J Membrane Biol (2008) 224:9–19
DOI 10.1007/s00232-008-9124-6

Kinetic Analysis of Permeation of Mitochondria-Targeted Antioxidants Across Bilayer Lipid Membranes

Tatyana I. Rokitskaya · Sergey S. Klishin · Inna I. Severina · Vladimir P. Skulachev · Yuri N. Antonenko

Received: 3 June 2008 / Accepted: 7 August 2008 / Published online: 20 September 2008

Abstract Mitochondria-targeted antioxidants consisting of a quinone part conjugated with a lipophilic cation via a hydrocarbon linker were previously shown to prevent oxidative damage to mitochondria in vitro and in vivo. In the present work, we studied the permeation of a series of compounds of this type across a planar bilayer phospholipid membrane. For this purpose, relaxation of the electrical current after a voltage jump was measured. With respect to the characteristic time of the relaxation process reflecting the permeation rate, hydrophobic cations can be ranked in the following series: 10(plastoquinonyl) decylrhodamine 19 (SkQR1) > 10-(6′-plastoquinonyl) decyltriphenylphosphonium (SkQ1) > 10-(6′-methylplastoquinonyl) decyltriphenylphosphonium (SkQ3) > 10-(6′-ubiquinonyl) decyltriphenylphosphonium (MitoQ). Thus, the permeation rate increased with (1) an increase in the size of the hydrophobic cation and (2) an increase in hydrophobicity of the quinone moiety. SkQ1 containing plastoquinone was shown to be more permeable through the membrane compared to MitoQ containing ubiquinone, which might be the reason for more pronounced beneficial action of SkQ1 in vitro and in vivo. The above approach can be recommended for the search for new antioxidants or other compounds targeted to mitochondria.

T. I. Rokitskaya (✉) · S. S. Klishin · I. I. Severina · V. P. Skulachev · Y. N. Antonenko
A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119992, Russia
e-mail: rokitskaya@genebee.msu.ru

T. I. Rokitskaya · S. S. Klishin · I. I. Severina · V. P. Skulachev · Y. N. Antonenko
Mitotechnology LLC, Moscow, Russia

Keywords Hydrophobic cation · Translocation rate · Antioxidant · Current relaxation · Bilayer lipid membrane

Introduction

Hydrophobic ions with delocalized charge represent an interesting class of molecules capable of permeating through the lipid part of cellular membranes in contrast to other charged molecules and inorganic ions (Liberman et al. 1969; LeBlanc 1969; Liberman and Skulachev 1970). They are used for measuring transmembrane electrical potential of subcellular organelles such as mitochondria (Grinius et al. 1970; Bakeeva et al. 1970; Rottenberg 1984). The mechanism of permeation of hydrophobic ions has been studied most intensively with tetraphenylborate, dipicrylamine (DPA), and some analogues of these molecules (Ketterer et al. 1971; Andersen and Fuchs 1975; Benz 1988; Melikyan et al. 1996; Zimmermann et al. 2008) by measuring relaxation of the electrical current across a bilayer lipid membrane (BLM) after applying a voltage. It is generally accepted that the rate constant of translocation of hydrophobic ions through a bilayer membrane is determined by the free energy profile near the center of the membrane. In the absence of surface potentials, the total free energy of transferring a hydrophobic ion from solution to a given position in the membrane can be approximated as $\Delta G^0 = \Delta G^0_{Born} + \Delta G^0_{Dipole} + \Delta G^0_{Hydro}$ (Flewelling and Hubbell 1986b), where ΔG^0_{Born} is the electrostatic "image" energy, which is positive for all ions (negative or positive) and is inversely proportional to the ionic radius; ΔG^0_{Hydro} is the hydrophobic energy of attraction; and having opposite signs for positive and negative ions, ΔG^0_{Dipole} emerges from the interaction of the ion charge with the membrane dipole potential and determines much higher free energy profile in

the membrane core for cations than for anions of similar structure. Therefore, the rate constant of translocation for anions greatly exceeds that for cations (10^4-fold and even more) (Flewelling and Hubbell 1986a; Pickar and Benz 1978).

In addition to slower permeation, hydrophobic cations exhibit substantially weaker binding to membranes than structurally similar anions (Flewelling and Hubbell 1986a). This apparently accounts for the appearance of only a few publications about the permeation of cations through lipid bilayers. In one of these, the translocation of the cationic fluorescent dyes octadecylrhodamine (R18) and indocarbocyanine (DiI) from one monolayer of a phospholipid bilayer membrane to the other was monitored by membrane current relaxation and fluorescent quenching measurements, and the estimated time constants were about 10^{-1} s (Melikyan et al. 1996). The translocation rate constant, k_i, of the spin-labeled tetraphenylphosphonium in liposomes was estimated by measuring time-dependent changes in the electron paramagnetic resonance spectrum, $7.4 \cdot 10^{-4}$ s^{-1} (Cafiso & Hubbell 1982). At the same time, tetraphenylphosphonium (TPP^+) was shown to increase the membrane conductivity (Liberman and Topaly 1969; Severina et al. 2007).

The mitochondrial interior is the only intracellular compartment negatively charged relative to its environment (i.e., the cytosol); therefore, on entering the cell, penetrating cations will be specifically accumulated within mitochondria. It was proposed that penetrating cations can be used by mitochondria as "electric locomotive molecules" for accumulation of uncharged substances attached to these cations (Severin et al. 1970). Recently, the principle of the "electric locomotive molecule" was used by M. P. Murphy for the addressed delivery of antioxidants, namely, vitamin E (Smith et al. 1999) and ubiquinone (Kelso et al. 2001), into mitochondria. So-called MitoQ (10-[6′-ubiquinonyl] decyltriphenylphosphonium), composed of ubiquinone and decyltriphenylphosphonium cation, seemed to be promising. In fact, MitoQ was shown to be accumulated and reduced by mitochondria and to protect them (as well as cell cultures) against oxidative stress (Kelso et al. 2001, 2002; Murphy and Smith 2007; Smith et al. 1999; James et al. 2005; Jauslin et al. 2003; Saretzki et al. 2003). We have confirmed the data of Murphy and coworkers and found that, at least under our conditions, the window between anti- and pro-oxidant concentrations of MitoQ is narrow (Skulachev 2007). It was recently shown by our group that plastoquinone-based SkQ1 exhibited higher antioxidant activity than MitoQ in aqueous solution and in a lipid environment (Antonenko et al. 2008b). In a system of mitochondria, the window between anti- and pro-oxidant activity of SkQ1 was shown to be substantially broader (Skulachev 2007; Antonenko 2008a, b).

Here, we investigated the permeation kinetics of antioxidant hydrophobic cations through a planar BLM for a series of structurally related compounds shown in Fig. 1. The process of permeation is an important step in the action of mitochondria-targeted antioxidants since the compound must be delivered to mitochondria in advance to exert antioxidant activity. Triphenylphosphonium cation was conjugated with a 10-carbon linker (C_{10}) to plastoquinone (SkQ1), methylplastoquinone (SkQ3) or ubiquinone (MitoQ). In SkQR1, the rhodamine 19 cation was linked through a C_{10} bridge to plastoquinone. For comparison, dodecyltriphenylphosphonium (C_{12}TPP) and R18 lacking redox groups were employed. Structure dependence of the estimated rate constants of translocation across the interface (k) and the interior of the membrane (k_i) for these compounds is discussed.

Materials and Methods

BLMs were formed from a 2% solution of *Escherichia coli* lipid extract, diphytanoylphosphatidylcholine (DPhPC) or a mixture of DPhPC and diphytanoyl phosphatidylglycerol (DPhPG) (all lipids were from Avanti Polar Lipids, Alabaster, AL) in *n*-decane on a hole in a Teflon partition separating two compartments of a chamber containing aqueous buffer solutions (Mueller et al. 1963). A chamber with a 0.8-mm-diameter hole was used in the current-relaxation experiments. Hydrophobic cations were added from stock solutions in ethanol into the bathing solutions on both sides of the BLM and routinely incubated for at least 10 min with constant stirring. MitoQ, SkQ1, SkQ3, SkQR1 and C_{12}TPP were synthesized by Drs. G. Korshunova and N. Sumbatyan in our group as described elsewhere (Skulachev 2007; Antonenko et al. 2008a). R18 was from Fluka (Buchs, Switzerland). In most of the experiments, the solution was 100 mM KCl, 10 mM Tris, 10 mM morpholinoethanesulfonic acid (MES, pH 7.0) or 10 mM KCl, 2 mM Tris, 2 mM MES (pH 7.0). All experiments were carried out at room temperature (23–25°C).

Electric currents (I) were recorded under voltage-clamp conditions. Voltage was applied to BLMs with Ag-AgCl electrodes placed directly into the experimental chamber. The current measured by means of a patch-clamp amplifier (OES-2; OPUS, Moscow, Russia) was digitized using an NI-DAQmx (National Instruments, Austin, TX) and analyzed with a personal computer, using WinWCP Strathclyde Electrophysiology Software designed by J. Dempster (University of Strathclyde, UK; http://spider.science.strath.ac.uk/sipbs/page.php?page=software_ses). In the current-relaxation experiments the voltage was switched

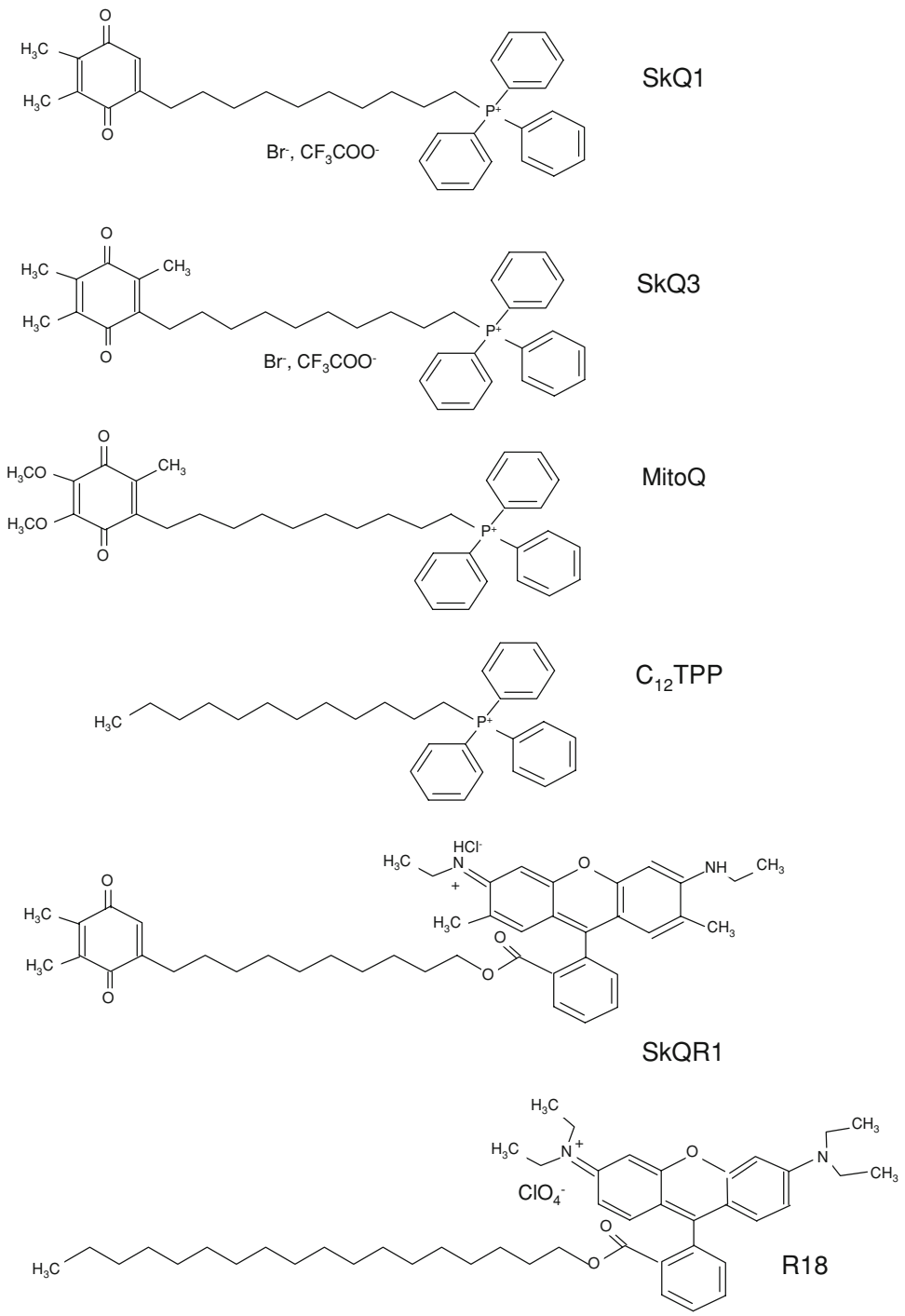

Fig. 1 Chemical structures of hydrophobic cations studied in the present work

from zero to some particular value of U (usually > 100 mV) at $t = 0$ and the current across the membrane (I[t]) started to decrease from an initial level, $I(0)$, to a steady-state level, $I(\infty)$. At the beginning of each experiment we recorded capacitance response of an unmodified membrane (a control recording of the current after a voltage jump). The recording in the presence of a hydrophobic cation was analyzed after subtraction of the control recording.

Results and Discussion

The system was incubated for 10 min with constant stirring after addition of a hydrophobic cation to the membrane-bathing aqueous solution to complete the binding of the cation to the BLM. The current across the membrane, I(t), which was maximal at the first moment after application of voltage U (usually > 100 mV), spontaneously decreased in

time from an initial level $I(0)$ to a steady-state level $I(\infty)$ (the current-relaxation process). This relaxation was accounted for by the redistribution of the cations between the two half-membrane leaflets after application of the voltage. Figure 2 shows the current relaxation after a voltage jump of $U = 150$ mV for six different hydrophobic cations taken at 0.5 μM concentration. Figure 2 also shows monoexponential fits of the experimental curves with $\tau = 7.5$ s (SkQR1), 36 s (SkQ1), 70 s (MitoQ), 47 s (SkQ3), 24 s (C_{12}TPP) and 0.38 s (R18). R18 was tested to compare our data with previously published results (Melikyan et al. 1996). Rather good monoexponential approximations were obtained in all cases. Interestingly, current relaxation was absent in the case of TPP^+ cation at concentrations up to 90 μM, although the membrane conductance increased to $4 \cdot 10^{-8}$ S/cm^2 (data not shown). Obviously, this resulted from low membrane affinity of TPP^+ and/or low value of k_i (Cafiso and Hubbell 1982). It should be noted that the addition of quinone-containing hydrophobic cations at micromolar concentrations led to membrane destabilization, especially at high voltages, which limited the resolution of our measurements.

The time course of the current across a BLM after application of voltage U at time $t = 0$ was shown to be a monoexponential curve (Ketterer et al. 1971):

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

and

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

where $u = \frac{U}{RT/F}$, c is the concentration of hydrophobic ions in aqueous solution, z is the valence of the lipid-soluble ion and γ is a ratio of surface and bulk concentrations of the ion (adsorption constant). The ratio of the initial and stationary currents should be

$$\frac{I(0)}{I(\infty)} = \frac{2k_i\cosh(zu/2) + k}{k} \quad (3)$$

The relaxation of the current was most pronounced in the case of SkQR1, exhibiting high amplitude and velocity. Figure 3a shows an example of a recording of the electrical current for SkQR1. The relaxation after switching off the potential can be called the "off" response. Figure 3b shows the relaxations at different U values ("on" responses) and the current relaxation after switching off the voltage to zero level ("off" responses). Amplitudes of "on" and "off" responses were similar in the case of $U = 50$ mV, whereas at $U > 100$ mV the amplitude of the "on" responses exceeded considerably that of the "off" responses. Figure 3c, d shows the dependence of τ and $I(0)/I(\infty)$ on the value of U for SkQR1. The magnitude of τ decreased with the increase in U, while $I(0)/I(\infty)$ increased with U. These data are in qualitative agreement with Eqs. 2 and 3, where the term depending on U, i.e., $k + 2k_i\cosh(zu/2)$, was in the denominator and the numerator, respectively.

Figure 4 shows similar data for SkQ1 having the TPP^+ group instead of rhodamine 19 (Fig. 1). To increase the signal-to-noise ratio, higher U values were applied (125, 150, 175 and 200 mV) in these experiments. The "off" responses approached the baseline for the voltages used except for $U = 200$ mV (curve 5 in Fig. 4a), showing that the "off" response was smaller in the case of SkQ1 compared to SkQR1. Again, the dependence of τ and $I(0)/I(\infty)$ on the value of U (Fig. 4b, c) was in qualitative agreement with Eqs. 2 and 3.

The studied hydrophobic cations (SkQR1, SkQ1, SkQ3 and MitoQ) increased the steady-state current leading to low values of $I(0)/I(\infty)$ (Figs. 2–4). These data differed from the results obtained for hydrophobic anions, where the value of $I(0)/I(\infty)$was about 100 or even higher (Andersen and Fuchs 1975). Equation 3 suggests that a low value of $I(0)/I(\infty)$requires $k_i < k$, which means that the limiting step of the whole process is the translocation of the cation from one surface of the membrane to the other. This conclusion also allows us to explain the differences in the amplitudes of the "on" and "off" responses. In fact, the translocation rate constant depends exponentially on the value of U, i.e. $k_i' = k_i \cdot \exp(\frac{zFU}{2RT})$, while the escape constant to the aqueous phase, k, is voltage-independent. At high voltages, k_i' exceeds k and a substantial portion of bound hydrophobic cations translocates to the other surface. The "off" response assumes $U = 0$ mV, and we have again $k_i' < k$, which favors release of the cation to the aqueous phase rather than translocation across the membrane.

The dependence of τ and $I(0)/I(\infty)$ on the value of U (Figs. 3b, c and 4b, c and other experiments) was fitted with Eqs. 2 and 3 as solid lines and corresponding values of k_i and k are summarized in Table 1. The table also contains the constants for SkQ3 and MitoQ. In agreement with the above consideration, the values of k_i were about 30 times lower than k in the case of SkQ1 and SkQ3, while the ratio of these constants was substantially lower for SkQR1 (about 10). The differences in the ratio of k_i and k for SkQR1 and SkQ1 can explain the differences in their "on" and "off" responses (Figs. 3a, 4a). The values of k for the compounds studied were substantially less than those of tetraphenylborate and DPA (Brock et al. 1981; Junges and Kolb 1983).

Figure 5 shows the dependence of membrane steady-state conductance on the concentration of SkQ1, SkQ3 and MitoQ. The conductance scales the concentration in agreement with Eq. 1, which gives the value of $\lambda_\infty = \frac{z^2F^2}{RT}\gamma \cdot c\frac{k \cdot k_i}{2k_i + k}$ in the limit of $t \to \infty$. The reduced conductance of SkQ3 can be associated with the low value of k_i

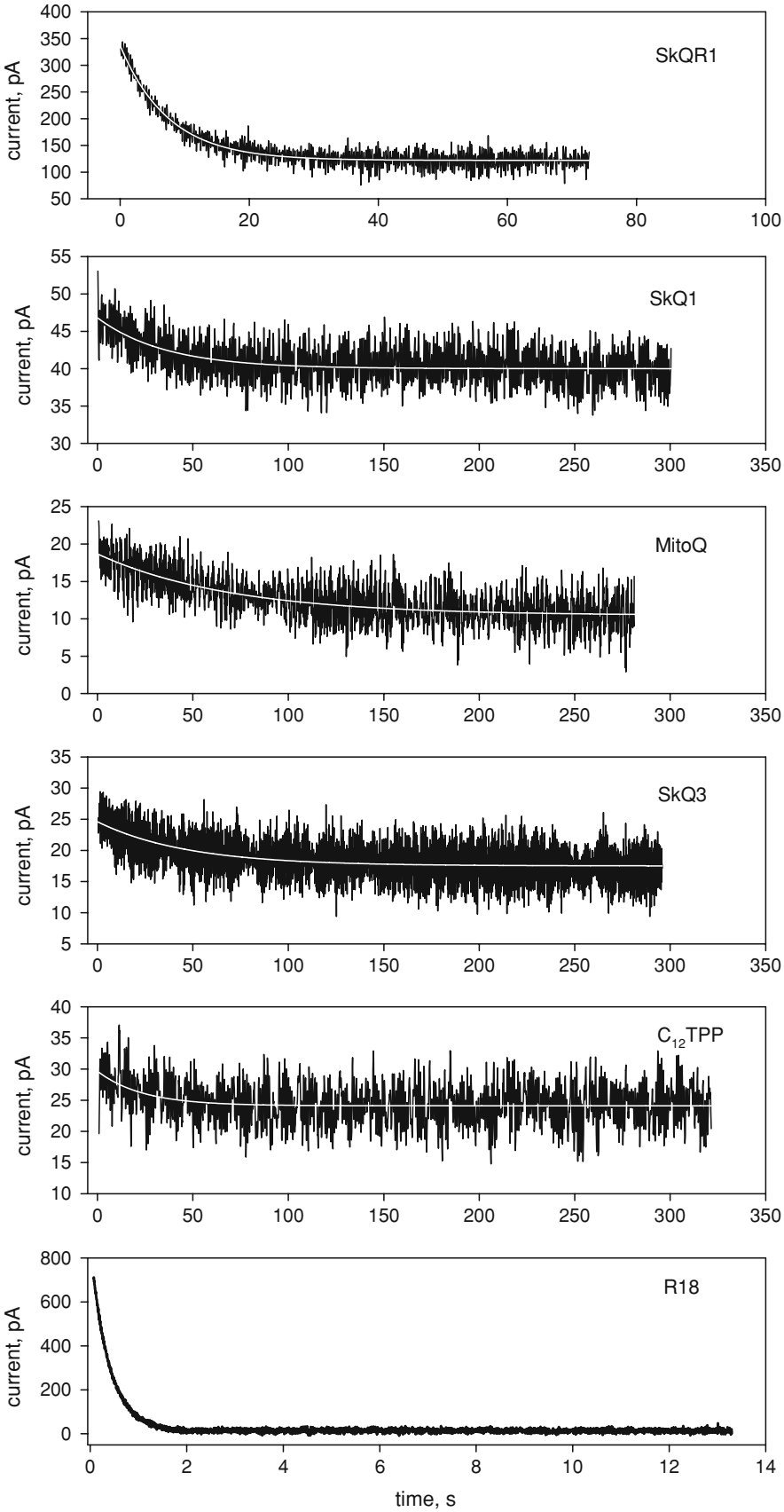

Fig. 2 Time courses of electrical current after application of a voltage jump of $U = 150$ mV (at $t = 0$, i.e., "on" response) in the presence of a 0.5 μM concentration of different hydrophobic cations (SkQR1, SkQ1, MitoQ, SkQ3, C_{12}TPP and R18) and their best fits by a monoexponentinal function with $\tau = 7.5$ s (SkQR1), $\tau = 36$ s (SkQ1), $\tau = 70$ s (MitoQ), $\tau = 47$ s (SkQ3), $\tau = 24$ s (C_{12}TPP) and $\tau = 0.38$ s (R18). Planar bilayers were formed from *E. coli* lipids. The solution was 10 mM MES, Tris, 100 mM KCl (pH 7.0)

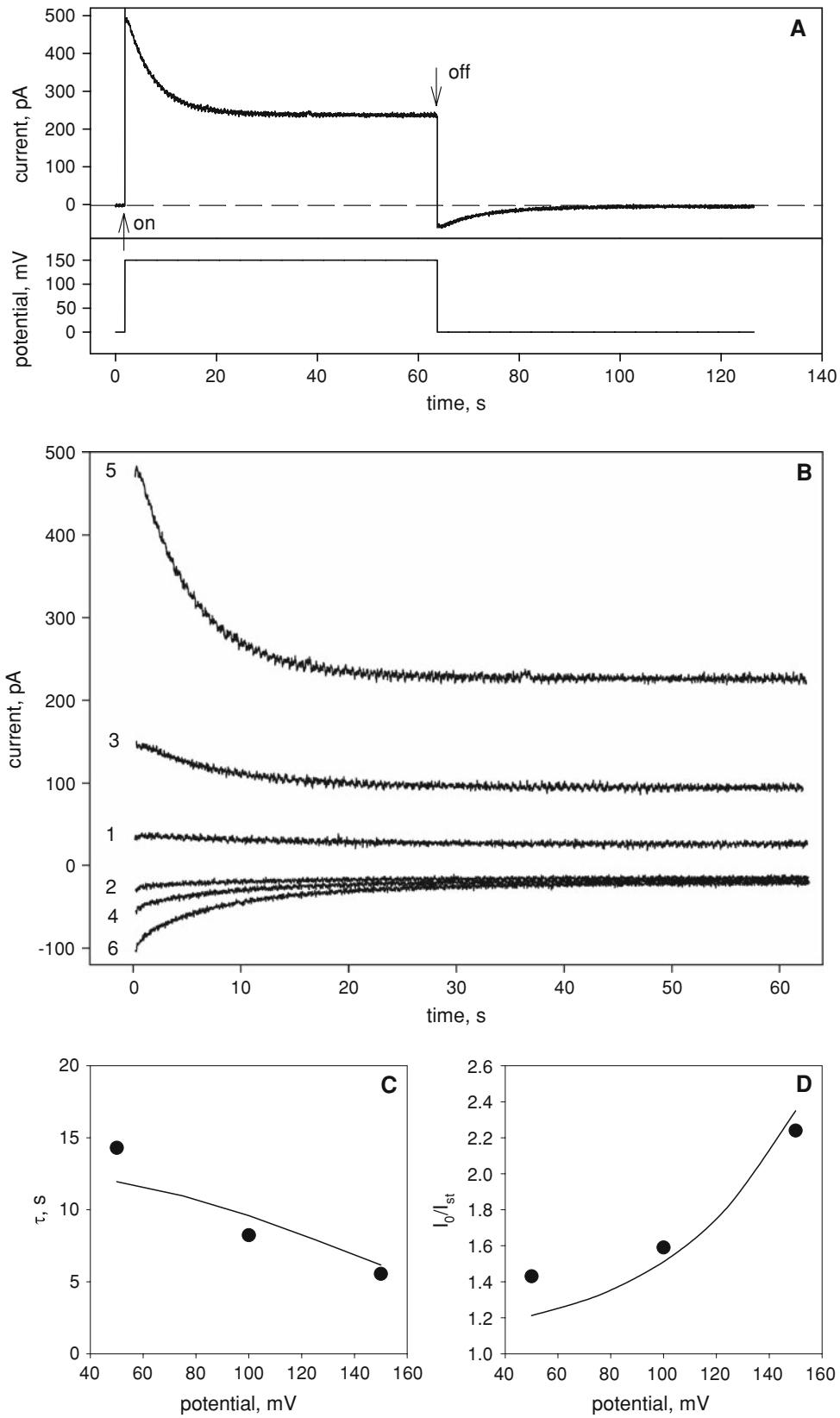

Fig. 3 (**a**) An example of a recording of the electric current (*top trace*) for SkQR1 in response to applied voltage (*bottom trace*). (**b**) "On" and "off" responses after voltage jumps of $U = 50$ m*V* (curves *1* and *2*), 100 m*V* (*3* and *4*) and 150 m*V* (*5* and *6*) in the presence of 0.5 μM SkQR1. Other conditions were the same as in the legend to Fig. 2. (**c**, **d**) Voltage dependence of the characteristic time τ (**c**) and the ratio I_0/I_∞ (**d**). *Solid curves* are best fits according to Eqs. 2 and 3

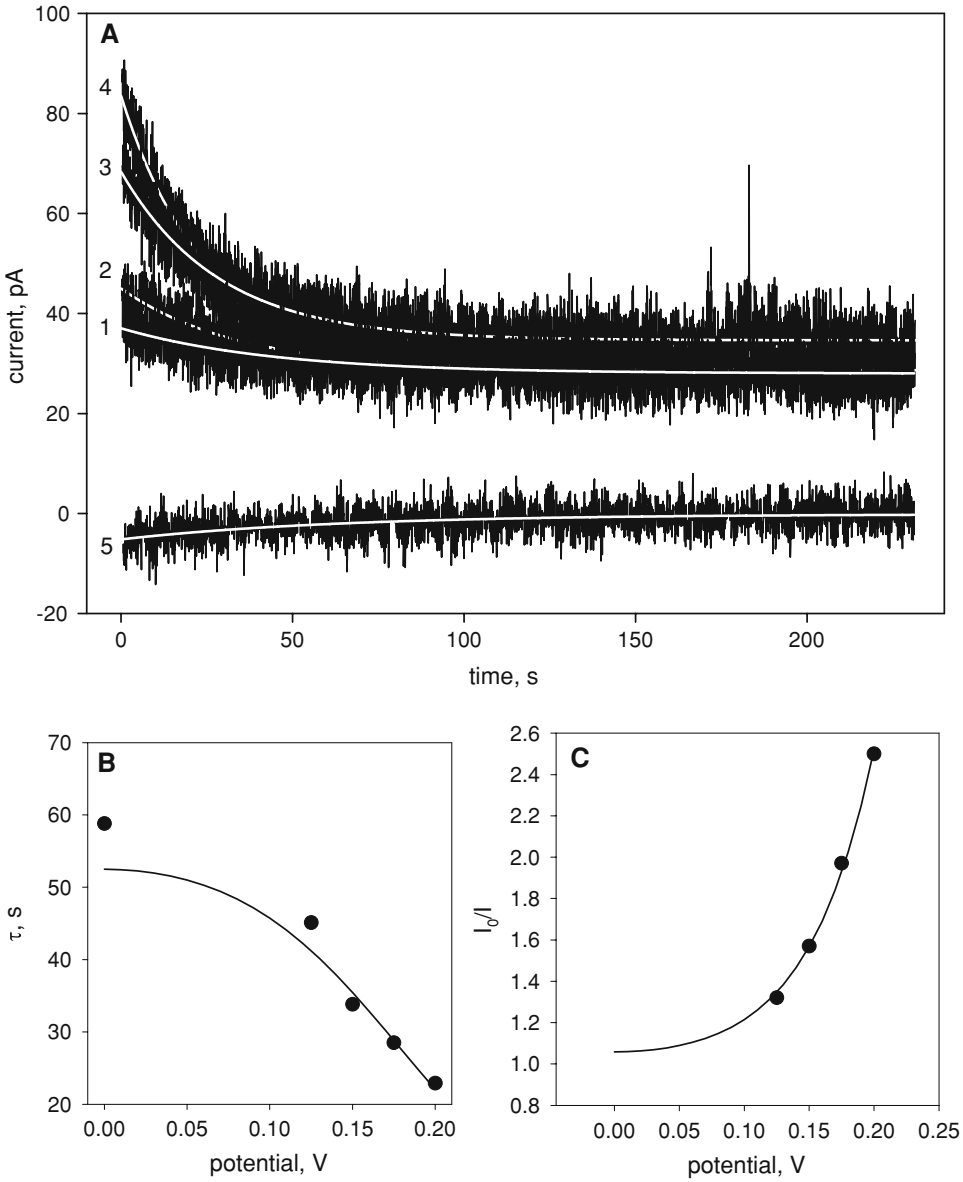

Fig. 4 (**a**) "On" responses after voltage jumps of $U = 125$ mV (curve *1*), 150 mV (*2*), 175 mV (*3*) and 200 mV (*4*) and "off" response after $U = 200$ mV (*5*) in the presence of 0.5 μM SkQ1. *White lines* are best fits by a monoexponential function with $\tau = 45.1$ s (*1*), $\tau = 33.8$ s (*2*), $\tau = 28.5$ s (*3*), $\tau = 22.9$ s (*4*) and $\tau = 58.5$ s (5). Planar bilayers were formed from *E. coli* lipids, and the buffer was 2 mM MES, Tris, 10 mM KCl (pH 7.0). (**b**, **c**) Voltage dependence of the characteristic time τ (**b**) and the ratio I_0/I_∞ (**c**)

Table 1 Translocation rate constants through the membrane barrier (k) and the interface (k_i) and adsorption constant (γ) for different lipophilic cations

	k (s^{-1})	k_i (s^{-1})	γ (cm)
SkQ1	$(1.8 \pm 0.1) \cdot 10^{-2}$	$(5.3 \pm 0.3) \cdot 10^{-4}$	$2 \cdot 10^{-2}$
SkQ3	$(1.8 \pm 1.0) \cdot 10^{-2}$	$(1.8 \pm 0.8) \cdot 10^{-4}$	$3 \cdot 10^{-2}$
SkQR1	$(6.9 \pm 0.9) \cdot 10^{-2}$	$(4.8 \pm 0.6) \cdot 10^{-3}$	$1.6 \cdot 10^{-2}$
MitoQ	$(9.5 \pm 2) \cdot 10^{-3}$	$(2.7 \pm 0.4) \cdot 10^{-4}$	$1.6 \cdot 10^{-2}$

(Table 1). Besides, the cation conductance depends on the adsorption constant γ. The values of γ were estimated using Eq. 1 for $I(0)$ and are shown in Table 1 for SkQ1, SkQ3, SkQR1 and MitoQ. The compounds can be ranked in the following series of the decrease of their membrane affinity: SkQ3 > SkQ1 > MitoQ ~ SkQR1. Our data suggested

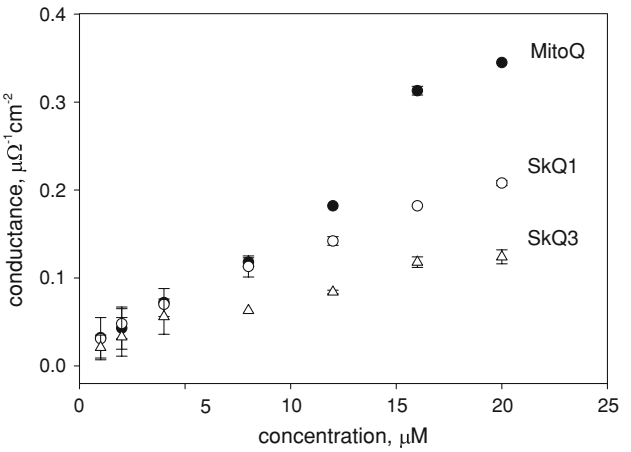

Fig. 5 Dependence of membrane conductance on the concentration of hydrophobic cation

that MitoQ has less membrane affinity than SkQ1 and SkQ3, in agreement with the higher hydrophobicity of plastoquinone and methylplastoquinone compared to ubiquinone (Rich & Harper, 1990).

In our voltage-jump experiments, we usually used hydrophobic cations at a concentration of 0.5 μM, which was far from the saturating concentration, as seen from Fig. 5. The fact that SkQ1 and SkQ3 can generate electrical potential across a planar membrane close to the theoretical (Nernstian) limit (Antonenko et al. 2008a) excludes the possibility of an increase in the electrical current due to nonspecific membrane perturbation. However, the diffusion potential of MitoQ was lower than Nernstian, especially at high concentrations, which indicated membrane leakage resulting in additional conductance shunting the MitoQ diffusion potential. This may explain why steady-state conductance was higher with MitoQ than with SkQ1 (Fig. 5).

The studied hydrophobic cations contained a quinone unit that can be in an oxidized or reduced form. All of the above data were obtained for the oxidized form in which the cations were synthesized. To obtain a reduced form of SkQ1, its water/ethanol (9:1) solution was incubated with $NaBH_4$. The voltage-jump experiment in the presence of reduced SkQ1 showed current relaxation of low amplitude that was hardly detectable (Fig. 6). Steady-state conductance was also reduced. These data agree with a substantially lower partition coefficient of reduced forms of quinol compared to quinone (Jemiota-Rzeminska et al. 2001; Rich and Harper 1990).

The experiments described above were performed on membranes made of a total lipid extract of *E. coli*. To gain insight into the dependence of the current relaxation on the lipid composition, SkQR1-mediated relaxation was studied in membranes made of DPhPC with varying amounts of negatively charged lipid DPhPG. The value of τ did not depend on the DPhPG content (data not shown), while the parameter $I(0)/I(\infty)$increased considerably with the DPhPG content (Fig. 7). It is worth noting that total lipid of *E. coli* contains a high percentage of negatively charged lipids (about 15% phosphatidylglycerol and 10% cardiolipin). It is generally believed that the translocation rate constant k_i does not depend on the surface potential of the membrane, being a function of the free energy profile across the membrane (Mclaughlin 1977; Sokolov and Mirsky 2004). We think that the presence of DPhPG in the BLM decreased the constant k and increased γ, leading to an increase in the concentration of hydrophobic cations in BLM. It was shown that in fact the binding of 2-(4-dimethylaminostyryl)-1-ethylpyridinium to the membrane increased in the case of a negatively charged membrane compared to a neutral membrane (Sedgwick and Bragg 1993). The sensitivity of the parameters $I(0)/I(\infty)$ and τ to the change in the ratio of k/k_i can be calculated using Eqs. 2 and 3. Such a calculation shows that when the k/k_i ratio increased sevenfold, the value of τ increased 1.5-fold, while the parameter $I(0)/I(\infty)$ increased 4.5-fold. Thus, the parameter $I(0)/I(\infty)$ is more sensitive to the change in parameter k compared to τ, consistent with the experimental data of Fig. 7.

Another important parameter that is used in the analysis of current transients is the transferred electrical charge, Q (Andersen and Fuchs 1975; Melikyan et al. 1996). Analysis of the dependence of Q on the applied voltage indicates that the movement of hydrophobic ions is influenced by a

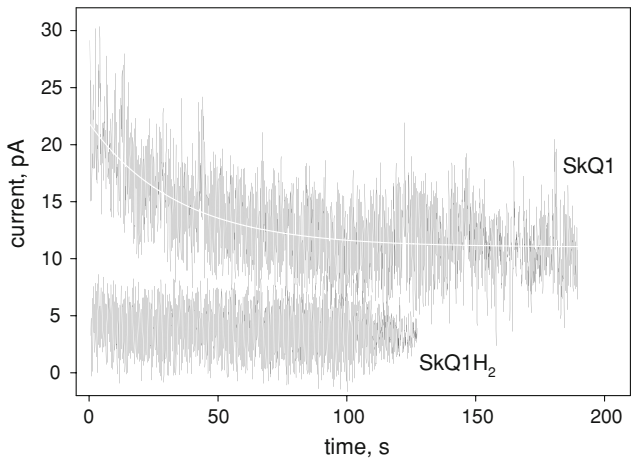

Fig. 6 "On" responses after voltage jumps of $U = 150$ mV in the case of 0.5 μM oxidized SkQ1 and reduced SkQ1 ($SkQ1H_2$). *White line* is the best fit by a monoexponential function with $\tau = 34.5$ s. Other conditions were the same as in the legend to Fig. 4

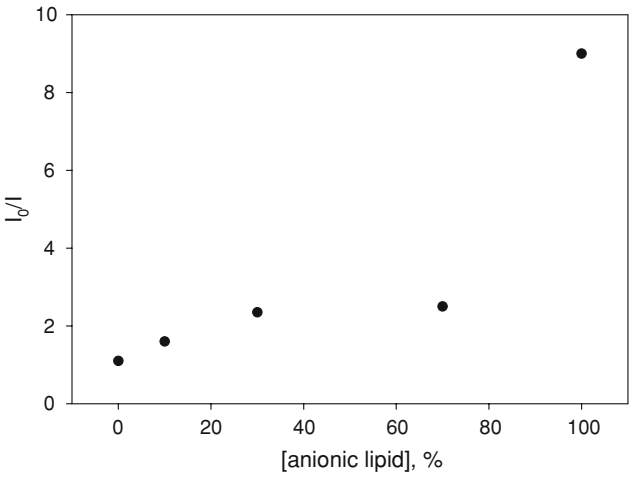

Fig. 7 Dependence of the ratio I_0/I_∞ on the anionic lipid content of DPhPG in the membrane-forming solution in the presence of 0.5 μM SkQR1 at $U = 150$ mV. Planar bilayers were formed from a mixture of lipids (DPhPC and DPhPG) with varying weight ratio; the solution was 10 mM MES, Tris, 100 mM KCl (pH 7.0)

certain fraction of the applied potential (β), which means that the ions are located at a certain distance from the membrane–solution interface. The dependence of Q on the applied voltage can be expressed as

$$Q = \tau \cdot (I(0) - I(\infty)) = 2zF\gamma ck_i \sinh(\beta zu/2) \frac{2k_i \cosh(\beta zu/2)}{(2k_i \cosh(\beta zu/2) + k)^2} \quad (4)$$

At $k = 0$ Eq. 4 transforms into

$$Q = 2zF\gamma ck_i \tanh(\beta zu/2) \quad (5)$$

This equation is used frequently to determine the value of β, thus estimating the location of the hydrophobic ion. It was shown that $\beta = 0.77$ for tetraphenylborate (Andersen and Fuchs 1975).

Figure 8a, b shows data on the voltage dependence of the transferred charge Q for R18 and SkQR1, respectively. The $Q(U)$ dependence for R18 was linear at low voltages and saturated at voltages higher than 100 mV, in agreement with previously published data (Melikyan et al. 1996). In contrast, the dependence for SkQR1 was strongly supralinear (Fig. 8b). This effect can be associated with the high value of k/k_i in the case of SkQR1. In fact, the data can be well fitted by Eq. 4 with $k/k_i = 7$ and $\beta = 0.76$. The experiment was carried out at T = 28°C in order to accelerate the relaxation process. The value of k/k_i at room temperature (23–25°C) was about 14 (Table 1), apparently due to the higher temperature sensitivity of k_i compared to k. The data for R18 were approximated by Eq. 5. It can be seen from Fig. 2 that $I_0 >> I_\infty$ and therefore $k_i >> k$. The fittings gave $\beta = 0.85$ for R18. The value of β is in good agreement with the published data (Melikyan et al. 1996), where β was estimated to be 0.85 for the case of DOPC/DOPE membranes. It can be concluded that the positively charged rhodamine part of the SkQR1 molecule is located somewhat deeper in the membranes compared to R18. However, the value of β was close to unity, suggesting predominantly surface localization of the cationic part of the SkQR1 molecule, which was in agreement with published data (James et al. 2007), where the localization of the TPP^+ part of MitoQ was studied.

Summarizing the current-relaxation data, hydrophobic cations can be ranked with respect to the characteristic time of the relaxation process and, hence, their permeation through the membrane in the following series: SkQR1 > SkQ1 > SkQ3 > MitoQ (Fig. 2). Table 1 shows that k_i of SkQR1 is about 10 times higher compared to SkQ1. These compounds differ in the cation moieties, namely, rhodamine 19 and TPP^+ in SkQR1 and SkQ1, respectively (Fig. 1). In the case of penetrating anions, k_i was shown to increase by two orders of magnitude with an increase in the effective radius of the compound differing for triphenylcyanoborate and tetraphenylborate (Benz 1988). The size

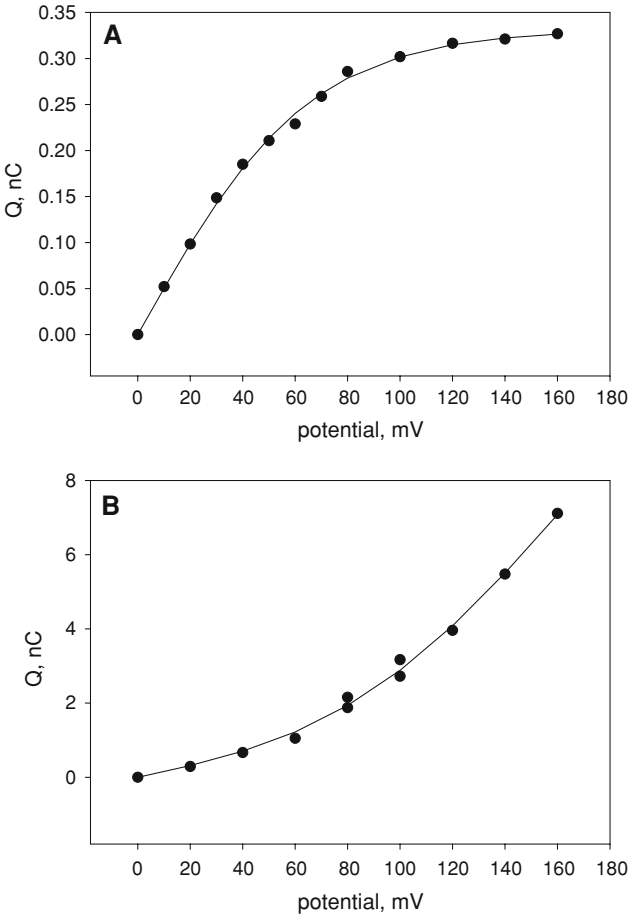

Fig. 8 Potential dependence of the transferred charge (*circles*) and fitting lines according to Eq. 4 for 0.5 μM R18 (**a**) and 0.5 μM SkQR1 (**b**) in the bathing solution

of rhodamine is obviously greater than that of TPP^+, which can explain the difference in the permeation ability of SkQR1 and SkQ1. As to electrically neutral molecules, their permeability decreases with the increase in the molecule size (Xiang and Anderson 1994; Walter and Gutknecht 1986). However, the electrostatic component of the free energy profile ΔG^0_{Born} decreases with the increase in the size of the molecule, which should facilitate the translocation (Benz 1988).

Our data show that small changes in the structure of a quinone unit of the compounds considerably affected their permeation across the membrane. The compounds $C_{12}TPP$, SkQ1, SkQ3 and MitoQ, which carry identical cationic moieties, differed in the permeation ability in the series $C_{12}TPP$ > SkQ1 > SkQ3 > MitoQ. It can be assumed that the membrane partition coefficient can significantly contribute to the rate of the translocation process because of the influence of ΔG^0_{Hydro} on the free energy profile of the molecule across the membrane. Consistent with this assumption, the partition coefficient of plastoquinone is higher compared to that of ubiquinone. Moreover, the

partition coefficient of oxidized quinone is higher than that of the fully reduced form (Jemiota-Rzeminska et al. 2001; Rich and Harper 1990), which can explain a strong decrease in the current relaxation of SkQ1 upon its reduction (Fig. 6). SkQ3 should be more hydrophobic compared to SkQ1 due to the presence of the methyl residue in the quinone moiety. However, the permeation of SkQ3 was slower than that of SkQ1. This suggests that hydrophobicity is not the only parameter affecting the transmembrane permeation of these compounds.

Two different mechanisms of permeation of molecules across the BLM are discussed in the literature, i.e., the solubility–diffusion theory and the flip–flop theory. The first mechanism which suggests the linear relationship between the rate of permeation and the partition coefficient on a logarithmic scale can be applied to comparatively small molecules lacking amphipathic properties (Orbach and Finkelstein 1980). The second mechanism that is discussed in relation to amphipathic compounds implies an inverse dependence on the partition coefficient. In fact, the rate of flip–flop of natural phosphatidylcholines and their lyso-forms was higher for the compounds carrying shorter hydrocarbon chains (Homan and Pownall 1988; Fujii et al. 1985). Based on the available experimental data, it is hardly possible at present to distinguish between the two mechanisms. The complex structure of the compounds having hydrophilic and hydrophobic parts favors the second mechanism, while the dependence of the permeation on the partition coefficient supports the first. Additional efforts involving more compounds are required to address this problem. The present work characterized the membrane permeation of several mitochondria-targeted antioxidants that are potentially important for pharmacology. It was shown, in particular, that SkQ1 is more permeable through the membrane compared to MitoQ, which can be a reason for its more pronounced protective action in vitro and in vivo (Skulachev 2007).

Acknowledgments We are grateful to Dr. Boris Chernyak, Dr. Lev Yaguzhinsky and Dr. Vadim Tashlitsky for helpful discussions. We also thank Dr. Elena Kotova for critical reading of the manuscript. This work was supported by Russian Foundation for Basic Research grants 06-04-48523, 08-04-01731 and 09-04-91272; Mitotechnology LLC, M. V. Lomonosov Moscow State University; the Vol'noe Delo Foundation (grant N 99F-06); and Russian Ministry of Education and Science grant 5762.2008.4 "Leading Scientific Schools."

References

Andersen OS, Fuchs M (1975) Potential energy barriers to ion transport within lipid bilayers. Studies with tetraphenylborate. Biophys J 15:795–830

Antonenko YN, Avetisyan AV, Bakeeva LE, Chernyak BV, Chertkov VA, Domnina LV, Ivanova OYu, Izyumov DS, Khailova LS, Klishin SS, Korshunov SS, Korshunova GA, Kovaleva NA, Lyamzaev KG, Muntyan MS, Nepryakhina OK, Pashkovskaya AA, Pletjushkina OYu, Pustovidko AV, Rokitskaya TI, Ruuge EK, Saprunova VB, Severina II, Simonyan RA, Skulachev IV, Skulachev MV, Sumbatyan NV, Sviryaeva IV, Tashlitsky VN, Tikhomirova NK, Vassiliev YM, Vyssokikh MYu, Yaguzhinsky LS, Skulachev VP (2008a) Mitochondria-targeted derivative of plastoquinone as a tool to interrupt execution of an aging program. Biochemistry (Mosc) (in press)

Antonenko YN, Roginsky VA, Pashkovskaya AA, Rokitskaya TI, Kotova EA, Zaspa AA, Chernyak BV, Skulachev VP (2008b) Protective effects of mitochondria-targeted antioxidant SkQ in aqueous and lipid membrane environments. J Membr Biol 222:141–149

Bakeeva LE, Grinius LL, Jasaitis AA, Kuliene VV, Levitsky DO, Liberman EA, Severina II, Skulachev VP (1970) Conversion of biomembrane-produced energy into electric form. II. Intact mitochondria. Biochim Biophys Acta 216:13–21

Benz R (1988) Structural requirement for the rapid movement of charged molecules across membranes. Experiments with tetraphenylborate analogues. Biophys J 54:25–33

Brock W, Stark G, Jordan PC (1981) A laser-temperature-jump method for the study of the rate of transfer of hydrophobic ions and carriers across the interface of thin lipid membranes. Biophys Chem 13:329–348

Cafiso DS, Hubbell WL (1982) Transmembrane electrical currents of spin-labeled hydrophobic ions. Biophys J 39:263–272

Flewelling RF, Hubbell WL (1986a) Hydrophobic ion interactions with membranes thermodynamic analysis of tetraphenylphosphonium binding to vesicles. Biophys J 49:531–540

Flewelling RF, Hubbell WL (1986b) The membrane dipole potential in a total potential model. applications to hydrophobic ion interactions with membranes. Biophys J 49:541–552

Fujii T, Tamura A, Yamane T (1985) Trans-bilayer movement of added phosphatidylcholine and lysophosphatidylcholine species with various acyl chain lengths in plasma membrane of intact human erythrocytes. J Biochem 98:1221–1227

Grinius LL, Jasaitis AA, Kadziauskas YP, Liberman EA, Skulachev VP, Topali VP, Tsofina LM, Vladimirova MA (1970) Conversion of biomembrane-produced energy into electric form. I. Submitochondrial particles. Biochim Biophys Acta 216:1–12

Homan R, Pownall HJ (1988) Transbilayer diffusion of phospholipids: dependence on headgroup structure and acyl chain length. Biochim Biophys Acta 938:155–166

James AM, Cocheme HM, Smith RA, Murphy MP (2005) Interactions of mitochondria-targeted and untargeted ubiquinones with the mitochondrial respiratory chain and reactive oxygen species. Implications for the use of exogenous ubiquinones as therapies and experimental tools. J Biol Chem 280:21295–21312

James AM, Sharpley MS, Manas AR, Frerman FE, Hirst J, Smith RA, Murphy MP (2007) Interaction of the mitochondria-targeted antioxidant MitoQ with phospholipid bilayers and ubiquinone oxidoreductases. J Biol Chem 282:14708–14718

Jauslin ML, Meier T, Smith RA, Murphy MP (2003) Mitochondria-targeted antioxidants protect Friedreich ataxia fibroblasts from endogenous oxidative stress more effectively than untargeted antioxidants. FASEB J 17:1972–1974

Jemiota-Rzeminska M, Latowski D, Strzalka K (2001) Incorporation of plastoquinone and ubiquinone into liposome membranes studied by HPLC analysis The effect of side chain length and redox state of quinone. Chem Phys Lipids 110:85–94

Junges R, Kolb HA (1983) Noise analysis and relaxation experiments of transport of hydrophobic anions across lipid membranes at equilibrium and nonequilibrium. Biophys Chem 17:301–312

Kelso GF, Porteous CM, Coulter CV, Hughes G, Porteous WK, Ledgerwood EC, Smith RA, Murphy MP (2001) Selective targeting of a redox-active ubiquinone to mitochondria within

cells: antioxidant and antiapoptotic properties. J Biol Chem 276:4588–4596

Kelso GF, Porteous CM, Hughes G, Ledgerwood EC, Gane AM, Smith RA, Murphy MP (2002) Prevention of mitochondrial oxidative damage using targeted antioxidants. Ann N Y Acad Sci 959:263–274

Ketterer B, Neumcke B, Läuger P (1971) Transport mechanism of hydrophobic ions through lipid bilayer membranes. J Membr Biol 5:225–245

LeBlanc OH (1969) Tetraphenylborate conductance through lipid bilayer membranes. Biochim Biophys Acta 193:350–360

Liberman EA, Skulachev VP (1970) Conversion of biomembrane-produced energy into electric form IV. General discussion. Biochim Biophys Acta 216:30–42

Liberman EA, Topaly VP (1969) Permeability of biomolecular phospholipid membranes for fat-soluble ions. Biofizika 14: 452–461

Liberman EA, Topaly VP, Tsofina LM, Jasaitis AA, Skulachev VP (1969) Mechanism of coupling of oxidative phosphorylation and the membrane potential of mitochondria. Nature 222:1076–1078

Mclaughlin S (1977) Electrostatic potentials at membrane solution interfaces. Curr Top Membr Transport 9:71–144

Melikyan GB, Deriy BN, Ok DC, Cohen FS (1996) Voltage-dependent translocation of R18 and DiI across lipid bilayers leads to fluorescence changes. Biophys J 71:2680–2691

Mueller P, Rudin DO, Tien HT, Wescott WC (1963) Methods for the formation of single bimolecular lipid membranes in aqueous solution. J Phys Chem 67:534–535

Murphy MP, Smith RA (2007) Targeting antioxidants to mitochondria by conjugation to lipophilic cations. Annu Rev Pharmacol Toxicol 47:629–656

Orbach E, Finkelstein A (1980) The nonelectrolyte permeability of planar lipid bilayer membranes. J Gen Physiol 75:427–436

Pickar AD, Benz R (1978) Transport of oppositely charged lipophilic probe ions in lipid bilayer membranes having various structures. J Membr Biol 44:353–376

Rich PR, Harper R (1990) Partition coefficients of quinones and hydroquinones and their relation to biochemical reactivity. FEBS Lett 269:139–144

Rottenberg H (1984) Membrane potential and surface potential in mitochondria: uptake and binding of lipophilic cations. J Membr Biol 81:127–138

Saretzki G, Murphy MP, von Zglinicki T (2003) MitoQ counteracts telomere shortening and elongates lifespan of fibroblasts under mild oxidative stress. Aging Cell 2:141–143

Sedgwick EG, Bragg PD (1993) Mechanism of uptake of the fluorescent dye 2-(4-dimethylaminostyryl)-1-ethylpyridinium cation (DMP^+) by phospholipid vesicles. Biochim Biophys Acta 1146:113–120

Severin SE, Skulachev VP, Yaguzhinskii LS (1970) Possible role of carnitine in the transport of fatty acids through the mitochondrial membrane [in Russian]. Biokhimiia 35:1250–1253

Severina II, Vyssokikh MY, Pustovidko AV, Simonyan RA, Rokitskaya TI, Skulachev VP (2007) Effects of lipophilic dications on planar bilayer phospholipid membrane and mitochondria. Biochim Biophys Acta 1767:1164–1168

Skulachev VP (2007) A biochemical approach to the problem of aging: "megaproject" on membrane-penetrating ions. The first results and prospects. Biochemistry (Mosc) 72:1385–1396

Smith RA, Porteous CM, Coulter CV, Murphy MP (1999) Selective targeting of an antioxidant to mitochondria. Eur J Biochem 263:709–716

Sokolov VS, Mirsky VM (2004) Electrostatic potentials of bilayer lipid membranes: basic research and analytical applications. In: Thin layer electrochemical sensors and biosensors: technology and performance. pp 255–291

Walter A, Gutknecht J (1986) Permeability of small nonelectrolytes through lipid bilayer membranes. J Membr Biol 90:207–217

Xiang TX, Anderson BD (1994) The relationship between permeant size and permeability in lipid bilayer membranes. J Membr Biol 140:111–122

Zimmermann D, Kiesel M, Terpitz U, Zhou A, Reuss R, Kraus J, Schenk WA, Bamberg E, Sukhorukov VL (2008) A combined patch-clamp and electrorotation study of the voltage- and frequency-dependent membrane capacitance caused by structurally dissimilar lipophilic anions. J Membr Biol 221:107–121