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ARTICLE

Theoretical evaluation of a possible nature of the outer membrane potential of mitochondria

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Abstract A possibility of generation of the outer membrane potential in mitochondria has been suggested earlier in the literature, but the potential has not been directly measured yet. Even its nature, metabolic impact and a possible range of magnitudes are not clear, and require further theoretical and experimental analysis. Here, using simple mathematical model, we evaluated a possible contribution of the Donnan and metabolically derived potentials to the outer membrane potential, concluding that the superposition of both is most probable; exclusively Donnan origin of the potential is doubtful because unrealistically high concentrations of charged macromolecules are needed for maintaining its relatively high levels. Regardless of the mechanism(s) of generation, the maximal possible potential seems to be less than 30 mV because significant osmotic gradients, created at higher values, increase the probability of the outer membrane rupture. New experimental approaches for direct or indirect determination of true value of the outer membrane potential are suggested here to avoid a possible interference of the surface electrical potential of the inner membrane, which may change as a result of the extrusion of matrix protons under energization of mitochondria.

Keywords Cell biophysics · Channels in organelles · General theory · Organelle metabolism · Surface · Energetics

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Introduction

Earlier, it has been suggested that the energy flux from mitochondria to the cytoplasm might be modulated by voltage-dependent anion channels (VDAC) of the outer membrane of mitochondria (OM) (Liu and Colombini 1992a; Saks et al. 1993; Rostovtseva and Colombini 1997; Hodge and Colombini 1997; Vander Heiden et al. 2000; Lemeshko and Lemeshko 2000; Lemeshko 2002; Rostovtseva et al. 2005; Lemasters and Holmuhamedov 2006). One of the ways for such modulation, additional to the allosteric regulation of VDAC (Colombini 2004; Lemasters and Holmuhamedov 2006), requires generation of the OM potential by some mechanism(s), such as the Donnan equilibrium potential (DP) (Liu and Colombini 1992b, 1992c; Porcelli et al. 2005), or several steady-state metabolically dependent mechanisms according to which the OM potential might be generated due to the difference in the OM permeability to various charged metabolites (Lemeshko and Lemeshko 2000, 2004a, b), or as a part of the inner membrane (IM) potential of mitochondria directly applied to the OM through the electrogenic contact sites between the inner and the outer membranes (Lemeshko 2002).

The progress in understanding the nature and physiological role of the OM potential could highly benefit from careful measurements of the potential in different metabolic states. Intriguing experimental data, suggesting a generation of the OM potential in intact cells, were recently obtained by Porcelli et al. (2005). Using the human ECV304 cells, which have been transiently transfected with the enhanced yellow fluorescent protein (EYFP) to measure pH in the mitochondrial intermembrane space (IMS), the authors

concluded that the IMS's pH is lower than the pH in the cytoplasm by the value of 0.7. Taking into account that OM is highly permeable to protons, the authors calculated that this pH difference is equilibrated with the electrical potential of about –43 mV across the OM. The origin of this potential has been attributed to the Donnan electrochemical equilibrium, but a contribution of metabolically derived potential (MDP) cannot be excluded because inhibitors of mitochondrial metabolism caused complete equilibration of the IMS's pH with that in the cytoplasm (Porcelli et al. 2005). Such magnitude of change of the pH difference across the OM would not be expected for the Donnan potential. Also, in the case of EYFP attached to the IM, local pH at the outer surface of the IM might contribute to the measured pH difference between IMS and the cytoplasm.

In this work, we used simple mathematical model to evaluate possible contribution of the DP and MDP to the OM potential. The obtained data demonstrate that unrealistically high concentrations of charged macromolecules are required to generate DP of relatively high values. The calculations also demonstrate that the superposition of DP and MDP is most probable, as it was suggested earlier (Lemeshko and Lemeshko 2000, 2004a, b). Regardless of the mechanism(s) of generation, the physiological maximal value for the OM potential seems to be less than 30 mV because high osmotic gradients across the OM, created at higher values of the potential, significantly increase the probability of the OM rupture. We also demonstrate that in the case of indirect estimation of the OM potential, for example, an indicator of the IMS pH attached to the IM (Porcelli et al. 2005), the difference of pH between the IMS and the cytoplasm may be over-estimated by local pH near the IM as result of the change of the IM surface potential during energization/de-energization of mitochondria. Some new experimental approaches are suggested for more adequate measurements of the OM potential in living cells and isolated mitochondria.

Materials and methods

The liposomal model for evaluation of the Donnan and metabolically derived membrane potentials

The model is based on the earlier published model demonstrating the principle of generation of MDP (Lemeshko and Lemeshko 2000). In this case, inside the liposome we also include the macromolecules carrying a charge z to generate DP in addition to MDP (Fig. 1). Liposomal membrane is assumed to be freely permeable for K^+ and Cl^-. An enzyme E inside the liposome catalyzes an essentially irreversible reaction of conversion of the substrate S^- into the product P^-. Membrane permeability for S^- is significantly higher than for P^-, thus the steady-state MDP, minus inside, depending on the activity of the enzyme E, will be generated. The final membrane potential results from superposition of DP and MDP, while it is not a simple algebraic sum of both (see Appendix for complete mathematical description).

Theory for the surface potential of the inner mitochondrial membrane

Let us consider a hypothetical liposome formed by non-inverted IM placed in the medium of 125 mM KCl. The IM may be theoretically considered as a perfect impenetrable plane surface with uniformly smeared charge over it, following approximation used in the work (Xu and Loew 2003). In frames of Gouy–Chapman–Graham theory of the electrical double layer (Xu and Loew 2003), the surface potential ϕ_0 on the Helmholtz's layer of the IM depends on the surface charge density σ and can be calculated using Graham's equation:

$$\sigma = -\left\{2RT\varepsilon\sum_i C_{i,\infty}[\exp(-z_i\varphi_0 F/RT) - 1]\right\}^{\frac{1}{2}}, \qquad (1)$$

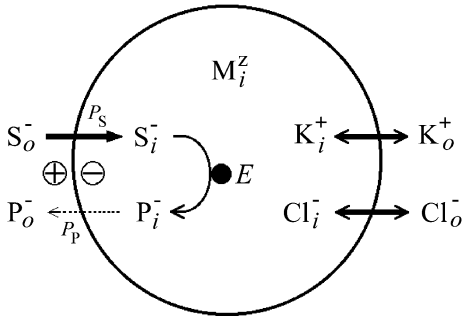

Fig. 1 The model for generation of the Donnan and metabolically derived membrane potentials. Liposome, containing macromolecules M_i^z with a charge z, is incubated in the medium with infinite volume and composed of the solutions of potassium salts of Cl^-, S^- and P^-. Liposomal membrane is characterized by infinitely high permeability for K^+ and Cl^-, and the permeability for S^- is significantly higher than for P^-. Inside the liposome, an enzyme E catalyzes an irreversible reaction of conversion of the entering substrate S^- into the product P^-, which releases from the liposome, thus the steady flux of charged metabolites generates the steady-state MDP across the membrane (Lemeshko and Lemeshko 2000). The final membrane potential results from the superposition of both DP and MDP

where F is the Faraday's constant, $C_{i,\infty}$ is the concentration of the ion i in the infinity, which can be taken as the concentration C of the one–one valent electrolyte KCl in the bulk phase, i.e., as the concentration of KCl in the system, R is the universal gas constant, T is the absolute temperature, ϵ is the absolute dielectric constant of water, z_i is the valence of the ion i that in our case is equal to +1 for potassium and –1 for chloride. Knowing σ and calculating ϕ_0, the electrical potential ϕ at the distance x from the Helmholtz's layer of the IM is described by the following equation:

$$\frac{\left(e^{\varphi F/2RT}-1\right)\left(e^{\varphi_0 F/2RT}+1\right)}{\left(e^{\varphi F/2RT}+1\right)\left(e^{\varphi_0 F/2RT}-1\right)}=e^{-\frac{x}{\delta}}, \tag{2}$$

which is the exact solution of the one-dimensional Poisson–Boltzman equation for the electrical double layer. Here, x is the distance from the membrane; δ is the Debye length expressed as

$$\delta=\sqrt{RT\varepsilon/2F^2C}, \tag{3}$$

where C is the concentration of KCl in the system. Note, only KCl electrolyte is present in the incubation medium for this model.

For de-energized IM, ϕ_0 results from the negatively charged phospholipids and the charged groups of the integral proteins of the membrane, somewhat compensated by counter-ions localized in the Helmholtz's layer, i.e., ϕ_0 results from σ. But, due to the proton extrusion from the mitochondrial matrix during energization of the IM, the surface charge density σ should increase by apparent value $kc\psi_m$, where ψ_m is the trans-membrane potential of the IM, c is the electric capacity of the membrane equal to 1 μF/cm^2 and k is the factor by which real surface of the inner membrane is higher than the smoothed outer membrane surface of mitochondria. This concept has been developed earlier (Lemeshko et al. 1981) to explain a possible mechanism by which mitochondria change their electrophoretic mobility with energization of the IM (Kamo et al. 1976). The surface charge density σ of the IM of de-energized rat liver mitochondria was evaluated as –0.7 μC/cm^2 (Kamo et al. 1976), i.e., 0.044 e/nm^2 that is in a good concordance with recently published data (Nichols-Smith and Kuhl 2005.). Thus we might change Eq. 1, rewriting it as

$$\sigma+kc\psi_m=-\left\{2RT\varepsilon\sum_i C[\exp\left(-z_i\varphi_0F/RT\right)-1]\right\}^{\frac{1}{2}}. \tag{4}$$

Here, we take σ = –0.7 μC/cm^2, k = 10, c = 1 μF/cm^2, ψ_m = 0 mV for the de-energized and ψ_m = –180 mV for the energized mitochondria, C = 125 mM KCl or other, where indicated. The relative dielectric constant of water ϵ_r, which is included in the absolute dielectric constant ϵ as $\epsilon_0\epsilon_r$, was equal to 78. According to the recently obtained data, the relative dielectric constant in the membrane–water interface may be equal to 4–6, increasing to 78 only at a distance of about 6 nm from the membrane, in a 0.1 M one–one valent electrolyte system (Cherepanov et al. 2003). As a crude estimation of the tendency of change of the near membrane potential profile, originated from the IM surface potential at lower dielectric constant, the calculations were also made using Eqs. 2–4 at ϵ_r = 33, the average value of the water dielectric constant for the near-membrane space of 0–5 nm.

All calculations were made by numerical methods using Mathcad Professional 2001i (MathSoft, Cambridge, MA).

Results

The liposomal model for evaluation of the Donnan and metabolically derived membrane potentials

If we assume that the liposome (Fig. 1) does not contain charged macromolecules ($[M^z]_i$ = 0 in Fig. 1 and Eq. 10), only MDP will be generated. Its value increases with an increase in the maximum activity of the enzyme E and strongly depends on the permeability coefficients P_S and P_P (Fig. 2). Lower permeability of the membrane (Fig. 2, curves a and b in comparison to c and d, respectively), as well as its higher selectivity for the anion S^- (Fig. 2, curves a and c in comparison to b and d, respectively) favor generation of relatively high MDP.

On inclusion of the charged impermeant macromolecule M^z into the liposome and taking v_m = 0 in Eq. 5, only DP will be generated: –10 mV at 10 mM M^{10^-}, –27 mV at 30 mM M^{10^-} and –43 mV at 60 mM M^{10^-} (Fig. 3A, b, c and d, respectively, for v_m = 0). The positive DP of the same values may be generated at corresponding concentrations of M^{10^+} (Fig. 3A, e, f and g, for v_m = 0). The obtained results also demonstrate that at high concentrations of polyanion, such as 60 mM for example (DP = –43 mV), the contribution of MDP in the total membrane potential is significantly lower (Fig. 3A, d) than at lower concentrations (Fig. 3A, c and b) or the absence of macromolecules (Fig. 3A, a). In the case of polycation M^{10^+}, in contrast,

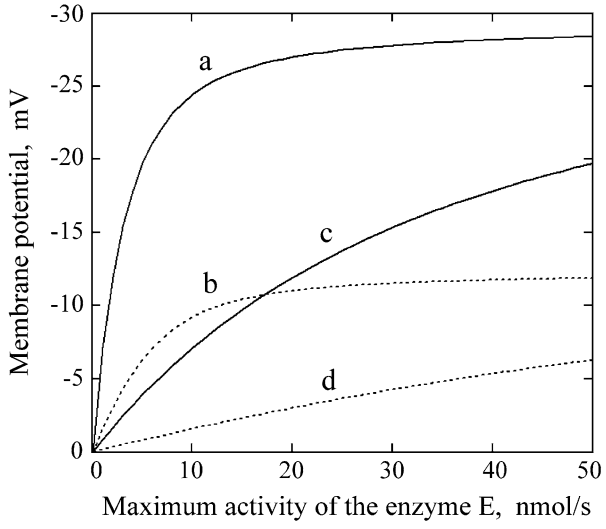

Fig. 2 The dependence of MDP on the maximum activity (v_m) of the enzyme E in the liposome at different permeabilities of the membrane for charged metabolites (P_S and P_P):$P_S = 1$, $P_P = 0.01P_S$ (*a*); $P_S = 1$, $P_P = 0.05P_S$ (*b*); $P_S = 10$, $P_P = 0.01P_S$ (*c*); $P_S = 10$, $P_P = 0.05P_S$ (*d*). Liposome (Fig. 1) is incubated in a medium of infinite volume composed of 125 mM K^+, 105 mM Cl^-, 10 mM S^- and 10 mM P^-. For these calculations, $[M_i^z] = 0$

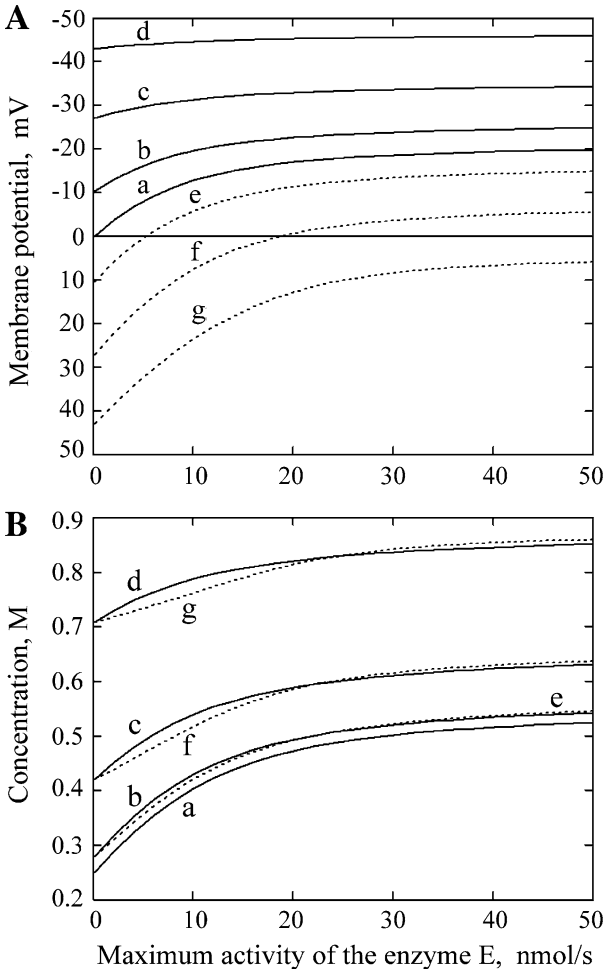

Fig. 3 The dependence of the membrane potential (*Panel A*) and of the concentration of osmotically active particles in the liposome (*Panel B*) on the maximum activity (v_m) of enzyme E, and on the concentration of charged macromolecules M_i^z in the liposome. Liposome (Fig. 1) is incubated in a medium of infinite volume composed of 125 mM K^+, 105 mM Cl^-, 10 mM S^- and 10 mM P^-. All calculations were made at $P_S = 2$, $P_P = 0.02P_S$; $[M_i^z] = 0$ (*a*); $[M_i^{10-}] = 10$ mM (*b*); $[M_i^{10-}] = 30$ mM (*c*);$[M_i^{10-}] = 60$ mM (*d*). $[M_i^{10+}] = 10$ mM (*e*); $[M_i^{10+}] = 30$ mM (*f*); $[M_i^{10+}] = 60$ mM (*g*)

the contribution of MDP significantly increased with an increase in its concentration (Fig. 3A, e–g). At relatively low 10 mM concentration of M^{10+}, the final membrane potential changed in the range from +10 mV, which is pure DP, to –15 mV when the MDP was also generated due to an increase of the maximum activity of the enzyme E (v_m in Eq. 5) from 0 to 50 nmol/s (Fig. 3A, e).

The increase in the calculated membrane potential (Fig. 3A) is accompanied by a significant increase in the concentration of osmotically active particles in the liposome (Fig. 3B), when compared to the concentrations in external medium (250 mM). Particularly, the concentrations of 420 and 710 mM in the liposome (Fig. 3B, c and d, at $v_m = 0$) are created at a DP of –27 and –43 mV, respectively (Fig. 3A, c and d, at $v_m = 0$). As well, the concentration of 520 mM (Fig. 3B, a, $v_m = 50$ nmol/s) is created at the MDP of –20 mV (Fig. 3A, a, 50 nmol/s), and the concentration of 540 mM (Fig. 3B, b, $v_m = 50$ nmol/s) resulted from the combined DP–MDP of –25 mV (Fig. 3A, b, $v_m = 50$ nmol/s). In general, the calculated data demonstrate that the generation of the membrane potential higher than 30 mV leads to a significant increase in the concentration of osmotically active particles in the liposome. The exact difference in the osmotic pressure between the internal and external volumes depends on the osmotic coefficients of accumulated salts, which for KCl, for example, is equal to approximately 0.9 for the range of the salt concentrations of 0.1–1.0 M (Hamer and Wu 1972). In the case of mitochondria, high osmotic gradients between the IMS and the external medium may lead to inhibition of mitochondrial matrix metabolism, as well as to the OM rupture. For example, the rupture of the OM of 40–50% of liver mitochondria, isolated from 12– to 24-months rats, was observed in a semi-isotonic KCl medium, although significantly less effect was observed for young 1- and 3-months rats (Lemeshko 1982).

The model for evaluation of the inner membrane surface potential

Taking into account that in the work of Porcelli et al. (2005) the pH-sensitive EYFP moiety was attached to the glycerol–phosphate dehydrogenase, an integral protein of the IM, it might partially reflect the pH value at the outer surface of the IM in addition to the bulk pH in the IMS. It is known that glycerol-3-phosphate dehydrogenase forms complexes with complex III of the respiratory chain by means of ubiquinone-binding structural components Qcr1p and Qcr2p (Grandier-Vazeille et al. 2001). Thus, the EYFP moiety, attached to this enzyme, might be localized in a proximity to the proton pump of the complex III (Fig. 4), with the pH-sensitive spot (Fig. 4, PS) oriented to the membrane. This pH-sensitive spot might be localized close to one side of the EYFP barrel; at least it was shown that directed mutagenesis of Thr203 to Tyr or His, which is adjacent to the chromophore and is peripherally situated, results in significantly red-shifted excitation and emission maxima (Ormo et al. 1996), i.e., in an enhanced yellow fluorescence of EYFP.

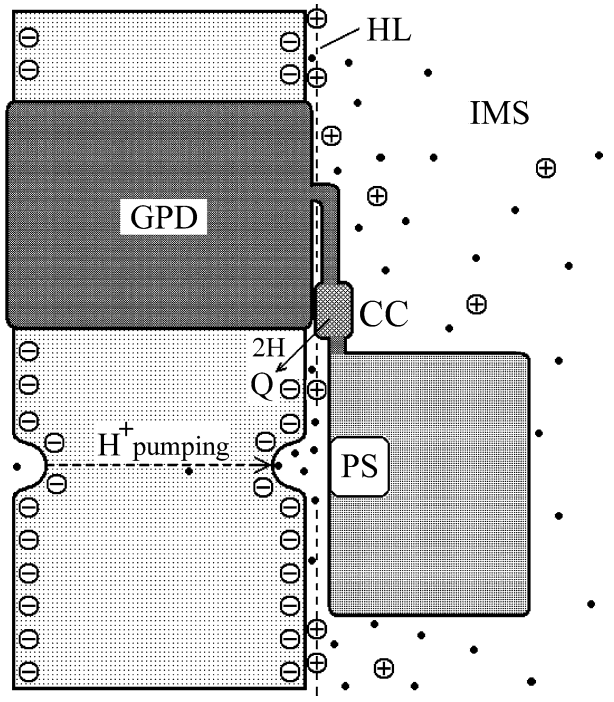

Fig. 4 Schematic presentation of a possible localization of the EYFP moiety, covalently attached to the mitochondrial glycerol-3-phosphate dehydrogenase (GPD) next to its catalytical center (CC). It is possible that the pH-sensitive spot (PS) on the side of EYFP is faced to the outer surface of the inner mitochondrial membrane in front of the mouth of the complex III proton pump. *HL* Helmholtz's layer, *Q* ubiquinone

Glycerol-3-phosphate dehydrogenase has three helices incorporated in the IM, with the relatively large fragment containing substrate-binding center exposed to the IMS (MacDonald and Brown 1996). The substrate-binding center is localized over a distance of more than 70 amino acid residues from the last helix, and it is very sensitive to the surface potential of the IM that is revealed in a significant influence of the calcium ions (MacDonald and Brown 1996) and of the IM surface charge on the accessibility of negatively charged substrate to the enzyme (Nalecz et al. 1980; Amler et al. 1990). Tight proximity of the catalytical center of this dehydrogenase (Fig. 4, CC) to the membrane surface should facilitate electron transfer to ubiquinones.

Moreover, the extruded protons might be maintained at relatively high concentration near the outer surface of the IM, being electrostatically attracted to it, and due to the high energetic barriers to equilibrate with the protons of the bulk phase, as it was determined experimentally (Cherepanov et al. 2004, and references therein). To estimate the possibility that the surface potential of IM contribute at least in part to the IMS's pH measured by Porcelli et al. (2005), the classic Gouy–Chapman–Graham's theory of electrical double layer was applied for analysis, although for detailed description of more complex systems, Monte Carlo simulations seem to be most convenient (Valisko et al. 2004). First, we estimated the surface potential (ϕ_0) according to the modified equation of Graham (Eq. 4) for energized ($\psi_m = -180$ mV) and de-energized ($\psi_m = 0$ mV) IM, and calculated potential profile at a distance of 0–5 nm from the outer plane of the IM Helmholtz's layer for $\epsilon_r = 78$ using Eq. 2. The values of the calculated surface potential significantly decreased upon the IM de-energization (from –30 mV to –9 mV) (Fig. 5a, b, respectively), as well as the values of the near membrane potentials decreased to almost zero at a distance of 3–5 nm, for both energized and de-energized states. The minimal distance between the IM and the OM for the orthodox configuration of mitochondria is even higher, 8–10 nm, according to the electron microscopic data (Frey and Mannella 2000).

If we take $\epsilon_r = 33$, an average relative dielectric constant of water for the space of 0–5 nm near a membrane (Cherepanov et al. 2003), a tendency of a significant increase in the value of the surface potential was observed. In this case, the magnitude of the near membrane potential drops to almost zero at a distance of 2–3 nm for both energized and de-energized IM (Fig. 5c, d, respectively) that is somewhat shorter than that obtained for $\epsilon_r = 78$ (Fig. 5a, b).

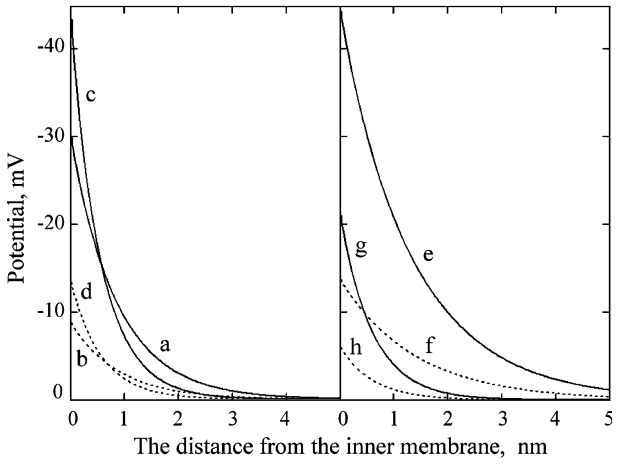

Fig. 5 Potential profile in electrical double layer of the external surface of hypothetical liposome formed by the inner mitochondrial membrane in energized (*a*, *c*, *e*, *g*) and de-energized (*b*, *d*, *f*, *h*) states, for different concentrations of KCl in an incubation medium and different relative dielectric constants of water near the membrane. *a*, *b* 125 mM KCl, $\epsilon_r = 78$; *c*, *d* 125 mM KCl, $\epsilon_r = 33$; *e*, *f* 50 mM KCl, $\epsilon_r = 78$; *g*, *h* 260 mM KCl, $\epsilon_r = 78$. The distance $x = 0$ corresponds to the Helmholtz's layer

The pH-sensitive EYFP-moiety, attached to the glycerol–phosphate dehydrogenase, might be situated in front of the mouth of proton channel of the complex III, to which the ions like potassium and chloride might have a limited access (Fig. 4). In this case, the values of the surface potential should be higher than that at free access of these ions. To model such a situation, we diminished the concentration of KCl in the bulk phase, arbitrarily from 125 to 50 mM, taking $\epsilon_r = 78$ that resulted in an increase of the value of ϕ_0 to –44 and –14 mV, for energized and de-energized IM, respectively (Fig. 5e, f), with the difference of ϕ_0 between energized and de-energized states equal to –30 mV.

On the other hand, the concentration of electrolytes in the IMS may be significantly higher due to the OM potential generation by any mechanism. For example, at OM equal to –20 mV and free permeability of the membrane to potassium, its equilibrium concentration in the IMS will be approximately 260 mM, if the concentration of KCl in the external medium is 125 mM. Under these conditions, the surface potential decreases to –21 mV for energized and –6 mV for de-energized IM (Fig. 5g, h, respectively), if KCl is freely accessible to the pH-sensitive spot of EYFP.

The most important result of the analysis of electrical double layer of IM is that the value of the IM surface potential may be relatively high, with a tendency to further increase with a decrease in the dielectric constant of water in the near membrane space, or in the case of a restriction of potassium and chloride access to the pH sensitive spot of EYFP. The difference in the surface potential for the energized and de-energized IM may reach several tens of mV. The values of the near membrane potentials for both energized and de-energized states of the IM, under conditions close to physiological, decrease almost to zero at a distance less then 5 nm from the IM. Thus in mitochondria, if an electrical potential indicator is localized at a distance less then 1–2 nm from the IM, the difference of electrical potentials between this point and a point in the bulk phase in the cytoplasm may theoretically reach 10–30 mV, or higher, without any OM potential.

Discussion

The outer mitochondrial membrane seems to play an important role in regulation of the cell energy metabolism due to the presence of VDAC, which might restrict energy flux between mitochondria and the cytoplasm, if the OM potential is generated by some mechanism(s). A direct measurement of the OM potential represents serious experimental problem. That is why in the literature there are no data of direct experimental measurements of its value, to the best of our knowledge. Theoretical analysis of the problem may be helpful in developing adequate methodological approaches for direct or indirect measurements of the true OM potential and in the interpretation of some indirect experimental data.

For example, an indirect evaluation of the OM potential of mitochondria in living cell was undertaken by Porcelli et al. (2005) using EYFP attached to the IM as indicator of pH in the IMS. Based on the difference in pH between the point where pH-sensitive spot of EYFP was localized and the cytoplasm, the authors calculated the OM potential to be –43 mV, assuming that the electrochemical equilibrium of protons is established. But, if a high energetic barrier exists to equilibrate proton electrochemical potential near the IM with that in the bulk phase (Cherepanov et al. 2004, and references therein), this assumption cannot be applied. The calculated difference of electrical potentials might also result from the electrical double layer of the IM, without a significant value of the OM potential. The calculated potential of –43 mV was attributed to DP, but in this case it is not clear why the inhibition of mitochondrial metabolism by rotenone and oligomycin caused its complete drop (Porcelli et al. 2005), or even induced the generation of a small positive potential, according to the calculations based on the determined pH difference. Thus, metabolically

derived potential might also be involved in the registered pH difference across the OM.

One may assume that the negative DP as high –43 mV is generated due to the presence of polyanions in the IMS, and that the value of DP might vary in some range as a result of the IMS volume change under well-known metabolically dependent orthodox/condensed conformational changes of mitochondria (Lang and Bronk 1978; Lloyd et al. 2002), although vice versa effect cannot be ruled out, i.e., conformational transitions of mitochondria as a result of MDP generation, accompanied with the creation of osmotic gradient across the OM. For DP of –43 mV, unrealistically high concentration of impermeant macromolecules carrying negative charge is needed. According to the calculations made (Fig. 3A, d for $\nu_m = 0$) for the liposomal model (Fig. 1) with hypothetical polyanion M^{10^-}, its concentration in the IMS should be equal to 60 mM that is almost two orders of magnitude higher than the concentration of cytochrome *c* (around 0.7–1.0 mM), one of the most abundant proteins of the IMS.

Even the prevalence of polyanions, in comparison to polycations in the IMS, is not evident. If the total negative charge of macromolecules and of membrane surfaces in the IMS is higher than the total positive charge, the value of DP across OM should decrease when the IMS volume increases, for example, under orthodox-to-condensed configuration transition of mitochondria. But the contrary behavior has been observed in the experiments of Cortese et al. (1992): the difference of pH across the OM was only 0–0.2 units for the orthodox configuration and increased to 0.4–0.5 units for the condensed state (see also Colombini 2004 for review).

The data of Cortese et al. (1992) might be interpreted as a result of the presence of positively charged macromolecules in the IMS at a concentration which is higher than that of polyanions with the same charge value. The FITC-dextran used in this work to measure pH in the IMS might be preferentially localized at the surface of the IM, reflecting the surface pH, because many data indicate that dextrans have certain affinity to biological membranes: to the plasma membrane of red blood cells (Ohlson et al. 2000; Neu and Meiselman 2002) and to mitochondria (Lemeshko et al. 2003). In the orthodox configuration of mitochondria, when the IMS volume is very small, the positively charged cytochrome *c* and other positively charged macromolecules might replace protons and other counter-ions at the negatively charged surface of the IM, or even almost completely compensate it, thus lowering the measured pH difference, as illustrated in Fig. 6. Here, the interaction of only cytochrome *c* with IM is shown for simplicity. Independent of possible interpretations, the data of Cortese et al. (1992) seem to indicate that the concentration of anionic groups in the IMS is not higher than the concentration of cationic groups.

Based on the estimations made, we suggest that not only DP, but also MDP and electrical double layer of the IM might contribute to the difference of electrical potentials (or pH) between the cytoplasm and the point in the IMS were an indicator of electrical potential (or pH) is situated (Fig. 7). For qualitative presentation in Fig. 7, we used the data for DP and MDP (Fig. 3A, e) calculated for a liposome containing 10 mM M^{10^+} (Fig. 1), as well as the data for the membrane potential profile near the energized and de-energized IM (Fig. 7a, b, respectively) calculated for $\epsilon_r = 78$ and 125 mM KCl in the external medium. The true OM potential, according to this scheme, results from DP-MDP (–15 mV) for maximally energized mitochondria (point E in comparison with point C), and from only DP (+10 mV) for de-energized mitochondria (point D in comparison with point C). If a hypothetical indicator of electrical potential is localized on the Helmholtz's layer, where the surface potential for energized mitochondria is equal to –30 mV with respect to point E in the bulk phase of the IMS, the registered electrical potential with respect to point C in the bulk phase of the cytoplasm will be equal to –45 mV. For de-energized mitochondria (Fig. 7b), in a similar manner, the registered potential with respect to the same point C will be equal to +1 mV. Thus, according to the scheme, if an electrical potential indicator is localized in the Helmholtz's layer, the detecting potential should change from –45 mV to +1 mV after inhibition of metabolic activity of imaginary mitochondria, although the true OM potential changes from –15 mV to +10 mV. The contribution of the electrical double layer will be significantly less, if a potential indicator is localized at the distance (x) from the Helmholtz's layer of the IM (Fig. 7, points A and B for energized and de-energized mitochondria, respectively).

Regardless of the mechanism(s) of generation of the OM potential, its real value seems to be less than 30 mV because at higher values the created osmotic gradient across the OM might cause an inhibition of mitochondrial metabolism and might also induce the OM rupture. On the other hand, for VDAC to be able to close at relatively low values of the OM potential, the factors amplifying the voltage sensitivity of VDAC, like a protein factor of the IMS (Liu and Colombini 1992c; Holden and Colombini 1993), *X* factor of the cytoplasm (Saks et al. 1995) and others (see Colombini (2004) for review), should be involved.

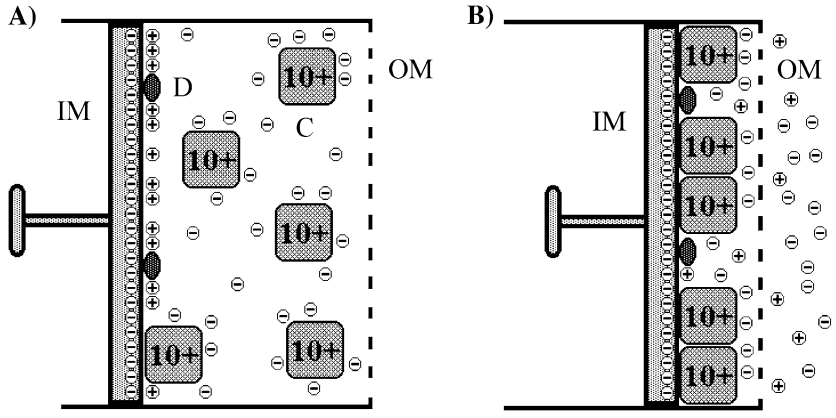

Fig. 6 A possible explanation of the data of Cortese et al. (1992) indicating the change of pH in the IMS under condensed(**a**)-to-orthodox(**b**) configuration transition of mitochondria. FITC-dextran (*D*) was used to evaluate the IMS pH. The possibility exists that FITC-dextran, having some affinity to biological membrane, preferentially reflects pH value at the surface of the IM. In the orthodox configuration of mitochondria, cytochrome *c* (*C*) and other positively charged macromolecules may replace protons near the IM due to the decrease in the IMS volume

The probability of osmotic damage of the OM at high values of the OM potential seems to be interesting with respect to possible mechanisms of permeabilization of the OM to activate mitochondrial pathways of apoptosis. Factors, which allosterically maintain VDAC in its open state, are anti-apoptotic (Colombini 2004; Lemasters and Holmuhamedov 2006). In the frame of our liposomal model (Fig. 1), it means that factors, which increase permeability coefficients, P_S and P_P (Fig. 2d) should decrease the value of generated MDP, thus decreasing osmotic gradient across the OM and, vice versa, factors which decrease these coefficients (Fig. 2a) should increase MDP, osmotic gradient and the probability of the OM rupture without a drop of the IM potential.

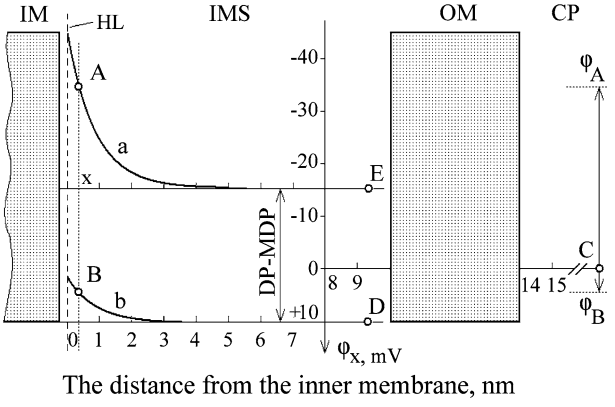

Fig. 7 Schematic presentation of possible profiles of electrical potential in the IMS with respect to the cytoplasm (*CP*), where electrical potential is equal to zero. Superposition of a potential in the electrical double layer of the IM and of the combined DP–MDP for energized (point *A* on the curve *a*) and de-energized (point *B* on curve *b)* mitochondria is shown for a distance *x* from the IM. The OM potential for energized mitochondria is equal to DP–MDP (point *E*), and it is changed to DP (point *D*) after de-energization. If for example, a potential indicator is localized in the Helmholtz's layer (*HL*), it indicates the electrical potential, which is algebraic sum of the surface potential and DP–MDP for energized mitochondria (–45 mV) and a sum of the surface potential and DP (+1 mV) for de-energized mitochondria

In conclusion, the nature, value and the dynamics of the OM potential are critical for our understanding of the role of the outer membrane in mitochondrial energy metabolism. Theoretical analysis of possible mechanisms of generation of the OM potential demonstrates that metabolically derived potential might complement the Donnan potential across the OM. With any combination of the mechanisms, the OM potential of extremely high amplitude should lead to a significant increase of osmotic pressure in the IMS that might endanger the integrity of the OM, thus inducing apoptosis. Electrical double layer might interfere with indirect estimations of the IMS electrical potential or pH, using some indicators. If for example, an indicator of pH is attached to the IM, as in the work of Porcelli et al. (2005), the electrical double layer of the IM could cause a significant over-estimation of the pH difference across the OM. Attaching EYFP, or other pH indicator, to different soluble proteins in the IMS, for example adenylate kinase, which is not bound to the mitochondrial membranes, could eliminate this type of uncertainties. For isolated mitochondria, the best possibility seems to be the employment of electrochromic chromophores, which are sensitive to a local intramembrane electric field associated with transmembrane potentials like di-4-ANEPPS or di-8-ANEPPS (Xu and Loew 2003), attaching them to

a 10 kDa or higher molecular mass Ficoll, dextrane or polyethylene glycol, thus avoiding the direct interference of the IM electrical double layer and allowing the interaction with exclusively outer membrane of intact mitochondria.

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Appendix: mathematical description of the liposomal model for generation of the Donnan and metabolically derived potentials

Let us assume that the liposome is placed in a medium of infinitely large volume composed of 125 mM K^+, 105 mM Cl^-, 10 mM S^- and 10 mM P^- (Fig. 1). The rate of an irreversible reaction of conversion of S^- into P^-, catalyzed by a liposomal enzyme E and characterized by simple first order Michaelis–Menten kinetics, may be described as

$$v = \frac{v_m [S^-]_i}{K_m + [S^-]_i}, \tag{5}$$

where v_m is the maximum rate, which can be varied to simulate various metabolic activities, and K_m is the Michaelis–Menten constant. For all calculations, $K_m = 2$ mM.

The steady-state fluxes of the ions S^- and P^- across the membrane due to the difference in their concentrations in the external medium and in generated membrane potential may be expressed by the Goldman equation:

$$J_S = P_S \frac{\Delta\psi F}{RT} \times \frac{[S^-]_i - [S^-]_0 e^{\frac{\Delta\psi F}{RT}}}{1 - e^{\frac{\Delta\psi F}{RT}}}, \tag{6}$$

$$J_P = P_P \frac{\Delta\psi F}{RT} \times \frac{[P^-]_i - [P^-]_0 e^{\frac{\Delta\psi F}{RT}}}{1 - e^{\frac{\Delta\psi F}{RT}}}, \tag{7}$$

where P_S is the membrane permeability coefficient for S^-, P_P is the permeability coefficient for P^-, F is the Faraday constant, $\Delta\psi$ is the membrane potential, R is the gas constant and $T = 310$ K.

At steady state, the rate v of conversion of S^- into P^- (Eq. 5), and the fluxes of S^- and P^- (Eqs. 6, 7) across the membrane must be equal (Lemeshko and Lemeshko 2000):

$$J_S = -J_P \tag{8}$$

$$J_S = v. \tag{9}$$

The liposome also contains macromolecules with the charge 10^- (polyanion) or 10^+ (polycation) at the concentration $[M^z]_i$, thus the electro-neutrality principle for the liposomal space is described as

$$[K^+]_i - [Cl^-]_i - [S^-]_i - [P^-]_i + z[M^z]_i = 0, \tag{10}$$

where z is the valence of a macromolecule.

Assuming that the liposomal membrane is highly permeability for K^+ and Cl^-, electrochemical equiliбriums for these ions can be presented by Nernst equations:

$$\Delta\psi = -(RT/F) \ln\left([K^+]_i/[K^+]_0\right), \tag{11}$$

$$\Delta\psi = (RT/F) \ln\left([Cl^-]_i/[Cl^-]_0\right). \tag{12}$$

Solving the system of Eqs. 5–12 allows for estimating the values of the DP potential (if $v_m = 0$), or the MDP (if $[M^z]_i = 0$), or the superposition of both.

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