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Effect of liposomes on energy-dependent uptake of the antioxidant SkQR1 by isolated mitochondria

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Abstract The mitochondria-targeted antioxidant SkQR1 composed of a plastoquinone part covalently bound to a cationic rhodamine 19 moiety via a decane linker was previously shown to effectively protect brain and kidney from ischemia injury accompanying generation of reactive oxygen species. In the present paper the energy-dependent SkQR1 uptake by isolated rat liver mitochondria was studied by fluorescence correlation spectroscopy peak intensity analysis (FCS PIA). This approach can be used to measure the number of fluorescent molecules per single mitochondrion. A large portion of SkQR1 appeared to be taken up by mitochondria in an energy-independent fashion because of its high affinity to membranes. Liposomes were found to compete effectively with mitochondria for the energy-independent SkQR1 binding, thereby facilitating, as an "SkQR1-buffer", observation of energy-dependent SkQR1 accumulation in mitochondria. The rate of energy-dependent SkQR1 uptake by mitochondria observed in the presence of liposomes was rather low (minutes) which was apparently due to slow redistribution of SkQR1 between liposomal and mitochondrial membranes. This can explain the low rate of staining of mitochondria by SkQR1 in living cells containing, besides mitochondria, other membrane components (endoplasmic reticulum, Golgi membranes, endosomes, lysosomes, etc.) which can compete with mitochondria for the energy-independent SkQR1 binding.

Keywords Mitochondria · Liposomes · Hydrophobic cations · Antioxidants · Bilayer lipid membranes

Abbreviations

$\Delta\psi$	Transmembrane electrical potential difference
BLM	Bilayer lipid membrane
C_8R1	Rhodamine 19 octyl ester
DPhPC	Diphytanoyl phosphatidylcholine
FCS	Fluorescence correlation spectroscopy
$G(\tau)$	Autocorrelation function
MES	2-(*N*-morpholino)ethanesulfonic acid
P_{ow}	Octanol–water partition coefficient
PIA	Peak intensity analysis
ROS	Reactive oxygen species
SkQR1	Compound containing plastoquinone linked to decylrhodamine 19
TMRE	Tetramethylrhodamine ethyl ester

Introduction

Mitochondria-targeted antioxidants (MTA) are a relatively new class of pharmacological compounds that are able to permeate through cellular membranes and specifically accumulate in mitochondria (Murphy and Smith 2007; Skulachev 2007; Skulachev et al. 2009; Skulachev et

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al. 2011; Hoye et al. 2008). The functioning of the respiratory chain proton pumps that transport protons out from the mitochondrial matrix to the intermembrane space of these organelles generates a negative-inside electrical potential difference ($\Delta\psi$) across the inner mitochondrial membrane. In fact, the mitochondrial interior is the only negatively-charged intracellular compartment. For this reason, all MTA contain a cationic group with a charge delocalized over aromatic residues, which aids permeation through the membranes and ensures many-fold accumulation in mitochondria compared to the cytoplasm. Also, most MTA are membranophilic compounds with a high octanol–water partition coefficient. Asin-Cayuela et al. (Asin-Cayuela et al. 2004) studied the accumulation of a series of conjugates of alkyltriphenylphosphonium with ubiquinone having hydrocarbon linker chains of different lengths (MitoQ series) into isolated mitochondria. It was shown that hydrophilic MTA are poorly accumulated by de-energized mitochondria, while MitoQs with a long linker chain exhibit significant mitochondria binding that is practically not enhanced upon energization. As to the popular MTA $MitoQ_{10}$, it has intermediate properties in this series, showing some $\Delta\psi$-dependent uptake along with nonspecific adsorption (Asin-Cayuela et al. 2004).

It has been recently found in our laboratory that rhodamine 19 can successfully substitute for cationic triphenylphosphonium in the conjugates delivering MTA into mitochondria (Skulachev et al. 2011; Antonenko et al. 2008), making it possible to visualize the distribution of this MTA within a cell similar to other fluorescent markers of mitochondrial membrane potential (Ehrenberg et al. 1988; Amchenkova et al. 1988; Chen 1988). Figure 1 shows a structure of a conjugate of plastoquinone and rhodamine 19 (SkQR1) as well as rhodamine 19 octyl ester, C_8R1 and TMRE, also used in the present work. According to recent data, SkQR1 can effectively protect brain and kidney from ischemic injury accompanied with the generation of ROS (Skulachev et al. 2011; Bakeeva et al. 2008; Plotnikov et al. 2010). Along with the antioxidant action observed at its nanomolar concentraions, SkQR1 was shown to have proton-transport activity inducing mild uncoupling of isolated mitochondria, when micromolar concentrations are used (Antonenko et al. 2011). Due to $\Delta\psi$ –dependence of ROS generation by mitochondria, a limited decrease in $\Delta\psi$ can be considered as an additional antioxidant action which have protective therapeutic significance (Skulachev 1998; Harper et al. 2001; Cunha et al. 2011; Nedergaard and Cannon 2003; Lanni et al. 2003). In the present paper we examined $\Delta\psi$-dependent SkQR1 uptake by isolated rat liver mitochondria using fluorescence correlation spectroscopy peak intensity analysis (FCS PIA) adapted for studying mitochondria (Perevoshchikova et al. 2008). This approach makes it possible to measure the number of fluorescent molecules per single mitochondrion. It was shown that liposomes compete with mitochondria for energy-independent SkQR1 binding, facilitating observation of the energy-dependent SkQR1 uptake by mitochondria. Such an effect explains slow kinetics of the SkQR1 staining of mitochondria in the living cells.

Materials and methods

SkQR1 and C_8R1 (Fig. 1) were synthesized at our institute by Drs. Natalia V. Sumbatyan and Galina A. Korshunova as described in (Antonenko et al. 2008; Rokitskaya et al. 2010).

Isolation of rat liver mitochondria Rat liver mitochondria were isolated by differential centrifugation (Johnson and Lardy 1967) in medium containing 250 mM sucrose, 10 mM MOPS, 1 mM EGTA, and bovine serum albumin (0.1 mg/ml), pH 7.4. The final washing was preformed in the same medium. Protein concentration was determined using bicinchoninic acid (Smith et al. 1985). Handling of animals and experimental procedures were conducted in accordance with the international guidelines for animal care and use and were approved by the Ethics Committee of A. N. Belozersky Institute of Physico-Chemical Biology at Moscow State University.

FCS experimental setup SkQR1 uptake by isolated mitochondria, which can be divided into an energy-dependent and energy-independent part (Rottenberg 1984; Demura et al. 1985; Scaduto and Grotyohann 1999), was measured by FCS. PIA analyses fluorescence time traces of suspensions of dye-doped mitochondria representing sequences of peaks of different intensity reflecting their random walk through the confocal volume (Perevoshchikova et al. 2008). 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 (Perevoshchikova et al. 2008). 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, Perkin Elmer 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 a second. The data acquisition time *T*

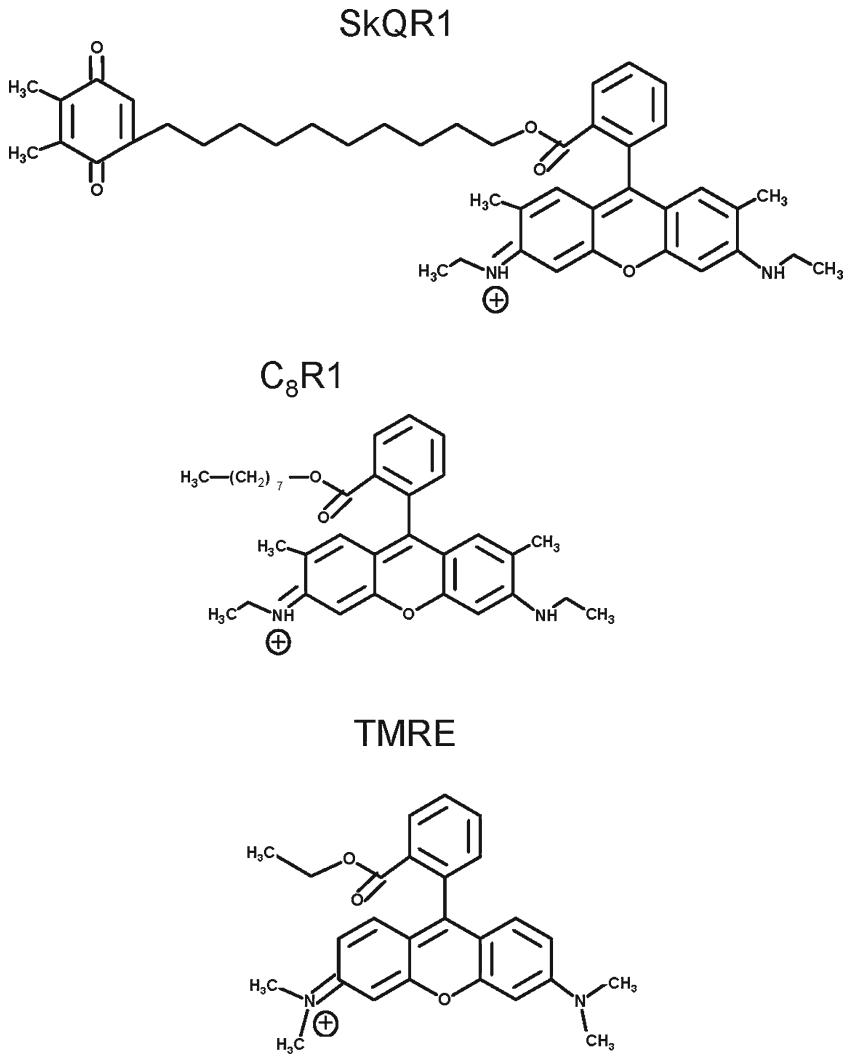

Fig. 1 Chemical structures of rhodamine 19 derivatives (SkQR1, C_8R1) and tetramethylrhodamine ethyl ester (TMRE) used in the present work

was 30 s. The card generated the autocorrelation function of the signal G(τ) defined as

$$G(\tau) = \frac{1}{T}\int_0^T F(t)F(t+\tau)dt = < F(t+\tau)F(t) >$$

Treatment of the fluorescence signal (PIA procedure) Fluorescence traces with sampling time of 25 μs were analyzed using the WinEDR Strathclyde Electrophysiology Software designed by J. Dempster (University of Strathclyde, UK). The software, originally designed for single-channel analysis of electrophysiological data, enables counting of the number of peaks ($n(F>F_0)$) of the FCS signal with amplitudes higher than the value F_0. A program of our own design with a similar algorithm (coined Saligat; provided on request) was also used.

Electrical current across planar lipid bilayers Planar bilayer lipid membrane (BLM) was formed from a 2 % solution of total lipids of *Escherichia coli* (Avanti Polar Lipids, Alabaster, AL) in *n*-decane on a hole (diameter 0.8 mm) in a Teflon partition separating two compartments of a chamber containing aqueous buffer solutions (Mueller et al. 1963). The electric current (I) was recorded under voltage-clamp conditions. Voltage was applied to the BLM with two Ag–AgCl electrodes placed on the two sides of the BLM. The current measured by a patch-clamp amplifier (OES-2, OPUS, Moscow) was digitized using an NI-DAQmx device (National Instruments, Austin, TX) and analyzed with a personal computer using WinWCP Strathclyde Electrophysiology Software also designed by J. Dempster.

Preparation of liposomes Liposomes were prepared from diphytanoyl phosphatidylcholine (DPhPC, Avanti Polar Lipids) in solution containing 10 mM Tris, 10 mM MES, 100 mM KCl, pH 7.4, or from phosphatidylserine (bovine brain, Sigma) by extrusion through a 100-nm filter (Avanti Mini-Extruder). To measure the redistribution of SkQR1,

the liposomes were diluted in the same buffer. The fluorescence at 560 nm (excitation at 530 nm) was monitored with a Panorama Fluorat 02 spectrofluorimeter (Lumex, Russia).

The octanol–water partition coefficient of SkQR1 was estimated using a method based on correlation of this parameter with retention time in a reverse-phase high performance liquid chromatography (HPLC) column (Lombardo et al. 2001). It was previously shown for related compounds (SkQ1 and MitoQ) that this method estimates the coefficient with satisfactory accuracy (Martyushin et al. 2008). The mobile phase consisted of 0.1 % trifluoroacetic acid in water and a gradient of acetonitrile from 5 to 95 % (11 min; a flow rate of 1.5 ml/min). The partition coefficient $logP_{ow}$ was calculated from the equation:

$$\log P_{ow} = 10.44 \times \log(t_R - t_0) - 25.48$$

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.

Results and discussion

Figure 2 shows recordings of SkQR1 (panel A) and tetramethylrhodamine ethyl ester (TMRE) (panel B) fluorescence measured in a suspension of rat liver mitochondria before (curve 1) and after (curve 2) addition of succinate. The records with SkQR1 did not depend on energization of the mitochondria, while in the case of TMRE the amplitude of signal peaks was substantially lower without succinate than in its presence, which is in line with our previous results (Perevoshchikova et al. 2008). Panels C and D of Fig. 2 show corresponding autocorrelation functions (G(τ)) of the above records. Although the dependence of G(τ) on τ was determined under stirring conditions, amplitudes of G(τ) were related to the mean number of fluorescent particles weighted by their brightness (Strakhovskaya et al. 2009). The amplitudes of G(τ) also depended on mitochondrial energization in the case of TMRE and were independent of energization in the case of SkQR1 (panels C and D, Fig. 2). The amplitude of G(τ) is determined by all fluorescent particles including TMRE and SkQR1 molecules in the medium, which complicates the analysis of their uptake by mitochondria. Therefore, we used peak intensity analysis (PIA). We suggested PIA previously to deal with high-amplitude peaks of fluorescence, i.e. with signals originating from stained mitochondria only (Perevoshchikova et al. 2008).

According to results of the PIA statistical treatment displayed in Fig. 3a, amplitudes of peaks corresponding to

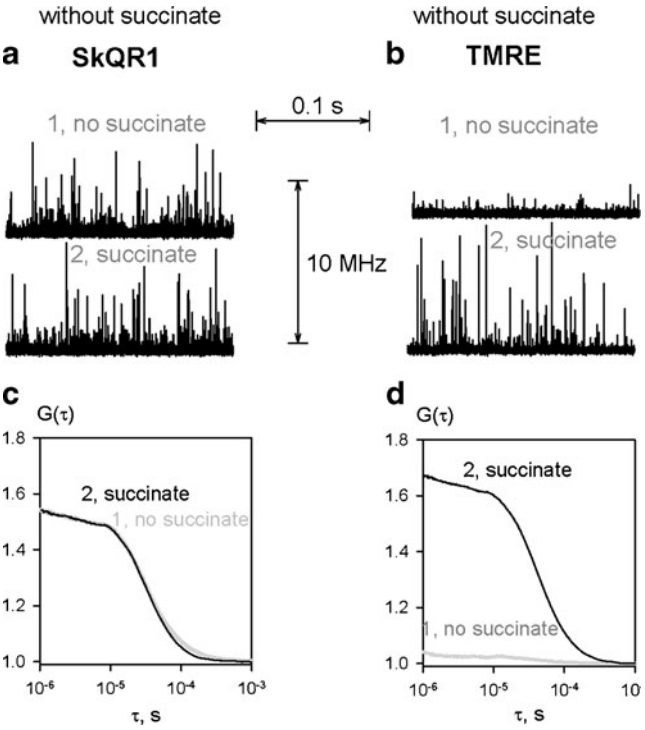

Fig. 2 a,b. FCS measurements of SkQR1- and TMRE-uptake by mitochondria. Time-resolved count rates of SkQR1 (20 nM, panel A) and TMRE (20 nM, panel B) from a suspension of rat liver mitochondria in incubation buffer (0.1 mg protein/ml) in the absence (curve 1) and in the presence (curve 2) of 5 mM succinate, respectively. Incubation mixture: 250 mM sucrose, 20 mM MOPS, 1 mM EGTA, 5 μM rotenone, 1 mg/ml BSA, pH 7.4. **c,d**. Corresponding autocorrelation functions G(τ)

fluorescence of SkQR1 taken up by mitochondria did not reveal a measurable dependence on energization. The data before the addition of succinate (closed circles) and after the addition of succinate (open circles) were nearly identical (Fig. 3a). This conclusion was also confirmed by the time course of the number of peaks with an amplitude exceeding 2 MHz ($n(F>2$ MHz), Fig. 3b). As shown in (Perevoshchikova et al. 2008), fitting of $n(F>F_0)$ by Eq. 1 can give a mean value of brightness of one stained mitochondrion (I_{single}):

$$n(F > F_0) = P_0 \cdot \left(\sqrt{\frac{I_{single}}{F_0}} - 1\right) \text{ for } F < I_{single}, \quad (1)$$

$$\text{and } n(F > F_0) = 0 \text{ for } F > I_{single}$$

Curves in Fig. 3a showed best fits with the following parameters: I_{single}=3.7 MHz (before addition of succinate); 4.2 MHz (after addition of succinate); 3.7 MHz (after addition of FCCP). The differences between these values of I_{single} were statistically not significant as the standard deviation of the measurements was about 10 %. Measurements of G(τ)

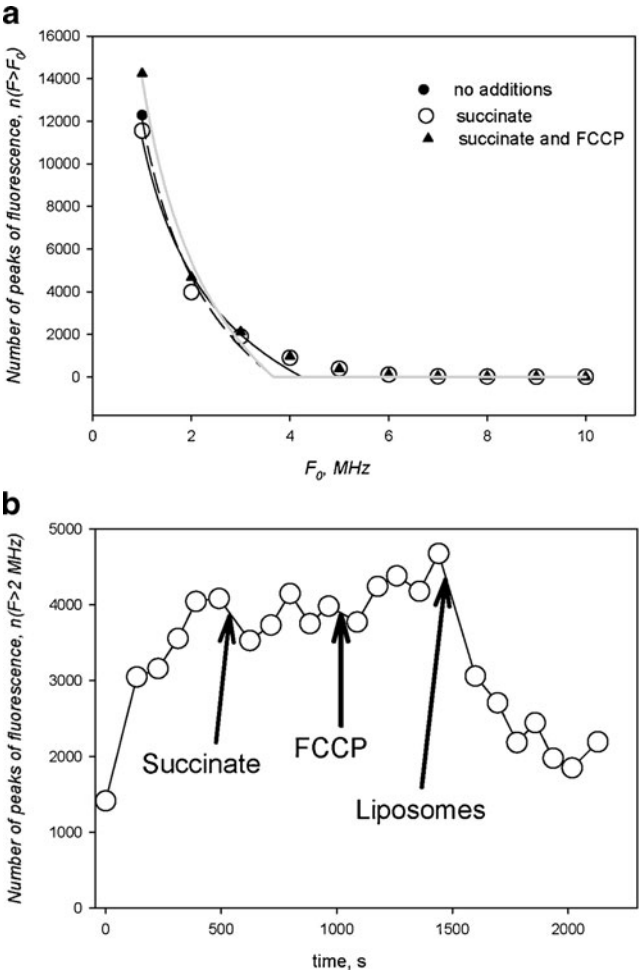

Fig. 3 **a** Dependence of the number of peaks $n(F>F_0)$ on F_0 for SkQR1 before the addition of succinate to mitochondria (curve 1, *closed circles*), in the presence of succinate (*open circles*), and after the addition of FCCP (*closed triangles*). Curves are best fits to Eq. 1 with mean fluorescence intensity per single mitochondron (parameter I_{single}). Panel **b.** Kinetics of SkQR1 uptake by mitochondria, estimated as the number of peaks of fluorescence exceeding amplitude of 2 MHz ($n(F>2$ MHz$)$), effect of liposomes. Additions, SkQR1 concentration, 20 nM; succinate, 5 mM; FCCP, 1 μM; liposomes, 0.4 mg phosphatidylcholine/ml

of rhodamine 6 G in solution suggested that one rhodamine molecule had brightness of 1.5 kHz under our experimental conditions. Therefore, the mean number of molecules of SkQR1 in a single mitochondrion was about 2,800 in the presence of succinate and about 2,470 in the presence of FCCP. Apparently, strong SkQR1 uptake by de-energized mitochondria can be accounted for by its high membranophilicity (see below, Table 1).

The SkQR1 uptake by mitochondria can be directly estimated by measuring fluorescence of butanol extracts of centrifuged mitochondria. About 50 % of added SkQR1 was detected in butanol at a mitochondrial protein concentration of 0.1 mg/ml. An increase in the protein concentration did not

Table 1 Estimation of octanol–water partition coefficient (P_{ow}) for several derivatives of rhodamine 19 by RP-HPLC

Compound	t_R, min	$\log P_{ow}$	P_{ow}
Rhodamine 6G	8.615	2.29	194
TMRE	7.68	1.69	50
C_8R1	10.827	3.44	2,754
SkQR1	11.493	3.74	5,500

lead to an increase in the butanol-extracted portion of SkQR1, suggesting that the rest of SkQR1 was bound to a light fraction of membranes which cannot be sedimented under our conditions. Similar experiments with TMRE showed that the presence of TMRE in butanol extracts was insignificant at these low protein concentrations.

The addition of liposomes at the end of the experiment presented in Fig. 3b led to a substantial decrease in the parameter n(F>2 MHz), i.e. to a decrease in the number of bright fluorescent organelles, which could be attributed to redistribution of SkQR1 from mitochondria to liposomes under the conditions of molar excess of liposome particles (about 100 nM) over SkQR1 (20 nM). In fact, the mean value of I_{single} decreased to 2.6 MHz at the end of the record.

As seen from Fig. 4, the presence of liposomes drastically changed the FCS-detected response of SkQR1 fluorescence to mitochondrial energization. Figure 4a (open circles) shows that the number of high fluorescence peaks n(F> 2 MHz) increased slowly after the addition of succinate and decreased after the addition of the uncoupler FCCP on the timescale of tens of minutes. In a control experiment in the presence of FCCP (closed triangles) the parameter n(F> 2 MHz) did not change upon the addition of succinate. Figure 4b displays the dependence of $n(F>F_0)$ on F_0 for three states of mitochondria. Best fits to Eq. 1 suggested the following values of mean brightness of a single mitochondrion: I_{single}=2.6 MHz before succinate; 4.4 MHz after succinate; 3.4 MHz after FCCP. The amplitude of the autocorrelation function increased upon mitochondrial energization in the presence of liposomes (insert to Fig. 4b) confirming that energization-induced redistribution of SkQR1 from liposomes to mitochondria.

Importantly, with C8R1, the quinone-less analog of SkQR1, the slow processes of succinate-induced accumulation and uncoupler-induced release from mitochondria were observed not only in the presence (Fig. 5b), but also in the absence of liposomes (Fig. 5a), the initial level of fluorescence of single mitochondrion being much lower when liposomes were present. As to the much less lipophilic cation TMRE, it exhibited fast responses to mitochondrial energization, which were independent of the presence of liposomes (Fig. 5). These results can be explained by lower P_{ow} coefficient for TMRE than for C8R1 (Table 1). The difference

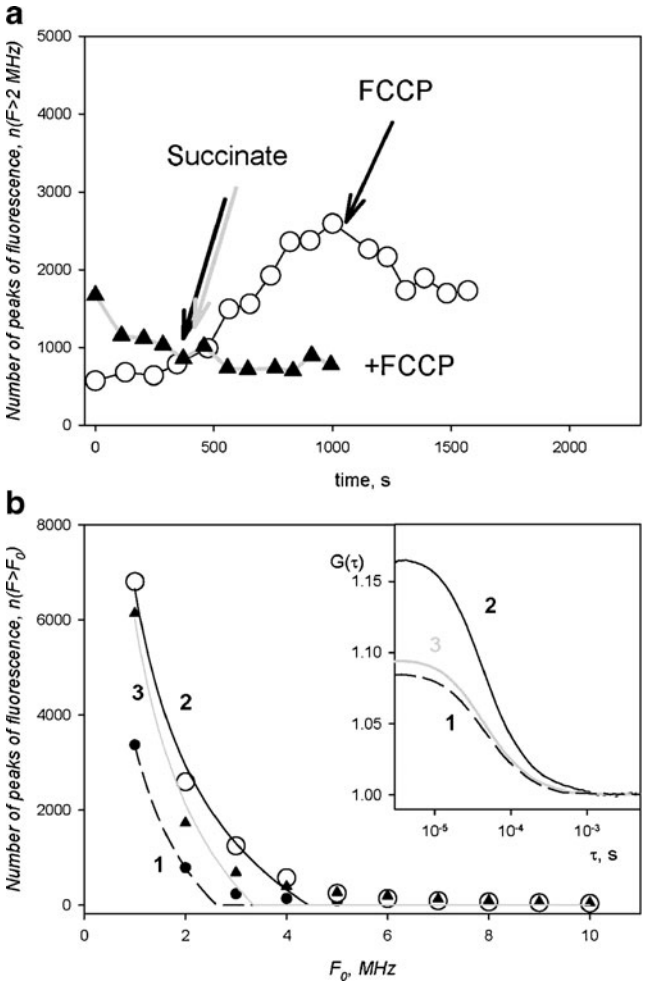

Fig. 4 **a** Kinetics of SkQR1 uptake by mitochondria upon their energization in the presence of liposomes (400 μg phosphatidylcholine/ml, *open circles*). *Closed triangles* were obtained in the presence of 1 μM FCCP. The uptake was estimated as the number of peaks of fluorescence exceeding an amplitude of 2 MHz (*n(F*> 2 MHz*)*). SkQR1 concentration, 20 nM. Panel **b**. Dependence of the number of peaks $n(F> F_0)$ on F_0 for SkQR1 before addition of succinate to mitochondria (curve 1, *closed circles*), in the presence of succinate (curve 2, *open circles*), and after addition of FCCP (curve 3, *closed triangles*). Curves are best fits to Eq. 1 with mean fluorescence intensity per single mitochondrion (I_{single}). Inset: Corresponding autocorrelation functions G(τ)

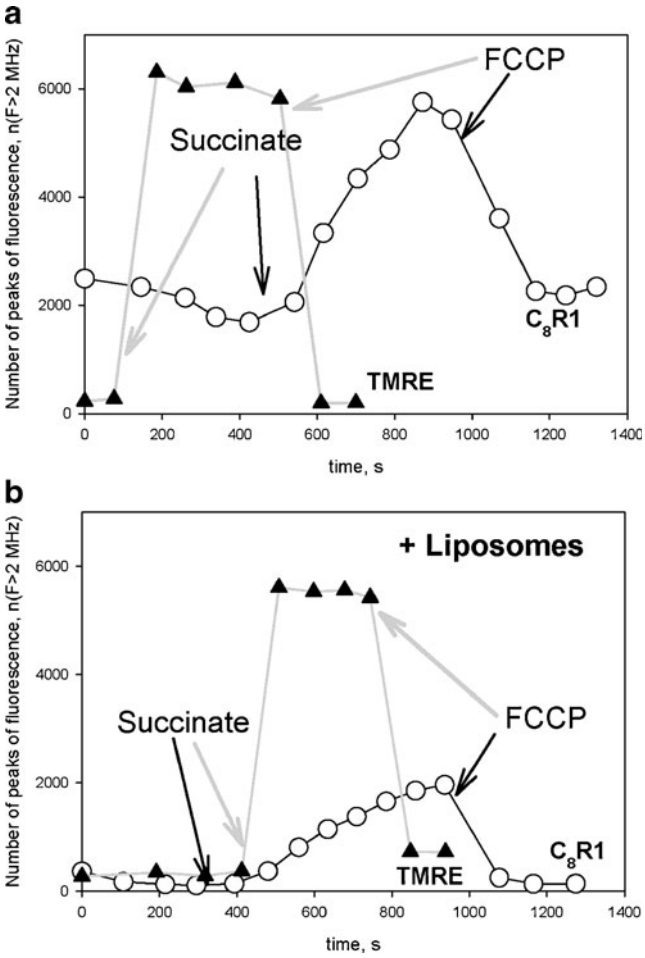

Fig. 5 Kinetics of TMRE uptake (*filled triangles*) and C8R1 uptake (*open circles*) by mitochondria upon their energization by 5 mM succinate in the absence (panel A) and in the presence (panel B) of liposomes (400 μg phosphatidylcholine/ml). The TMRE and C8R1 concentrations, 20 nM

between SkQR1 and C8R1 can also be accounted for the fact that P_{ow} for SkQR1 was the highest among penetrating cations tested (Table 1). Slow kinetics of C8R1 response to energization without liposomes may well be a result of redistribution of this cation between mitochondria and small membrane particles contaminating the mitochondrial fraction.

An alternative explanation of the slow kinetics of accumulation of our caions in mitochondria upon their energization could be a low rate of SkQR1 permeation through the inner membrane driven by electrical potential. According to our estimation of the rate of SkQR1 translocation across a planar bilayer lipid membrane (BLM) by measuring relaxation of transmembrane electrical current, the typical half-time of this process is 10 s (Rokitskaya et al. 2008). It was interesting to compare the rate of translocation of TMRE and SkQR1 in this system. The hydrophobic cations SkQR1 and TMRE were incubated for 10 min after addition to the membrane-bathing aqueous solution with constant stirring to complete their binding to the BLM. The current across the membrane *I(t)*, which was maximal immediately after application of the voltage *V*, spontaneously decreased in time from the initial level *I(0)* to the steady-state level *I* (∞). This current relaxation process was obviously associated with redistribution of the cations between two half-membrane leaflets of the BLM. Figure 6a shows current relaxation after a voltage jump of V=150 mV ("on" response) and backward relaxation to V=0 mV ("off" response) for SkQR1 at a concentration of 2 μM. The gray curves in Fig. 6a show monoexponential fits of the experimental curves with τ_{on}=1.67 s and τ_{off}=2.52 s. Figure 6b

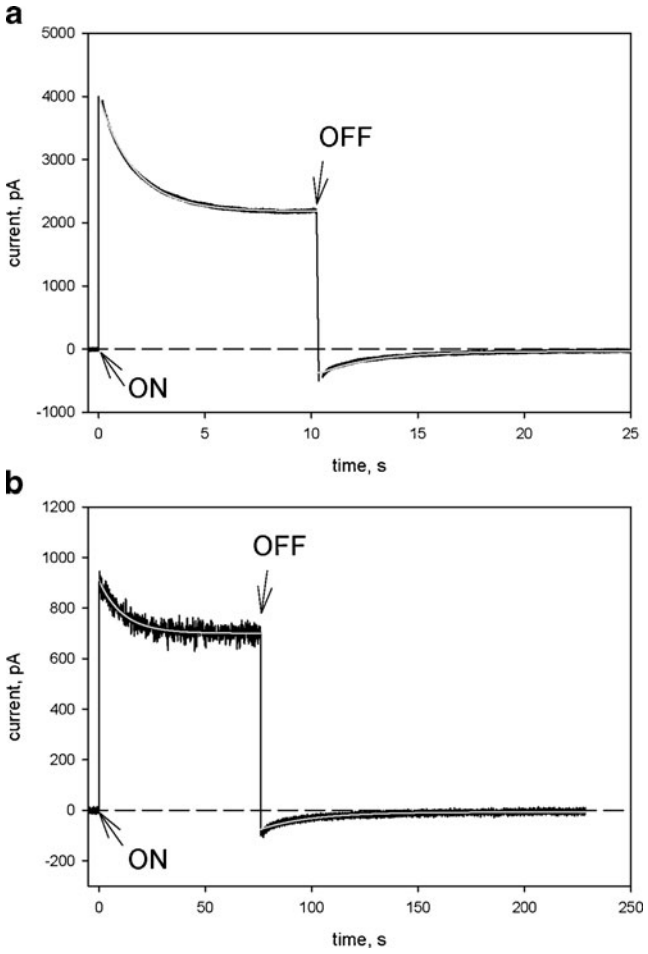

Fig. 6 Time courses of electrical current through BLM after application of voltage of V=150 mV (at t=0, i.e. "on" response) and relaxation with switching voltage to zero ("off" response) in the presence of 0.5 μM of SkQR1 (panel A) and 1 μM TMRE (panel B, curve 2), and their best fits by a single exponential function (*gray curves*) with τ_{on}=4.2 s, τ_{off}=8.3 s (SkQR1), τ_{on}=24 s τ_{off}=35 s (TMRE). Planar phospholipid membrane was formed from *E. coli* lipids in decane. The solution contained 2 mM Tris, 2 mM MES, 10 mM KCl, pH 7.0. Temperature 27 °C

shows demonstrates results of similar experiments with 2 μM TMRE. TMRE also exhibited current relaxation upon application of 150 mV, although it was lower in amplitude and substantially slower compared to that of SkQR1 (note different time scales in panels A and B). The gray curves in Fig. 6b show monoexponential fits with τ_{on}=11.3 s and τ_{off}=23.7 s. It can be concluded that TMRE lacking a hydrophobic residue had a slower translocation rate through the lipid bilayer than SkQR1. These data indicated that the rate of transmembrane movement of penetrating cations did not limit the overall process of cation accumulation in mitochondria, described in this paper.

Large uptake of SkQR1 by de-energized mitochondria observed in the present study is presumably associated with the high partition coefficient P_{ow} of this hydrophobic molecule. Let us compare the binding observed by FCS with the value of P_{ow} for SkQR1 determined by RP-HPLC (Table 1). To estimate SkQR1 partitioning into mitochondrial membranes from the mean number of SkQR1 molecules bound per single mitochondrion (about 2,500), one should know the volume of its membrane phase. According to electron microscopy of isolated rat liver mitochondria, the mean inner membrane area is 6.5 μm^2 (Schwerzmann et al. 1986). Assuming the membrane thickness to be 40 Å and believing the area of the inner membrane to be much larger than the area of the outer membrane, we estimated the membrane volume of an isolated rat liver mitochondrion as 0.026 μm^3 and the inner membrane concentration of SkQR1 as $\frac{2500}{6.03\cdot10^{23}\cdot0.026\cdot10^{-15}} = 0.16\ mM$, which gave us the lowest limit of the ratio (P_{mw}) of the inner membrane concentration of SkQR1 to its concentration in the aqueous solution (20 nM in our experiments): P_{mw}=7,900. This value is comparable with the octanol–water partition coefficient P_{ow}=5,500.

Let us consider the effect of addition of liposomes on SkQR1 binding to mitochondria in de-energized and energized states. In the de-energized state, a substantial part of SkQR1 should be bound to liposomes, because the concentration of lipid in liposomes (0.4 mg/ml) under our experimental conditions was higher than the concentration of mitochondrial protein (0.1 mg/ml), while the partition coefficients of SkQR1 for membranes of liposomes and mitochondria were supposed to be similar. Note that the protein/lipid ratio of rat liver mitochondria is known to be 2.4 (Getz et al. 1962). The addition of succinate leading to generation of potential on the inner mitochondrial membrane should shift the equilibrium distribution of SkQR1 from liposomes to mitochondria because of an additional driving force arising from the ability of SkQR1 to permeate across mitochondrial membrane in cationic form.

Recent studies of (Skulachev et al. 2011; Fetisova et al. 2010; Bakeeva et al. 2008) have revealed selective accumulation of SkQR1 in mitochondria in cells or tissues, which can be abolished upon the addition of uncouplers. The kinetics of the accumulation was slow and reached saturation after about 60 min (Fetisova et al. 2010). Based on our data, it can be assumed that the energy-dependent accumulation of SkQR1 is a process that depends on the presence in cells of different membrane components other than mitochondria competing with mitochondria for taking up SkQR1. These components may play the role of liposomes in our system.

Let us consider the role of lipophilicity of penetrating cations in targeting them to mitochondria in cells. In case of an imaginary highly hydrophilic penetrating cation, the degree of its accumulation in mitochondria of, e.g., rat liver cells, can be estimated knowing the portion of mitochondrial

volumes in cell volumes and a value of $\Delta\psi$. According to (Alberts et al. 2002), the mitochondrial volume comprises approximately 10 % of the total cell volume. Assuming mitochondrial $\Delta\psi$=180 mV that results in a 1000-fold higher concentration of the cations inside the matrix, the majority of the cations should be inside mitochondria. Considering the opposite case of a highly lipophilic penetrating cation, one can expect its $\Delta\psi$-dependent accumulation inside the inner leaflet of the inner mitochondrial membranes. The portion of the cations locating inside mitochondria should depend on the fraction of mitochondrial membranes in the total intracellular membrane pool. Assuming this portion to be similar to the fraction of mitochondrial lipids that comprises up to 20 % of the total lipid (Alberts et al. 2002), we conclude that the mitochondrial fraction of the cations should be also predominant in this case. Therefore, high lipophilicity of the cations should not decrease their targeting to mitochondria in cells. This consideration can be relevant to the distribution of SkQR1 in living cells and tissues which is important for the understanding of the mechanism of its protection from ischemia injury accompanying generation of reactive oxygen species (Skulachev et al. 2011; Bakeeva et al. 2008; Plotnikov et al. 2010).

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References

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P (2002) Molecular biology of the cell, 4th edn. Garland Science, New York

Amchenkova AA, Bakeeva LE, Chentsov YS, Skulachev VP, Zorov DB (1988) J Cell Biol 107:481–495

Antonenko YN, Avetisyan AV, Bakeeva LE, Chernyak BV, Chertkov VA, Domnina LV, Ivanova OY, Izyumov DS, Khailova LS, Klishin SS, Korshunova GA, Lyamzaev KG, Muntyan MS, Nepryakhina OK, Pashkovskaya AA, Pletjushkina OY, Pustovidko AV, Roginsky VA, Rokitskaya TI, Ruuge EK, Saprunova VB, Severina II, Simonyan RA, Skulachev IV, Skulachev MV, Sumbatyan NV, Sviryaeva IV, Tashlitsky VN, Vassiliev JM, Vyssokikh MY, Yaguzhinsky LS, Zamyatnin AA Jr, Skulachev VP (2008) Biochemistry (Mosc) 73:1273–1287

Antonenko YN, Avetisyan AV, Cherepanov DA, Knorre DA, Korshunova GA, Markova OV, Ojovan SM, Perevoshchikova IV, Pustovidko AV, Rokitskaya TI, Severina II, Simonyan RA, Smirnova EA, Sobko AA, Sumbatyan NV, Severin FF, Skulachev VP (2011) J Biol Chem 286:17831–17840

Asin-Cayuela J, Manas AR, James AM, Smith RA, Murphy MP (2004) FEBS Lett 571:9–16

Bakeeva LE, Barskov IV, Egorov MV, Isaev NK, Kapelko VI, Kazachenko AV, Kirpatovsky VI, Kozlovsky SV, Lakomkin VL, Levina SB, Pisarenko OI, Plotnikov EY, Saprunova VB, Serebryakova LI, Skulachev MV, Stelmashook EV, Studneva IM, Tskitishvili OV, Vasilyeva AK, Victorov IV, Zorov DB, Skulachev VP (2008) Biochemistry (Mosc) 73:1288–1299

Chen LB (1988) Annu Rev Cell Biol 4:155–181

Cunha FM, Caldeira da Silva CC, Cerqueira FM, Kowaltowski AJ (2011) Curr Drug Targets 12:783–789

Demura M, Kamo N, Kobatake Y (1985) Biochim Biophys Acta 820:207–215

Ehrenberg B, Montana V, Wei MD, Wuskell JP, Loew LM (1988) Biophys J 53:785–794

Fetisova EK, Avetisyan AV, Izyumov DS, Korotetskaya MV, Chernyak BV, Skulachev VP (2010) FEBS Lett 584:562–566

Getz GS, Bartley W, Stirpe F, Notton BM, Renshaw A (1962) Biochem J 83:181–191

Harper JA, Dickinson K, Brand MD (2001) Obes Rev 2:255–265

Hoye ATDJ, Wipf P, Fink MP, Kagan VE (2008) Acc Chem Res 41:87–97

Johnson D, Lardy H (1967) Methods Enzymol 10:94–96

Lanni A, Moreno M, Lombardi A, Goglia F (2003) FEBS Lett 543:5–10

Lombardo F, Shalaeva LY, Tupper KA, Gao F (2001) J Med Chem 44:2490–2497

Martyushin AA, Tcarev DA, Grigorenko MA, Fedorov II, Ramenskaya GV, Tashlitsky VN, Skulachev VP (2008) Pharmacia 5:23–29

Mueller P, Rudin DO, Tien HT, Wescott WC (1963) J Phys Chem 67:534–535

Murphy MP, Smith RA (2007) Annu Rev Pharmacol Toxicol 47:629–656

Nedergaard J, Cannon B (2003) Exp Physiol 88:65–84

Perevoshchikova IV, Zorov DB, Antonenko YN (2008) Biochim Biophys Acta 1778:2182–2190

Plotnikov EY, Silachev DN, Chupyrkina AA, Danshina MI, Jankauskas SS, Morosanova MA, Stelmashook EV, Vasileva AK, Goryacheva ES, Pirogov YA, Isaev NK, Zorov DB (2010) Biochemistry (Mosc) 75:145–150

Rokitskaya TI, Klishin SS, Severina II, Skulachev VP, Antonenko YN (2008) J Membr Biol 224:9–19

Rokitskaya TI, Sumbatyan NV, Tashlitsky VN, Korshunova GA, Antonenko YN, Skulachev VP (2010) Biochim Biophys Acta 1798:1698–1706

Rottenberg H (1984) J Membr Biol 81:127–138

Scaduto RC, Grotyohann LW (1999) Biophys J 76:469–477

Schwerzmann K, Cruz-Olive LM, Eggman R, Sanger A, Weibel ER (1986) J Cell Biol 102:97–103

Skulachev MV, Antonenko YN, Anisimov VN, Chernyak BV, Cherepanov DA, Chistyakov VA, Egorov MV, Kolosova NG, Korshunova GA, Lyamzaev KG, Plotnikov EY, Roginsky VA, Savchenko AY, Severina II, Severin FF, Shkurat TP, Tashlitsky VN, Shidlovsky KM, Vyssokikh MY, Zamyatnin AA, Zorov DB, Skulachev VP (2011) Curr Drug Targets 12:800–826

Skulachev VP (1998) Biochim Biophys Acta 1363:100–124

Skulachev VP (2007) Biochemistry (Mosc) 72:1385–1396

Skulachev VP, Anisimov VN, Antonenko YN, Bakeeva LE, Chernyak BV, Erichev VP, Filenko OF, Kalinina NI, Kapelko VI, Kolosova NG, Kopnin BP, Korshunova GA, Lichinitser MR, Obuchova LA, Pasyukova EG, Pisarenko OI, Roginsky VA, Ruuge EK, Senin II, Severina II, Skulachev MV, Spivak IM, Tashlitsky VN, Tkachuk VA, Vyssokikh MY, Yaguzhinsky LS, Zorov DB (2009) Biochim Biophys Acta 1787:437–461

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) Anal Biochem 150:76–85

Strakhovskaya MG, Antonenko YN, Pashkovskaya AA, Kotova EA, Kireev V, Zhukhovitsky VG, Kuznetsova NA, Yuzhakova OA, Negrimovsky VM, Rubin AB (2009) Biochemistry (Mosc) 74:1305–1314