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Biochimica et Biophysica Acta 1828 (2013) 208–212

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Biochimica et Biophysica Acta

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Cytochrome *c* produces pores in cardiolipin-containing planar bilayer lipid membranes in the presence of hydrogen peroxide

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ARTICLE INFO

Article history:
Received 5 May 2012
Received in revised form 21 September 2012
Accepted 3 October 2012
Available online 19 October 2012

Keywords:
Cytochrome c
Cardiolipin
Hydrogen peroxide
Peroxidase activity
Membrane permeability

ABSTRACT

Interaction of cytochrome *c* with cardiolipin in the presence of hydrogen peroxide induces peroxidase activity in cytochrome *c* and the ability to oxidize membrane lipids. These cytochrome *c* properties play a substantial role in the cytochrome *c*-mediated apoptotic reactions. In the present study the electric properties (specific capacitance and integral conductance) of the cardiolipin-containing asolectin planar bilayer lipid membranes (pBLM) in the presence of cytochrome *c* and hydrogen peroxide were studied. Cytochrome *c* interaction with cardiolipin-containing pBLM in the presence of hydrogen peroxide resulted in the dramatic increase of the conductance, pore production, their growth up to 3.5 nm diameter and subsequent membrane destruction. In the absence of hydrogen peroxide cytochrome *c* demonstrated almost no effect on the membrane capacitance and conductance. The data obtained prove the pivotal role of cytochrome *c* and membrane lipids in the permeabilization of pBLM. Correlation of apoptotic reactions and cytochrome *c*-mediated membrane permeability is discussed.

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1. Introduction

Studies of the apoptosis molecular and cellular mechanisms clearly showed the detailed basic reactions of the process [1–3]. It became obvious that the interaction of positively charged cytochrome *c* (+8) with negatively charged phospholipids, and first of all cardiolipin (−2), results in the lipid-protein complex formation, subsequent modification of cytochrome *c* active cite and finally cytochrome *c* peroxidase activity induction [4–6]. Being converted into a peroxidase cytochrome *c* acquires the ability to oxidize various substrates, and what is most important — membrane lipids (cardiolipin and other negatively charged phospholipids) [7,8]. Oxidation of phospholipids can involve membrane permeability increase and pore formation. In the present study the cytochrome *c* and hydrogen peroxide effects on the electric properties (specific capacitance and integral conductance) of the cardiolipin-containing asolectin planar bilayer lipid membranes were investigated. We attempted to clarify the molecular mechanisms of the membrane electric parameter alterations and give quantitative evaluation. These studies of the electric membrane properties are of great importance from the point of view of the elucidation of the molecular reactions taking place in mitochondria membranes in apoptosis.

2. Materials and methods

2.1. Reagents

To produce planar bilayer lipid membranes asolectin from soybean (27 mg/ml, Sigma) or a mixture of asolectin and cardiolipin (4:1, totally 27 mg/ml, Avanti Polar Lipids) solutions in decane were used. Azolectin from soybean comprised roughly equal proportions of phosphatidylcholine, cephalin and phosphatidylinositol. It contained about 24% saturated fatty acids, 14% mono-unsaturated and 62% polyunsaturated fatty acids. Lipid solutions in decane were applied on the 0.8 mm hole in the wall of Teflon chamber immersed in Tris–HCl buffer (2.5 mM, pH 7.4) prepared with double distilled deionized water (resistance 18 MΩ) and containing KCl (5 mM). To start the reaction cytochrome c (Sigma) and hydrogen peroxide (Sigma) were added.

2.2. pBLM experimental setup

To study the electric properties of the planar bilayer lipid membranes (pBLM) an original experimental setup was used. It was described by V.F. Antonov et al. [9] in details. Briefly, the experimental setup consisted of two main units: measurement unit, containing the Teflon beaker with the membrane, and electronic unit, containing triangle-wave generator, amplifier, analog-digital converter and computer. The measurement unit had two aqueous compartments — an external glass beaker (7 ml), containing buffer and internal Teflon chamber (3 ml) with a hole 0.8 mm in the wall, containing the

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http://dx.doi.org/10.1016/j.bbamem.2012.10.002

membrane. Every compartment had individual Ag–Cl electrode to measure the integral conductance of the membrane.

2.3. General characteristics of the pBLM

The pBLM used was assumed to be a symmetrically polarized membrane. The effective noise current in the frequency range 0–1 kHz was less than 0.25 pA. The electric feedback resistance of the amplifier was 100 MΩ. The current conversion factor into the output voltage was 10 mV/pA. The relative error of the current conversion factor was less than ±10%. Experimental setup for current measurement had low frequency 6th level Bessel filter. The maximal filtering frequency was set to 0.3 kHz.

Capacitance current and conductance current were evaluated according to the triangle-shaped pulses as it is shown in Fig. 1. The frequency of the signal polarity triggering was 4 Hz. The output analog signal was converted into the digital one by means of the analog-digital unit LA-1.5 PCI. The signal had two components: V_c and V_g, that represented capacitance and conductance currents of the pBLM respectively. Final capacitance and conductance of the membrane were calculated according to the following formulas:

$$C_m = \frac{\Delta V_c}{2fV_a R_o}$$

$$G_m = \frac{\Delta V_g}{R_o V_a}$$

where C_m — pBLM capacitance, G_m — pBLM conductance, f — applied signal frequency, R_0 — resistance, and V_a — generator applied voltage.

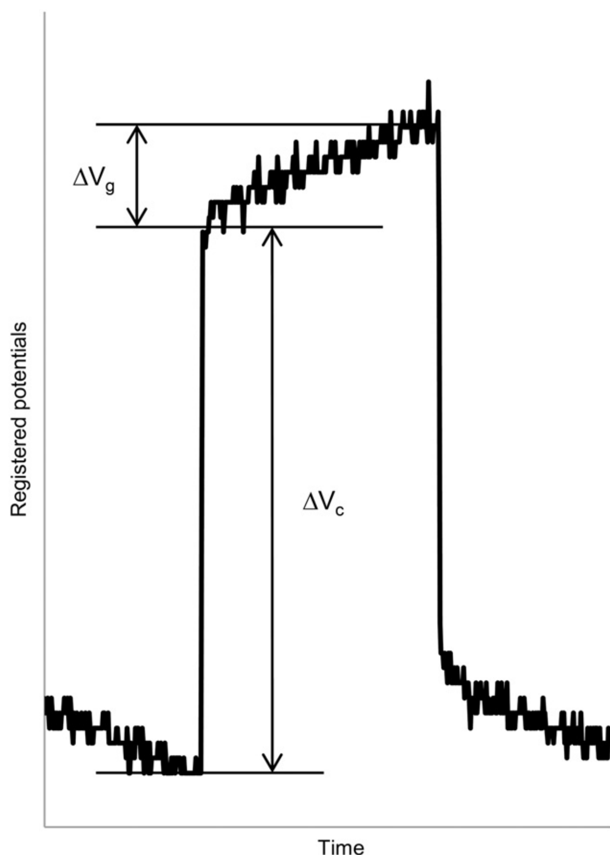

Fig. 1. Integral conductivity signal and its characteristics. ΔV_g- and ΔV_c-membrane conductive and capacitive potentials, correspondingly.

The specific capacitance of the membrane was calculated as:

$$C_s = \frac{C_m}{S_m} = \frac{C_m}{\pi r_m^2}$$

where C_s — specific capacitance, S_m — membrane area, and r_m — pBLM radius (torus subtracted).

Signal sampling frequency was 1 kHz. The experimental data obtained was originally processed with the ADC Lab software (Rudnev-Shilyaev company) and then Origin Pro 8 was applied for analysis and plotting.

2.4. Calculation of the pore diameter

To calculate pore diameter a conductance jump amplitude was used. The conductance variation was assumed as a result of the individual pore production in pBLM. The most adequate physical model used was "membrane hole filled with electrolyte". In the case that the membrane pore produced was cylindrical its diameter can be calculated as:

$$d = \frac{G}{2g} + \sqrt{\frac{G^2}{4g^2} + \frac{4Gl}{\pi g}}$$

where d — pore diameter, G — conductance surge amplitude, l — membrane thickness, and g — specific conductivity of the media. The conductivity of the solution inside the pore was assumed to be the same as a conductivity of the bulk electrolyte. The formula takes into account the access resistance of a cylindrical pore. Experimental results were analyzed by means of the "Statistica 8" software.

3. Experimental results

As it was mentioned above, cytochrome c interaction with cardiolipin induces in cytochrome c peroxidase activity as a result of cytochrome c-cardiolipin complex formation and subsequent active cite remodeling. To detect and evaluate the cytochrome c-cardiolipin complex peroxidase activity a chemiluminescence assay can be applied in the presence of hydrogen peroxide and luminol. The results of this experiment are presented in Fig. 2. One can see that cytochrome c in the presence of cardiolipin-containing liposomes and hydrogen peroxide gives an intensive chemiluminescence. If liposomes contained only phosphatidylcholine (lacking cardiolipin) the chemiluminescent

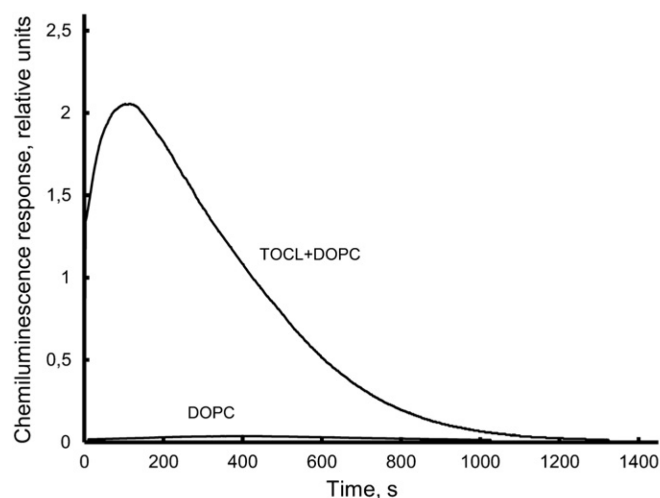

Fig. 2. Cytochrome c chemiluminescence kinetics in the presence of cardiolipin–phosphatidylcholine liposomes, hydrogen peroxide and luminol. Liposomes contained dioleoyl phosphatidylcholine (DOPC) or dioleoyl phosphatidylcholine:tetraoleoyl cardiolipin mixture in 1:4 ratio (DOPC + TOCL), and total lipid concentration 500 μM, cytochrome c — 10 μM, H_2O_2 — 100 μM and luminol — 500 μM.

response was practically absent. These results prove that formation of cytochrome c-cardiolipin complex really emerges a peroxidase activity in cytochrome c and luminol can serve as a substrate of this reaction to produce chemiluminescence.

The next question to be solved was: can the membrane phospholipids be the substrate of cytochrome c peroxidase reaction too, and if they can, what are the consequences of this peroxidase activity on the electric properties of the membrane? It was found that at room temperature and in Tris–HCl (2.5 mM, pH = 7.4) buffer media, containing KCl (2.5 mM) cardiolipin-containing asolectin (1:4) pBLM and asolectin pBLM, lacking cardiolipin, have similar specific capacitance values, equal to 0.31 ± 0.02 μF/cm^2 and 0.37 ± 0.02 μF/cm^2 and almost negligible conductance. Addition of cytochrome c (10 μM) and hydrogen peroxide (1 mM) to the systems resulted in the dramatic increase in the conductance in cardiolipin-containing pBLM as it is shown in Fig. 3A and B. It can be seen, that after a 30 minute lag period conductance of the pBLM starts sharp raise.

Extracting capacitance and conductance components from the integral conductance we can observe individual capacitance and conductance kinetics. These results are presented in Fig. 4. It is seen that when hydrogen peroxide was added to the system, containing cardiolipin enriched pBLM and cytochrome c, a stepwise increase in conductance was observed. Alterations in capacitance kinetics were negligible.

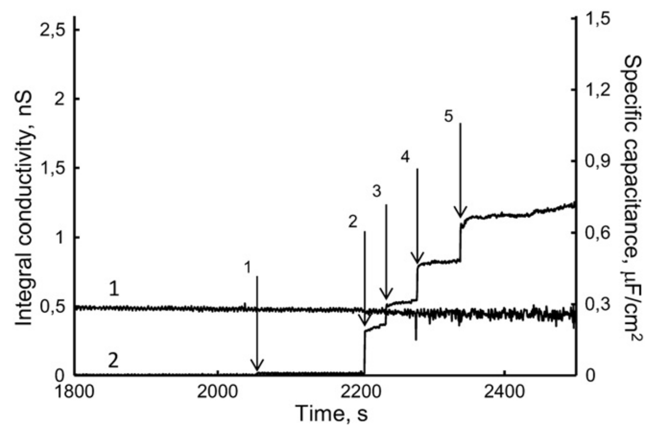

Fig. 4. Conductivity and capacity current kinetics in asolectin/cardiolipin pBLM in the presence of cytochrome c and hydrogen peroxide. Experimental conditions are as in the legend to Fig. 3(A). Arrows indicate the time points of conductivity increase and are the same as in Fig. 3(A).

The results of reference experiments, when the system was lacking hydrogen peroxide, cardiolipin or cytochrome c, are not presented. In these experiments both integral conductivity and capacitive and conductive component anamorphosis did not show any

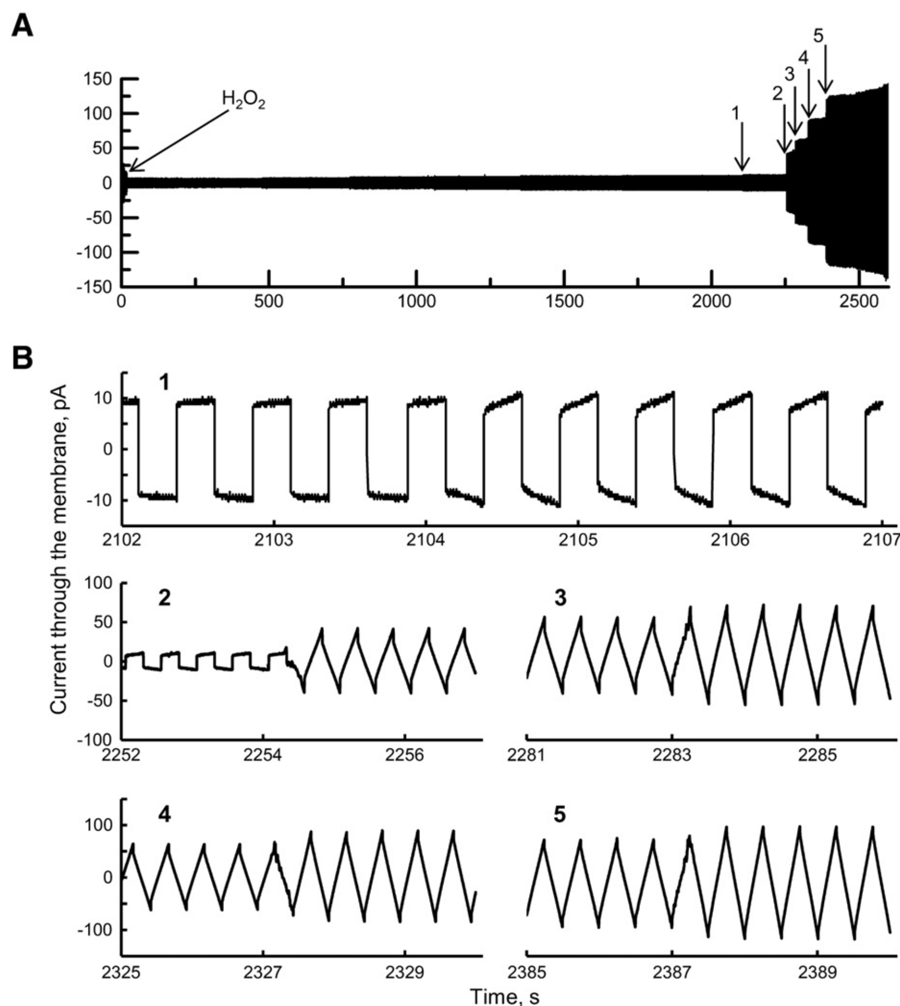

Fig. 3. Conductivity signal monitored in the experiment, when system contained asolectin cardiolipin enriched planar bilayer membranes, cytochrome c and hydrogen peroxide. A — full record of the experiment. Membrane was formed in the presence of cytochrome c (4 μM) and contained 20% of cardiolipin and 80% of asolectin. Arrows depict time points when H_2O_2 (1 mM) was added and conductivity surges (1–5). Incubation period before the increase of conductivity took about 30 min. B (1–5) — detailed records of individual conductivity surges.

alterations for the time period of 15–50 min. The removal of tetraoleoyl cardiolipin from the asolectin membranes, or cytochrome c or hydrogen peroxide from the system or replacement the unsaturated oleoyl residues for saturated myristoyl residues in cardiolipin did not affect the conductivity of the pBLM. This experimental data proves that alterations in conductivity arrive only when peroxidase activity of cytochrome c is present, i.e. when we have cytochrome c, cardiolipin with unsaturated fatty acid residues and hydrogen peroxide. When at least one of these components is lacking no peroxidase activity can be observed and no alterations in the electrical properties of the membrane are present. In some cases we do observe tiny changes in the conductivity of the membrane in the absence of hydrogen peroxide, but this observation needs additional studies.

Current–voltage characteristics for the pBLM, containing cardiolipin, cytochrome c and hydrogen peroxide, were plotted at various current values through the membrane. Results of this experiment are presented in Fig. 5. It can be seen that current–voltage dependencies are linear and this fact proves the symmetry of conductivity (there is no cation–anion selectivity). This kind of dependencies is relevant to the pores with diameter higher 1 nm.

Analysis of the membrane conductivity and current surge amplitude made it possible to calculate pore distribution and plot the distribution of pore diameters. The results of these calculations are presented in Fig. 6. One can see, that most likely pore diameter is 3.5 nm.

Concluding the experimental data analysis we can state that the interaction of cytochrome c with cardiolipin and hydrogen peroxide induces pore formation in the planar bilayer lipid membrane. These pores possess high stability, have average diameter of 3.5 nm and have symmetrical cation–anion conductivity. It is very likely that this pore is a toroidal pore with walls built with protein–lipid complexes.

4. Discussion

It is well known that cytochrome c is a mitochondrial protein, located on the outer side of the inner mitochondrial membrane and is kept on the membrane by electrostatic forces [1,2]. Cytochrome c interaction with cardiolipin results in the reconfiguration of the cytochrome c active cite and acquiring by cytochrome c peroxidase activity [3,5,6]. Being converted into a peroxidase the cytochrome c gains the ability to oxidize various substrates, including phospholipids and other membrane lipids, which, can enhance membrane permeability and pore formation. Really, cardiolipin-containing asolectin membranes substantially increase their permeability for ions, being subjected to cytochrome c peroxidase activity. We can assume that the essential mechanism of this process is the peroxidative oxidation of phospholipids, phospholipid peroxide production and membrane failure [7,10,11]. It is important to mention that the substrates for the cytochrome c peroxidase activity must be unsaturated phospholipids with linoleic or linolenic fatty acid residues, as oleoyl residues are not oxidized.

On the other hand, we cannot underestimate the fact that the increase of lipid bilayer polarity as a result of peroxidation will increase the cytochrome c adsorption on the membrane, accelerate pore formation and enhance cytochrome c involvement into the membrane pore building. Similar processes were observed in the interaction of mitochondrial protein Bax with lipid bilayer [12]. The following hypothesis seems to be reasonable: on the first step the production of cytochrome c-phospholipid complex is produced giving birth to cytochrome c peroxidase activity. On the next step, membrane lipid oxidation takes place, that results in the membrane polarity increase and membrane defects with enhanced permeability formation. Oxidation advancing may cause the real pore formation and increasing of the pore diameter. Embedding of the cytochrome c into the lipid stabilizes toroidal pores. This kind of pores is characterized by a rather long lifespan and the absence of the ion selectivity. Production of the similar pores in the presence of Bax protein was demonstrated by Fuertes et al. [13].

As it was shown by Hirota et al., H_2O_2 can induce cytochrome c oligomer production, containing from 2 to 3 up to 40 monomers [14]. These oligomers can embed into the lipid bilayer and create pores, that can be described by the “barrel-stave” model. These pores are characterized by low-amplitude and minimal dispersion currents. In our experiments we did not observe this kind of pore electrical activity. As it was presented in Fig. 3A and B we monitored current surges with wide amplitude variations. This kind of pore electrical activity can be better described by a toroidal model of the membrane pore.

In the present study we attempted to demonstrate the molecular mechanisms of pore formation in the lipid membranes when peroxidase activity is induced in the cytochrome c. These mechanisms appear to be essential if apoptotic processes are considered. This kind of large membrane pores can, probably, support the release of cytochrome c from the mitochondria in apoptosis.

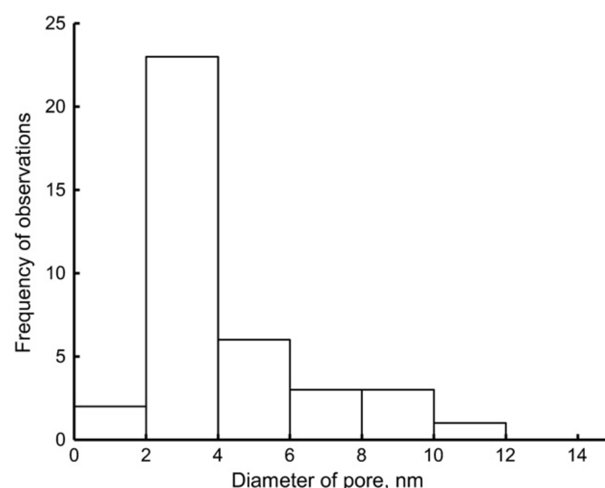

Fig. 6. Distribution of asolectin/cardiolipin pBLM pore diameters produced in the experiments with cytochrome c and hydrogen peroxide. Average diameter of the pores was equal to 3.5 ± 0.4 nm.

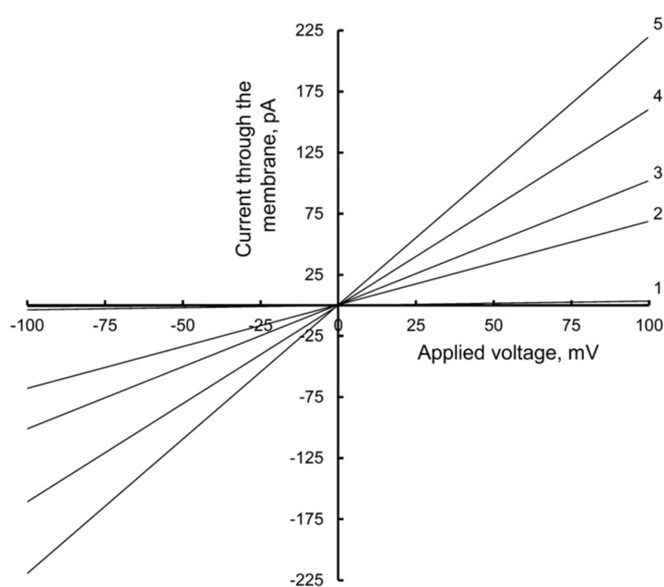

Fig. 5. Instant current–voltage characteristics of pBLM. Experimental conditions are as in the legend to Fig. 3(A). Line numbers correspond to arrow numbers presented in Fig. 3(A). Each line represents integral conductivity of the time period between the arrows in Fig. 3(A).

Acknowledgments

We would like to thank professor Valery F. Antonov for technical support, useful comments and fruitful discussions.

This study was supported by the Russian Foundation for Basic Research Grant # 10-04-01147-a (PI AN Osipov).

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