochondria can be associated with the presence of the pH gradient of approximately 0.5–1 units on the inner mitochondrial membrane, with matrix being more alkaline [76]. It has long been known that the pH difference across the mitochondrial membrane results in accumulation of anions of weak acids inside mitochondria [77]. Moreover, the unsubstituted fluorescein was found to accumulate in mitochondria in an energy-dependent manner [33,34]. Our experiments showed high affinity of C_{12}-FL, C_8-FL and C_{18}-FL to isolated mitochondria. The enhancement of C_4-FL and C_8-FL binding to mitochondria upon energization with succinate could be ascribed to formation of a pH gradient across IMM, which promotes additional accumulation of the weak acids inside mitochondria.

Limited uncoupling was shown to lead to favorable therapeutic action owing to slightly decreased membrane potential and reduced ROS production in mitochondria [6,8,78]. In fact, low concentrations of the protonophore DNP exhibited protective effects in several laboratory models of various pathologies and increased lifespan of yeast [79], *Drosophila* [80] and mice [81]. The uncoupling action of C_{12}-FL on isolated mitochondria can be of interest for the experiments in vivo. To this end, low toxicity of fluorescein [82,83] is of high importance. An obvious advantage of C_{12}-FL as an uncoupler is its bright fluorescence which allows for visualizing its localization. However, prior to in vivo, further in vitro studies are required to characterize more extensively the effects of the compound on several cellular and mitochondrial (dys)function, i.e. mitochondrial permeability transition, ATPase activity, cellular viability or cell death. Besides, the possibility that C_{12}-FL can be hydrolyzed by tissue or cell esterases should be also tested.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2013.09.011.

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