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Mitochondria-targeted penetrating cations as carriers of hydrophobic anions through lipid membranes

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ABSTRACT

High negative electric potential inside mitochondria provides a driving force for mitochondria-targeted delivery of cargo molecules linked to hydrophobic penetrating cations. This principle is utilized in construction of mitochondria-targeted antioxidants (MTA) carrying quinone moieties which produce a number of health benefitting effects by protecting cells and organisms from oxidative stress. Here, a series of penetrating cations including MTA were shown to induce the release of the liposome-entrapped carboxyfluorescein anion (CF), but not of glucose or ATP. The ability to induce the leakage of CF from liposomes strongly depended on the number of carbon atoms in alkyl chain (n) of alkyltriphenylphosphonium and alkylrhodamine derivatives. In particular, the leakage of CF was maximal at n about 10–12 and substantially decreased at $n=16$. Organic anions (palmitate, oleate, laurylsulfate) competed with CF for the penetrating cation-induced efflux. The reduced activity of alkylrhodamines with $n=16$ or $n=18$ as compared to that with $n=12$ was ascribed to a lower rate of partitioning of the former into liposomal membranes, because electrical current relaxation studies on planar bilayer lipid membranes showed rather close translocation rate constants for alkylrhodamines with $n=18$ and $n=12$. Changes in the alkylrhodamine absorption spectra upon anion addition confirmed direct interaction between alkylrhodamines and the anion. Thus, mitochondria-targeted penetrating cations can serve as carriers of hydrophobic anions across bilayer lipid membranes.

1. Introduction

It is generally accepted that mitochondria are an important source of reactive oxygen species (ROS) that are generated by the respiratory chain. Mitochondrial ROS are closely linked to various diseases, including cancer, diabetes, and Alzheimer's and other neurodegenerative disorders; this makes mitochondria an important target for delivery of ROS scavengers. The strategy of design of mitochondria-targeted antioxidants (MTAs) is based on the assumption that mitochondrial membrane potential (negative inside mitochondria) should drive accumulation of permeating cations into these organelles [1,2]. If a penetrating cation carries some antioxidant moiety, it can be an effective MTA. During recent years a series of such MTAs have been synthesized and successfully studied [3–10]. Along with targeted transport of antioxidants, permeating cations are capable to deliver covalently linked NO-donors into mitochondria [11,12], red-ox probes [13], and some other molecules [14–16].

The delivery of cationic antioxidants to mitochondria involves permeation through plasmalemma and mitochondrial membranes composed of lipid bilayer and proteins. Membrane permeation of hydrophobic ions with delocalized charges has been studied most intensively with tetraphenylborate, dipicrylamine (DPA), and some analogs of these molecules [17–21]. The rate of translocation of hydrophobic delocalized cations across an artificial bilayer lipid membrane (BLM) is about 3–4 orders of magnitude lower than that of hydrophobic anions due to the effect of dipole potential at the membrane-water interface [18,22]. Besides, cations bind to membranes substantially more weakly than structurally similar anions [23]. This apparently accounts for the appearance of only a few publications about the permeation of the cations through lipid bilayers. For example, the translocation of cationic fluorescent dyes R18 and DiI from one

Abbreviations: BLM, bilayer lipid membrane; CF, carboxyfluorescein; C_2R1, ethylrhodamine 19 or rhodamine 6G; C_8R1, octylrhodamine 19; C_{10}R1, decylrhodamine 19; C_{12}R1, dodecylrhodamine 19; C_{16}R1, octadecylrhodamine 19; C_{12}R4, dodecylrhodamine B; C_{18}R4 or R18, octadecylrhodamine B; C_{12}TPP, dodecyltriphenylphosphonium; DPhPC, diphytanoylphosphatidylcholine; EYPC, egg yolk phosphatidylcholine; MES, 2-(*N*-morpholino)ethanesulfonic acid; MTA, mitochondria-targeted antioxidant; MitoQ, compound of ubiquinone and decyl triphenylphosphonium; ROS, reactive oxygen species; SkQ1, compound of plastoquinone and decyl triphenylphosphonium; SkQR1, compounds of plastoquinone and decylrhodamine 19; SRB, sulforhodamine B; TPP, tetraphenylphosphonium

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monolayer of a phospholipid bilayer membrane to the other was monitored by membrane current relaxation and fluorescence quenching measurements, and the estimated time constants were about 10^{-1} s [20], which was about 3–4 orders of magnitude faster than that for tetraphenylphosphonium (TPP) [24]. Recent study of membrane permeation of cationic mitochondria-targeted antioxidants in our group [25] led to estimation of translocation rate constants of the order of 10^{-2} to 10^{-1} s^{-1} depending on the particular compound.

According to earlier works, some hydrophobic cations facilitate solubilization of hydrophilic anions in organic solvents [26] and also transport of anions across artificial and natural membranes [27–29]. There are several other groups of compounds that facilitate anion transport across lipid membranes, e.g. synthetic polyarginine derivatives, a phosphatidylcholine derivative, and molecular umbrella amphiphiles [30–33]. Mitochondria-targeted antioxidants, being hydrophobic delocalized cations, can possess this property as well. In fact, it has been recently shown in our group that SkQR1, SkQ1, and C_{12}TPP can mediate the transport of palmitate and carboxyfluorescein (CF) anions across planar bilayers and liposomal membranes [34]. As a protonated form of palmitic acid can cross the lipid membrane, a pair of a penetrating cation and a fatty acid can transport protons through artificial and natural membranes [34]. In the present paper, the phenomenon of anion translocation by penetrating cations was studied in detail using a wide range of chemical analogs differing in cationic headgroups and hydrocarbon tails. Moreover, competition between different organic anions for the cationic carrier-mediated translocation across a membrane was studied.

2. Materials and methods

Earlier a series of mitochondria-targeted antioxidants having substituted 1,4-benzoquinone rings conjugated to hydrophobic triphenylphosphonium or the rhodamine cation through the decyl linker were synthesized [5,35]. Structures of SkQ1, MitoQ and SkQR1 are shown in Fig. 1A. Also, an alkyl analog of SkQ1 and MitoQ lacking the quinone moiety (dodecyltriphenylphosphonium, C_{12}TTP) was prepared [35]. Here we report synthesis and anion transport properties of alkyl esters of novel rhodamines differing in the number of carbon atoms in the alkyl chain (general formula C_nR1) where n takes on values 2, 8, 10, 12 and 16 (Fig. 1B). Rhodamine 19 ethyl ester (C_2R1 or Rhodamine 6G) and Rhodamine B octadecyl ester (C_{18}R4 or R18) were purchased from Sigma. Rhodamine was modified by alkyl substituents by esterification of its free carboxylic group by two methods (Fig. 1B). An octyl ester of rhodamine 19 (C_8R1) was prepared as described earlier [36] by method I based on interaction of octyl alcohol with rhodamine 19 in the presence of sulfuric acid at 70 °C during 3 h (Supporting Information); the yield of C_8R1 was rather low. Decyl-, dodecyl- and hexadecyl esters of rhodamine 19 (C_{10}R1, C_{12}R1, C_{16}R1, correspondingly) and also dodecylrhodamine B (C_{12}R4) were prepared by method II, consisting of 72-h reaction between bromalkanes and the previously obtained Cs-salt of rhodamine 19, in dimethylformamide at 60 °C, the yields being almost quantitative. Target substances (C_8R1, C_{10}R1, C_{12}R1, C_{16}R1 and C_{12}R4) were purified by column chromatography on silica gel in solvent mixtures containing chloroform and methanol at different ratios and characterized by HPLC, NMR and mass spectrometry (Supporting Information).

Liposomes loaded with 5,6-carboxyfluorescein (CF, Sigma) were prepared by extrusion through a 100-nm filter (Avanti Mini-Extruder) from egg yolk phosphatidylcholine (Avanti Polar Lipids) in solution containing 100 mM CF titrated with Tris-base. The unloaded CF was then removed by passage through a Sephadex G-50 coarse column using 100 mM KCl, 10 mM Tris, pH 7.4 as the eluting buffer. To measure the rate of CF efflux, the liposomes were diluted in buffer containing 100 mM KCl, 10 mM Tris, pH 7.4. The fluorescence at 520 nm (excitation at 490 nm) was monitored with a Panorama Fluorat 02 spectrofluorimeter (Lumex, Russia). At the end of each recording, 0.1% Triton-X100 was added to complete the efflux process.

Liposomes loaded with ATP were prepared as described above in solution of 10 mM ATP, 100 mM KCl, 10 mM Tris, pH 7.6. The unloaded ATP was then removed by passage through a Sephadex G-50 coarse column using 100 mM KCl, 10 mM Tris, pH 7.6 as the eluting buffer. ATP efflux was measured by LKB-1250 luminometer in the presence of 20 μg/ml luciferin and 10 μg/ml luciferase. Melittin was used for induction of full release of ATP because Triton-X100 partially quenched the luminescence.

Liposomes loaded with glucose were prepared according to Haxby [37] from a mixture of egg PC and cholesterol (77:23 weight %) to diminish intrinsic leakage. Dried lipid mixture was hydrated in 300 mM glucose, and the mixture was vortexed, passed through a cycle of freezing and thawing, extruded through 0.1-μm pore size polycarbonate membranes, and passed through a Sephadex G-50 column using 100 mM Tris, 130 mM NaCl, 3.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, pH 8.5 as the eluting buffer. The measurements were carried out in the above buffer supplemented with 2 mM ATP, 1 mM NADP, 10 μg/ml hexokinase and 100 μg/ml glucose-6-phosphate dehydrogenase (37 °C). The leakage of glucose was recorded by means of the NADPH fluorescence at 460 nm (excitation at 345 nm).

The extent of the CF efflux and efflux of other markers was calculated as $(F_t - F_0)/(F_{100} - F_0)$, where F_0 and F_t represent the initial fluorescence and the fluorescence at time t, and F_{100} is the fluorescence after complete disruption of the liposomes by addition of the detergent Triton-X100 (final concentration, 0.1 % w/w).

Planar bilayer lipid membrane (BLM) was formed from a 2% solution of diphytanoylphosphatidylcholine (DPhPC, 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 [38]. 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 designed by J. Dempster (University of Strathclyde, UK).

3. Results

In the first series of experiments, it was found that mitochondria-targeted antioxidants, as well as their alkyl analogs lacking the quinone moiety, are competent in inducing efflux of CF from large unilamellar vesicles (liposomes). Such an effect was monitored by an increase in CF fluorescence (Fig. 2) which resulted from its dequenching upon dilution in the incubation medium of the previously liposome-entrapped, highly concentrated CF. The cationic compounds lacking quinones were even more active in this system: C_{12}R1>SkQR1, C_{12}TPP>SkQ1 (Fig. 2A). Alkylrhodamine derivatives were more potent in releasing CF from liposomes than the triphenylphosphonium ones: C_{12}R1>C_{12}TPP, SkQR1>SkQ1 (Fig. 2A), consistent with our data on their permeation through planar bilayer lipid membranes [25].

To distinguish between transport mechanisms (pore or carrier), we prepared glucose-loaded liposomes and measured NADPH formed in the presence of hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6PDH) as a result of coupled reactions [37]:

$$\text{Glucose} + \text{ATP} \rightarrow \text{Glucose}-6-\text{phosphate} + \text{ADP} \quad (\text{HK})$$
$$\text{G6P} + \text{NADP} \rightarrow 6-\text{phosphogluconate} + \text{NADPH} \quad (\text{G6PDH})$$

In the case of the pore-type efflux, our hydrophobic cation must induce the release of glucose with a rate comparable to that of CF, while the carrier-type efflux must be effective with anionic CF and

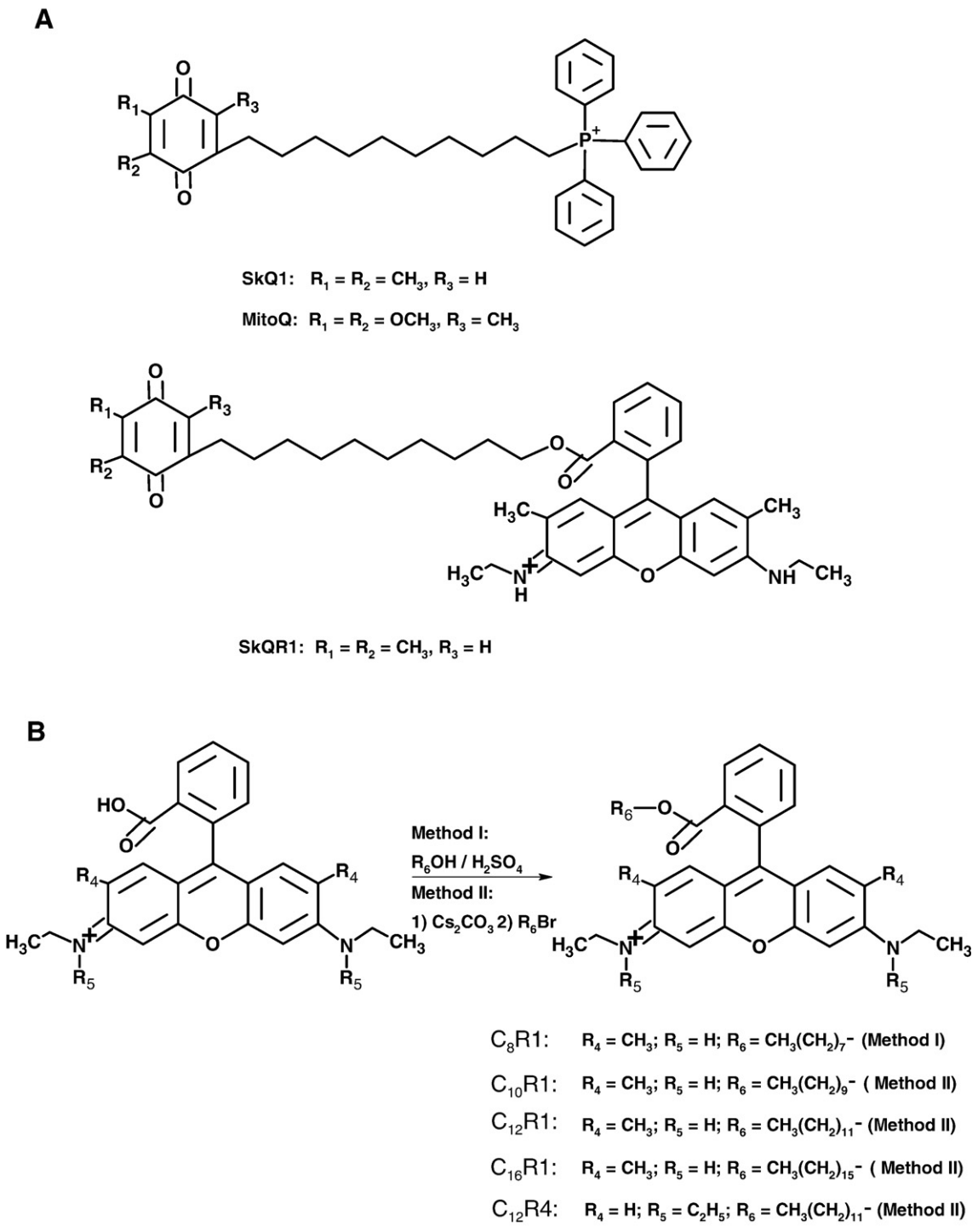

Fig. 1. (A) Mitochondria-targeted antioxidants containing 1,4-benzoquinone moieties. (B) Scheme of synthesis of alkylrhodamines.

ineffective with neutral glucose. It was shown that rhodamine derivative $C_{12}R1$ was ineffective in promoting glucose efflux (Fig. 2B), thereby favoring the carrier-type mechanism. The cation-mediated transport proved to be unspecific to organic anion structure. Not only CF anions, but also sulforhodamine B (which is also used as leakage assay [39]) and calcein were released by $C_{12}R1$, $C_{12}TPP$ and SkQ1 from liposomes loaded with these dyes (data not shown). It should be noted that Yang and co-workers [40] observed calcein leakage from liposomes induced by a series of short-chain triphenylalkylphosphonium, however they attributed this process to a membrane perturbation by these compounds.

It was interesting to check the ability of the carriers to induce the efflux of ATP, one of the most physiologically important anions. Fig. 2C shows the effect of SkQR1 on ATP efflux measured by the luciferin-luciferase assay and CF efflux under similar conditions. In contrast to CF, SkQR1 was ineffective in transporting ATP from ATP-loaded liposomes. This difference might be explained by the fact that ATP is more hydrophilic than CF.

The carrier-type mechanism of transport implies formation of complexes between a substrate (i.e. CF anion) and a carrier. The interaction between $C_{12}R1$ and organic anions was studied in aqueous solution by measuring $C_{12}R1$ absorption spectra (Fig. 3). The addition of micromolar

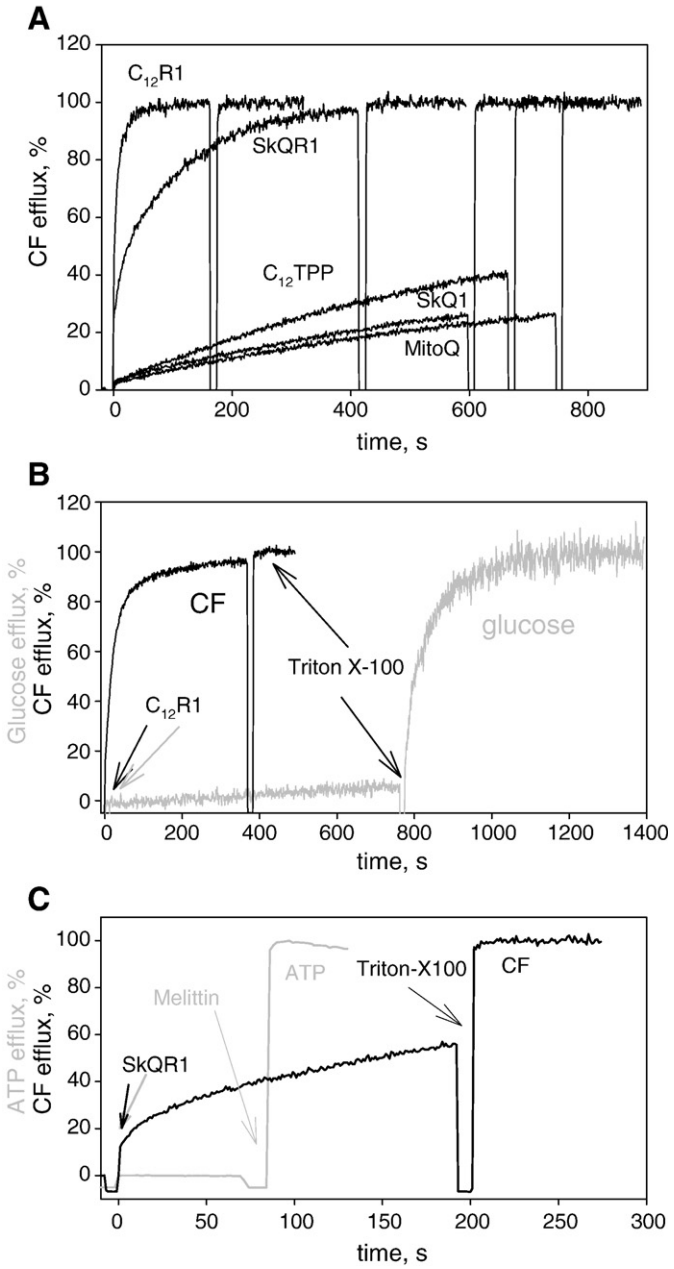

Fig. 2. (A) CF efflux from liposomes mediated by mitochondria-targeted antioxidants and their analogs. Fractional change in CF emission ($\lambda_{ex}=490$ nm, $\lambda_{em}=520$ nm) as a function of time after addition of 0.5 μM of different cations to egg yolk phosphatidylcholine (EYPC) liposomes (7 μM lipid, 10 mM Tris, 100 mM KCl, pH 7.4). (B) $C_{12}R1$ (added at $t=0$ s) induced CF efflux from liposomes (black line) but did not induce glucose efflux (gray line). Fractional change in CF emission ($\lambda_{ex}=490$ nm, $\lambda_{em}=520$ nm, black line) and NADPH emission ($\lambda_{ex}=345$ nm, $\lambda_{em}=460$ nm, gray line) as a function of time after addition of 10 μM $C_{12}R1$ to EYPC-cholesterol (77:23 by weight) liposomes (7 μM lipid for CF curve and 70 μM lipid for glucose curve). The solution for glucose measurement was 100 mM Tris, 130 mM NaCl, 3.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, 1 mM NADP, 2 mM ATP, 0.5 mM 1,4-dithiothreitol, pH 8.5 in the presence of 10 μg/ml Hexokinase and 100 μg/ml G6PDH. (C) SkQR1 (added at $t=0$ s) induced CF efflux from EYPC liposomes (black line) but did not induce ATP efflux (gray line). Fractional change in CF emission (curve 1, $\lambda_{ex}=490$ nm, $\lambda_{em}=520$ nm) or luminescence in the luciferin-luciferase assay (curve 2) as a function of time after addition of 0.5 μM SkQR1 to EYPC liposomes (7 μM lipid, 10 mM Tris, 100 mM KCl, pH 7.6). Triton or melittin were added at the end of the experiments in order to induce full leakage of the liposomes.

concentrations of laurylsulfate led to a substantial increase in the ratio of absorbance at 490 nm and 530 nm (Fig. 3, curves 1–4) corresponding to dimer and monomer absorption maxima of rhodamine 19 [41]. Several organic anions exerted similar effects (see, e.g. curve 1 of the inset to

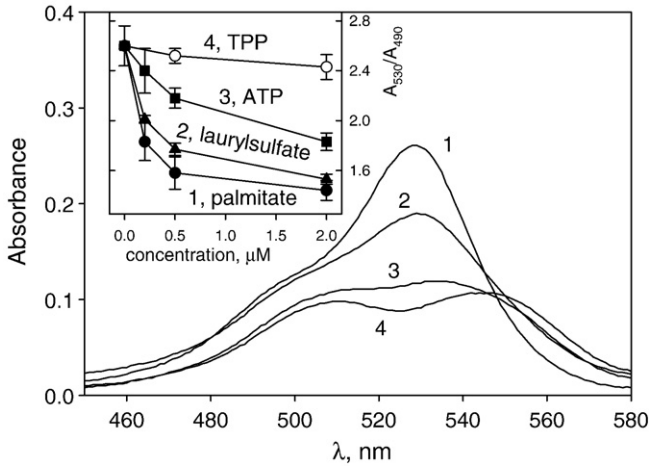

Fig. 3. Absorption spectra of $C_{12}R1$ (5 μM) in aqueous solution (1 mM Tris, 1 mM MES, pH = 7.4) in the absence (curve 1) and in the presence of 0.2 μM, 0.5 μM, and 2.0 μM palmitate (curves 2–4, respectively). Inset: ratio of absorption at 530 nm and 490 nm (A_{530}/A_{490}) as a function of concentration of palmitate (curves 1), laurylsulfate (curve 2), ATP (curve 3), and tetraphenylphosphonium (curve 4).

Fig. 3, where palmitate anion was used). ATP affected the spectrum of $C_{12}R19$ under these conditions as well (Fig. 3, inset, curve 3), which suggests that the failure of SkQR1 to transport ATP is associated with ineffective translocation of a carrier–ATP complex across a membrane rather than with poor binding of ATP to a carrier (Fig. 2C). Control experiments showed that the hydrophobic cation tetraphenylphosphonium did not alter the spectrum of $C_{12}R1$ (Fig. 3, inset, curve 4).

Fig. 4 shows the dependence of the initial rate of the CF efflux on the concentration of $C_{12}R1$. Obviously, the dependence is nonlinear. CF can have up to three negative charges at pH 7.4 [42], suggesting up to 3 to 1 stoichiometry of the complex with monocation $C_{12}R1$. The data were fitted well with a quadratic concentration dependence $[C_{12}R1]^2$ (solid curve in Fig. 4) suggesting that the predominant complex consisted of one CF and two $C_{12}R1$. This complex could be negatively charged or neutral at pH 7.4, i.e. the transmembrane flux of the CF-$C_{12}R1$-$C_{12}R1$ complex as well as the backward translocation of $C_{12}R1$ should be electrogenic, both generating positive charging of the liposome interior.

To study the dependence of the activity of hydrophobic cations on membrane potential of lipid vesicles, inside-positive or inside-negative diffusion potentials were generated across the liposomal membranes. For this, the K^+ carrier valinomycin was added to vesicles with transmembrane potassium gradient formed by using KCl buffer

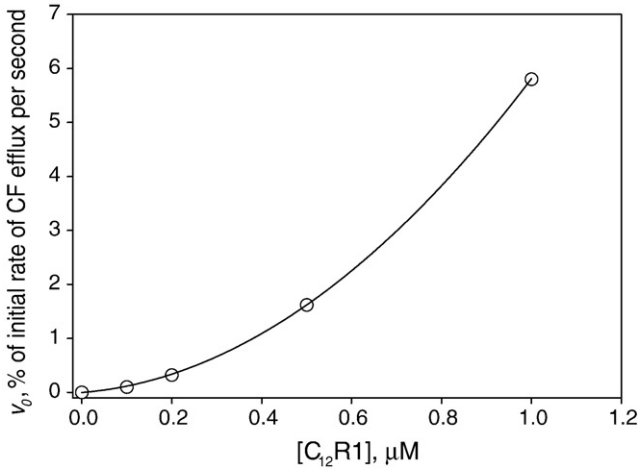

Fig. 4. Initial rate of CF efflux from EYPC liposomes as a function of $C_{12}R1$ concentration. Solid line is the best fit to the equation $v_0 = A\cdot[C_{12}R1]^2$. For conditions, see Fig. 2A.

and NaCl buffer, outside and inside, and vice versa, respectively. Importantly, in the case of NaCl buffer outside, liposomes contained KCl buffer inside. It was found that inside-positive potential blocked CF efflux induced by 0.1 μM C_{12}R4 (Fig. 5A), while inside-negative potential accelerated the CF efflux (Fig. 5B). Similar data were obtained with another hydrophobic cation, C_{12}TPP (data not shown). These data suggested that the overall process of CF transport by C_{12}R4 was electrogenic and can be accounted for by the following effects: (i) stimulation of efflux of negatively charged CF–C_{12}R4–C_{12}R4 complex, (ii) inhibition of influx of positively-charged C_{12}R4, or (iii) combination of (i) and (ii).

The CF-efflux assay was also used to study competition between CF and other organic anions. The experiments were carried out at non-saturating concentrations of C_{12}R1 (0.5 μM) which led to efflux of about 50 % of the CF during 30 s (Fig. 6, curve "no addition"). We tested the effect of 20 μM oleate, 10 μM dodecylsulfate, and 100 μM pyrenebutyrate on the C_{12}R1-mediated CF efflux. All of them strongly inhibited the CF efflux (Fig. 6). Palmitate blocked the efflux completely, while the effect of stearate was less pronounced (not shown). Apparently, these hydrophobic anions formed high-affinity complexes with the hydrophobic cation which hindered the interaction of the cation with CF. Consistent with this assumption, the absorption spectra of 5 μM C_{12}R1 was changed substantially by submicromolar concentrations of palmitate (Fig. 3, inset, curve 3). All anions listed above are highly hydrophobic. As to more hydrophilic anions, they were without inhibitory effect on the C_{12}R1-mediated CF efflux, like 100 μM 3-indolylacetate (Fig. 6), or naphthylacetate and MES (not shown).

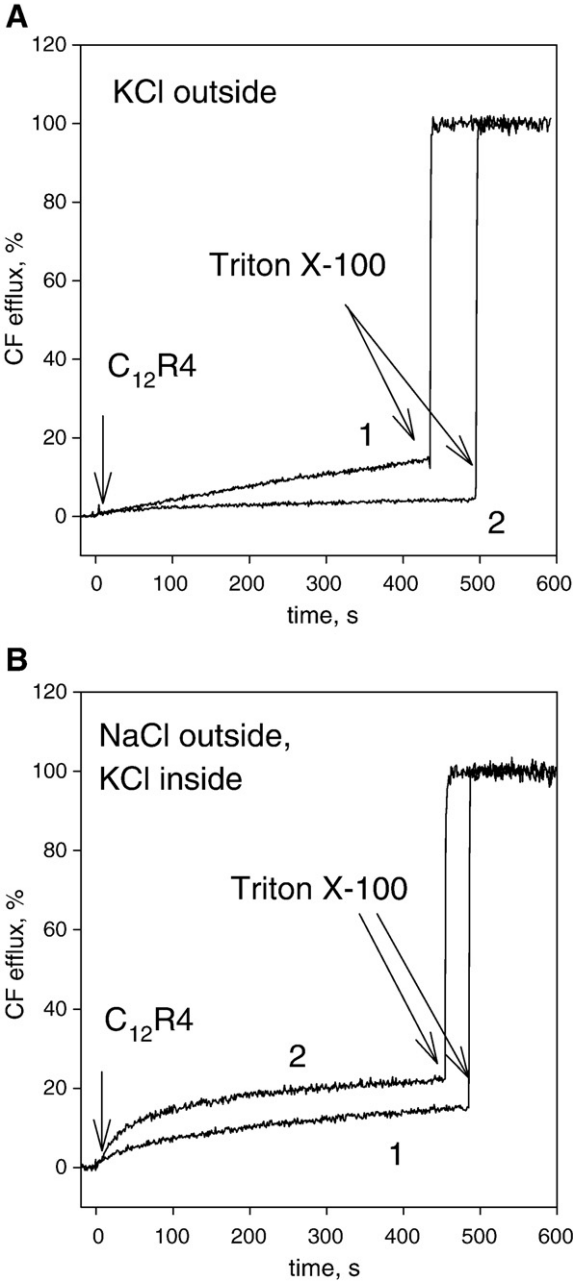

Fig. 5. Effect of transmembrane voltage on CF efflux from EYPC liposomes induced by C_{12}R4 (0.1 μM). (A) The inside buffer was CF-Tris mixture, outside buffer was 100 mM KCl, 10 mM TRIS, pH 7.4. (B) The inside buffer was CF-Tris mixture and 100 mM KCl, outside buffer was 100 mM NaCl, 10 mM TRIS, pH 7.4. Curve 1, without valinomycin; curve 2, with 20 nM valinomycin.

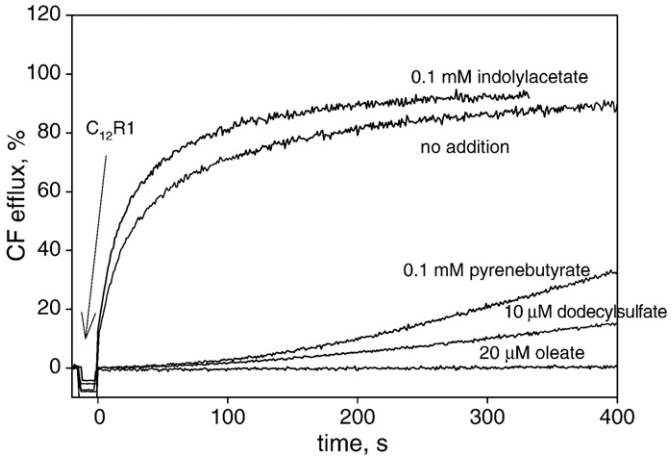

Fig. 6. Effect of different organic anions on CF efflux from EYPC induced by 0.5 μM C_{12}R1. Additions, 20 μM oleate, 20 μM dodecylsulfate, 0.1 mM pyrenebutyrate, 0.1 mM 3-indolylacetate. For conditions, see Fig. 2A.

Fig. 7 shows CF leakage data for a series of alkylrhodamines with different alkyl chain length. The extent of leakage induced by alkylrhodamines had a maximum at $n=12$ (compound C_{12}R1). Consistent with this finding, C_{12}R4 was 10-fold more effective than commercial octadecylrhodamine B (i.e. C_{18}R4). The increase in the activity with n at low n can be associated with an increase in binding of the cations to liposomes. The decrease in the activity at high n might result from 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 [43], which was attributed to increased restriction of transmembrane mobility at high n. By analogy with flip-flop, it can be assumed that the decrease in the CF transport at high n can be ascribed to restricted transmembrane mobility of alkylrhodamines. However, the following data contradicted this proposal.

According to our previous work, voltage application to a planar BLM in the presence of SkQ1 and related compounds results in relaxation of electric current [25] associated with redistribution of the cations between the two half-membrane leaflets. The time course of a change in the current across a BLM after application of voltage V at time $t=0$ was shown to represent a monoexponential curve

$$I(t) = 2zF\gamma ck_i \sinh(zu/2)\frac{2k_i\cosh(zu/2)e^{-t/\tau}+k}{2k_i\cosh(zu/2)+k} \quad (1)$$

and

$$\tau = \frac{1}{k+2k_i\cosh(zu/2)}, \quad (2)$$

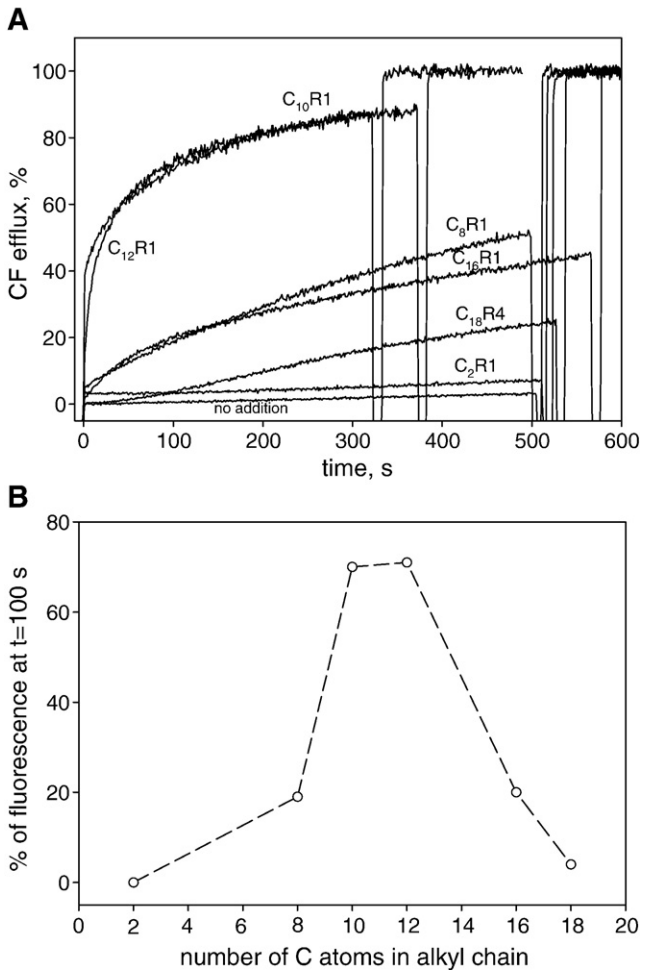

Fig. 7. (A) CF transport data for a series of 0.5 μM alkylrhodamines 19 with different alkyl chain length. (B) Dependence of extent of CF efflux at 100 s on the number of carbon atoms in the alkyl chain, *n*, of the rhodamine derivatives. The data for $n=18$ corresponded to $C_{18}R4$. For conditions, see Fig. 2A.

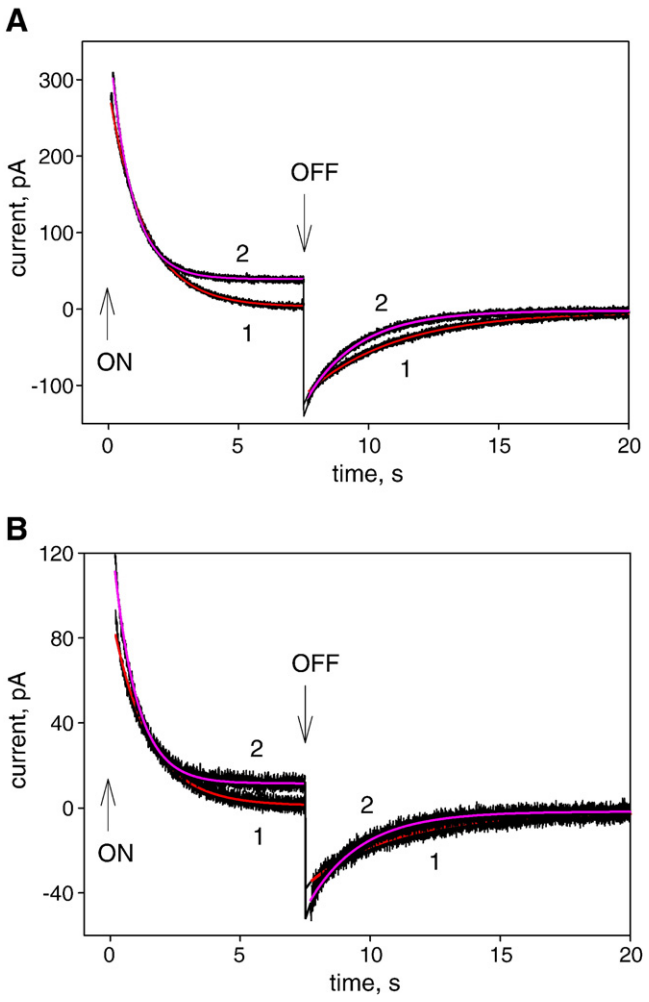

Fig. 8. Time courses of electrical current after application of voltage of $V=100$ mV (at $t=0$, i.e. "on" response) and relaxation with switching voltage to zero ("off" response) in the presence of 0.1 μM of $C_{12}R4$ (curve 1, panel A) and $C_{18}R4$ (curve 1, panel B), and their best fits by a monoexponential function (red curves) with $\tau_{on}=1.38$ s, $\tau_{off}=3.4$ s ($C_{12}R4$), $\tau_{on}=1.41$ s $\tau_{off}=3.3$ s ($C_{18}R4$). Curves 2 shows the records in the presence of 10 μM palmitate. Pink curves are their best fits by a monoexponential function with $\tau_{on}=0.85$ s, $\tau_{off}=2.0$ s ($C_{12}R4$), $\tau_{on}=0.9$ s $\tau_{off}=2.0$ s ($C_{18}R4$). Planar bilayers were formed from DPhPC in decane. The solution was 10 mM Tris, 100 mM KCl, pH 7.4.

where $u=\frac{V}{RT/F}$, c is the concentration of hydrophobic ions in aqueous solution, z is the valence of the lipid-soluble ion, γ is a partition coefficient, k and k_i are rate constants of translocation across the interface and the interior of the membrane, respectively [17]. We incubated the hydrophobic cations $C_{12}R4$ and $C_{18}R4$ for 10 min after addition to the membrane-bathing aqueous solution with constant stirring to complete their binding to BLM. Current across the membrane $I(t)$, which was maximal at the first moment after application of the voltage V, spontaneously decreased in time from the initial level $I(0)$ to a steady-state level $I(\infty)$ (the current relaxation process). Fig. 8 (curves 1) shows the current relaxation after a voltage jump of $V=100$ mV ("on" response) and backward relaxation to $V=0$ mV ("off" response) for $C_{12}R4$ (panel A) and $C_{18}R4$ (panel B) at a concentration of 0.1 μM. Fig. 8 also shows monoexponential fits of the experimental curves (red curves) with $\tau_{on}=1.38$ s ($C_{12}R4$) and $\tau_{on}=1.41$ s ($C_{18}R4$). Statistical analysis of several experiments gave $\tau_{on}=1.35\pm0.1$ s, $\tau_{off}=3.4\pm0.2$ s ($C_{18}R4$) and $\tau_{on}=1.4\pm0.2$ s, $\tau_{off}=3.2\pm0.3$ s ($C_{12}R4$) suggesting that the translocation kinetics of these two cations were similar. This conclusion is in line with a weak dependence of activation energy of triphenylalkylphophonium transport on the alkyl chain length for short chain alkyls [44]. The increased value of τ_{off} compared to τ_{on} resulted from the voltage dependence of the relaxation kinetics as described by Eq. (2). Thus, the reduced activity of $C_{18}R4$ compared to $C_{12}R4$ toward the CF efflux (Fig. 7) was not accounted for by a lower rate of $C_{18}R4$ backward translocation.

Relaxation experiments can be used for evaluating partition coefficients of hydrophobic ions γ. At $t=0$ equation 1 transforms into

$$I(0) = 2zF\gamma ck_i \sinh(zu/2) \quad (3)$$

thereby showing that the initial current is proportional to γ. The values of k_i for $C_{12}R4$ and $C_{18}R4$ should be similar because of the very close values of τ_{on} and τ_{off} for $C_{12}R4$ and $C_{18}R4$. Fig. 8 shows that $I(0)$ for $C_{12}R4$ was about three-fold higher than that for $C_{18}R4$ suggesting that approximately the same ratio holds for corresponding γ (Eq. (3)). This conclusion is surprising because $C_{18}R4$ has substantially longer hydrocarbon chain known to increase the octanol/water partition coefficient. Based on the literature data, it can be assumed that partitioning into lipid bilayers substantially differs from the octanol/water partitioning [45,46]. In particular, partitioning of alkylrhodamines into lipid bilayers exhibits saturation for compounds with hydrocarbon chains having more than twelve C-atoms.

It is worth noting that the records of transmembrane current shown in Fig. 8A do not have jumps and/or steps of the current, characteristic for pore formation in lipid bilayers. This is in line with our conclusion of carrier-type mechanism of anion translocation. Functioning of an ion carrier also suggests the induction of steady-

state transmembrane current upon the addition of the transporting anion. Fig. 8A (curve 2) shows an increase in the $C_{12}R4$-mediated steady-state current observed after the addition of palmitic acid (10 μM). Besides, palmitate induced acceleration of $C_{12}R4$-mediated current relaxation after the application of voltage (+100 mV, from $\tau_{on}=1.4$ s to $\tau_{on}=0.9$ s) which is also in line with carrier-type functioning. In fact, the current relaxation of $C_{12}R4$ is determined not only by the translocation rate constant k_i but also by back-translocation of its complex with palmitate. The phenomenon of current relaxation acceleration upon the addition of permeating ions was observed previously in experiments with the proton carrier CCCP [47,48] and the potassium ion carrier valinomycin [49,50]. With the $C_{18}R4$-mediated current, the effect of palmitate on the current relaxation pattern was approximately the same (Fig. 8B) as in the case of $C_{12}R4$, i.e. the similar effect on the steady-state current and the acceleration of the kinetics was observed (a decrease of τ_{on} from 1.4 s to 0.9 s). Therefore, the activity of long-chain alkylrhodamine on the planar bilayer lipid membrane was not suppressed with respect to medium-chain alkylrhodamines in contrast to the liposomal system (Fig. 7).

Another reason for the reduced activity of long-chain alkylrhodamines in liposomal membranes could be a low rate of partitioning and redistribution of the carriers between different vesicles. In fact, the addition of liposomes led to a rapid decrease in $C_{12}R1$ fluorescence at low liposome concentration, and subsequent rapid increase in the fluorescence was observed after the addition of high liposome concentration (Fig. 9A). However, the changes in the fluorescence in the case of $C_{16}R1$ proceeded in the timescale of tens of minutes (Fig. 9B). Fig. 9C shows the dependence of the steady-state fluorescence of different alkylrhodamines on the concentration of liposomes. The decrease in the fluorescence at low lipid concentration apparently resulted from self-quenching due to an increase in the local cation concentration upon binding to the membrane surface as shown for $C_{18}R4$ [20]. This conclusion is in agreement with the fluorescence increase observed at higher lipid concentrations (Fig. 9C), which may be explained by redistribution of rhodamines leading to dissociation of rhodamine stacking aggregates. The minimum level of fluorescence at an intermediate lipid concentration correlated with the alkyl chain length of the alkylrhodamines and obviously was associated with better binding ability of more hydrophobic compounds. The fluorescence of $C_{16}R1$ was substantially quenched even in the absence of liposomes, this effect being apparently due to formation of $C_{16}R19$ micelles.

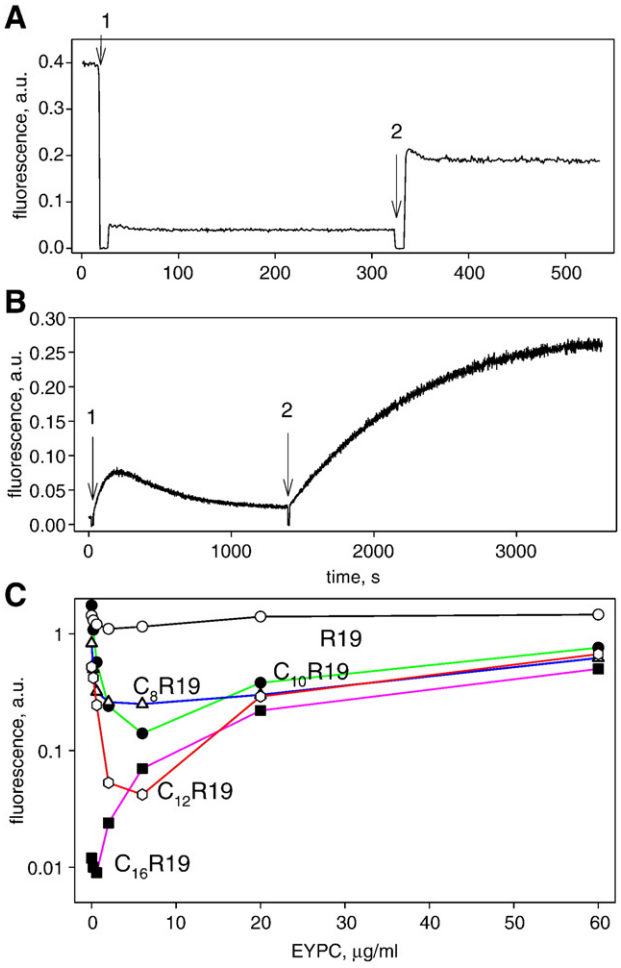

Fig. 9. Effect of addition of EYPC liposomes (addition 1, 5 μg/ml; addition 2, 15 μg/ml) on fluorescence of 1 μM $C_{12}R1$ (panel A) and 1 μM $C_{16}R1$ (panel B). The solution was 10 mM Tris, 100 mM KCl, pH 7.4, $\lambda_{ex}=530$ nm, $\lambda_{em}=560$ nm. (C) Emission intensity at 560 nm (excitation at 530 nm) of different alkylrhodamines as a function of EYPC concentration.

4. Discussion

Thus, it has been shown in the present work that SkQ1 and other penetrating cations are able to transport organic anions across lipid membranes due to the presence of a cationic group capable of permeating through membranes and forming complexes with the anions. It has been shown previously that negative hydrophobic ions can facilitate transport of hydrophobic cations by formation of electrically neutral complexes in lipid membranes [51]. The nature of these complexes is still unclear. In the case of CF transport, the possible stoichiometry of the carrier to CF was 2:1. This complex can be stabilized by electrostatic as well as hydrophobic interactions, the contribution of which can differ in aqueous solution and in the membrane. According to recent molecular dynamics simulations done in our group, application of adaptive biasing force to $C_{12}TPP$ results in a situation when its charged headgroup penetrated into the lipid phase and the carboxyl group of palmitate followed the movement of $C_{12}TPP$ through the membrane core, usually remaining at a distance of 4–6 Å apart. However, occasionally the distance increases up to 8–10 Å without irreversible dissociation of the ion pair [34].

Several lines of evidence in favor of the carrier mechanism of transport can be summarized as follows: (1) CF transport by penetrating cations was not accompanied by unspecific leakage of liposomal membranes (glucose or ATP leakage, Fig. 2). (2) Direct interaction between penetrating cations and different organic anions was shown (Fig. 3). (3) Competition between different anions for the penetrating cation-mediated transport was shown (Fig. 6). (4) The records of transmembrane current across planar lipid bilayers shown in Fig. 8 do not have jumps and/or steps of the current, characteristic for pore formation in lipid bilayers. (5) Carrier hypothesis predicts the appearance of steady-state current across planar bilayers upon the addition of palmitic acid anions. This prediction was in fact confirmed in our experiments (Fig. 8).

A study of transport selectivity of the carriers toward different organic anions was beyond the scope of the present work. It was shown, however, that some anions can be transported (CF, SRB, calcein) by these carriers, while another cannot (ATP). It should be stressed that inhibition of the transport of CF by several hydrophobic anions (Fig. 6) can hardly be used as a criterion of the carrier specificity of our cations. In fact, high carrier activity requires intermediate affinity of a substrate to a carrier, and the activity decreases if the affinity is too high [52]. By contrast, competition of some particular anion is proportional to the affinity to the carrier. ATP was shown to change substantially the absorption spectrum of $C_{12}R1$, suggesting the binding of ATP to the carrier. Probably the ATP molecule is too hydrophilic to be transported through the interior of the lipid membrane even in a complex with highly hydrophobic $C_{12}R1$.

Mitochondria-targeted antioxidants composed of hydrophobic delocalized cations linked to antioxidant quinone moieties produce a number of health benefitting effects by protecting cells and organisms from oxidative stress [6–8,10]. Anion-transport activity shown in the present work may be important for the choice of a particular compound for preclinical trials. The distribution of organic anions, which are typical cellular metabolites, between different compartments is crucial for correct cell functioning. Besides, some hormones and mediators are good candidates for substrates of the transport mediated by lipophilic penetrating cations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamem.2010.05.018.

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