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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 its 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 its ability to protect cells from damage in a number of physiological models leading to the idea of its 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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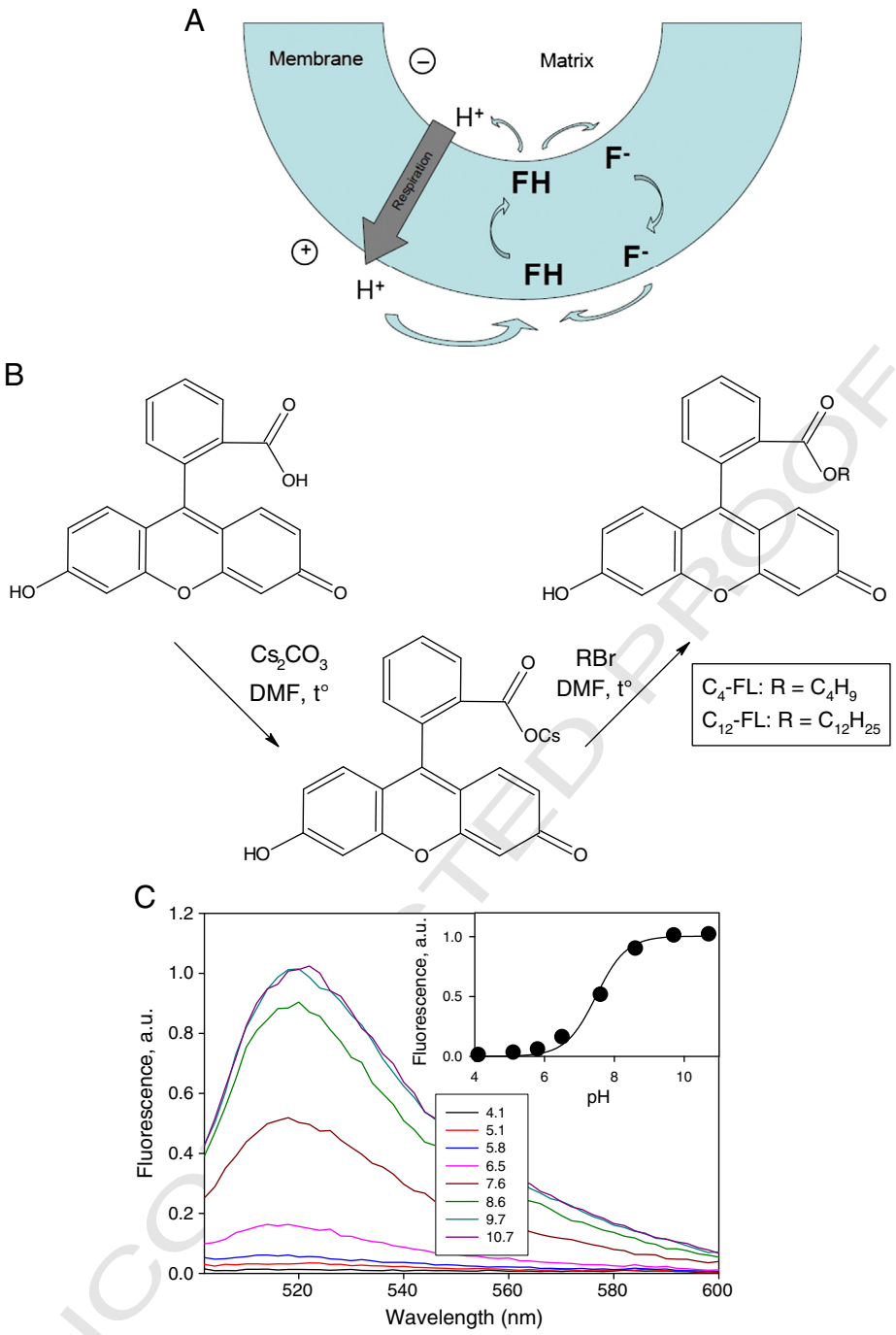

Fig. 1. A. Scheme of protonophore-mediated uncoupling on the inner mitochondrial membrane. B. Scheme of synthesis of alkyl-substituted fluoresceins. C. Emission spectra of C_{12}-FL at different pH. Inset: pH-dependence of C_{12}-FL fluorescence and a fitting curve according to Henderson–Hasselbalch 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

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