Bioelectrochemistry 119 (2018) e7966

Contents lists available at ScienceDirect

Bioelectrochemistry

journal homepage: www.elsevier.com/locate/bioelechem

Investigation of the chemical mechanisms involved in the electropulsation of membranes at the molecular level

Marie Breton, Lluis M. Mir *

Vectorology and Anticancer Therapies, UMR8203, CNRS, Univ. Paris-Sud, Gustave Roussy, Université Paris-Saclay, Villejuif F-94805, France

ARTICLE INFO

Article history:
Received 5 April 2017
Received in revised form 9 September 2017
Accepted 9 September 2017
Available online 11 September 2017

Keywords:
Electropulsation
Membrane phospholipids
Lipid oxidation
Reactive oxygen species
Electroporation
Antioxidant
Electropermeabilization

ABSTRACT

The chemical consequences of electropulsation on giant unilamellar vesicles (GUVs), in particular the possible oxidation of unsaturated phospholipids, have been investigated by mass spectrometry, flow cytometry and absorbance methods. Pulse application induced oxidation of the GUV phospholipids and the oxidation level depended on the duration of the pulse. Light and O_2 increased the level of pulse-induced lipid peroxidation whereas the presence of antioxidants either in the membrane or in the solution completely suppressed peroxidation. Importantly, pulse application did not create additional reactive oxygen species (ROS) in GUV-free solution. Lipid peroxidation seems to result from a facilitation of the lipid peroxidation by the ROS already present in the solution before pulsing, not from a direct pulse-induced peroxidation. The pulse would facilitate the entrance of ROS in the core of the membrane, allowing the contact between ROS and lipid chains and provoking the oxidation. Our findings demonstrate that the application of electric pulses on cells could induce the oxidation of the membrane phospholipids since cell membranes contain unsaturated lipids. The chemical consequences of electropulsation will therefore have to be taken into account in future biomedical applications of electropulsation since oxidized phospholipids play a key role in many signaling pathways and diseases.

1. Introduction

The exposure of cells to intense electric pulses of short duration induces the permeabilization of the cell membrane for periods of time that are orders of magnitude longer than the pulse duration [1,2]. The term electropulsation will be used in the text to describe both the exposure to electric pulses and its short term and long term consequences, respectively the electroporation of the cell membrane and the electropermeabilization of the cells. Thanks to this phenomenon, the use of microsecond and millisecond pulses on cells has generated many biomedical applications such as gene electrotransfer [3] or electrochemotherapy [4]. Electrochemotherapy is based on the combined use of electropulsation and chemotherapy on tumor cells. This technique allows increasing the efficiency of the anticancer drug by a factor of 1000 to 10,000 depending on the drug [5]. Efficient electropulsation processes have also been developed in the food and environment industries [6]. Ultra-short pulses called nanopulses, with a very high amplitude (tens of kV/cm) and duration of a few nanoseconds have also emerged in recent years as a promising tool for medicine [7,8].

The physical mechanisms underlying the interaction of electric pulses with cell membranes have been extensively studied. In particular, it has been demonstrated that the application of an electric field on a cell induces a transmembrane potential (TMP), which will add to the resting TMP of the cell. If the total TMP reaches a certain threshold value, the cell membrane will be permeabilized [9,10]. According to molecular dynamics simulations of lipid bilayers, nanometer-sized aqueous pores are formed inside the lipid bilayers during the pulse application [11,12]. The kinetics of the opening and closure of these pores appear to be on the order of nanoseconds. These theoretical results have been experimentally confirmed in the case of nanosecond pulses [13]. However, this purely physical description is not completely satisfactory since it only considers nanoseconds kinetics whereas electric pulses have displayed long-term effects on membrane permeability [14] and conductivity [15] of cells and tissues.

In order to explain these long-term effects, it seems essential to consider the chemical mechanisms of electropulsation. The potential chemical modifications induced by the application of electric pulses have been poorly considered yet. Nevertheless, it has already been detected that, after pulsing, the level of reactive oxygen species (ROS) increases at the periphery of the cell, which demonstrates a strong presence of oxidizing species close to the membrane [16,17]. Up to now, a few studies point to an oxidation of the membrane phospholipids [18–20]. Considering that in mammalian cell membranes, phospholipids are mostly polyunsaturated [21] and that these lipids are prone to oxidation, it is necessary to further investigate the possible oxidation of the phospholipids of the membrane due to electropulsation. These chemical processes could be critical in the mechanisms of cell electropulsation since the

* Corresponding author.
E-mail addresses: mariebreton@orange.fr (M. Breton), luis.mir@cnrs.fr (L.M. Mir).

http://dx.doi.org/10.1016/j.bioelechem.2017.09.005
1567-5394/

presence of oxidized lipids in the cell membrane has been demonstrated to increase the permeability of the cell membrane [22,23]. The oxidized phospholipids also play a key role in many signaling pathways and diseases [24].

The aim of this article is therefore to investigate chemical processes occurring at the molecular level due to electropulsation. We have studied the oxidation of phospholipids with various unsaturation degrees and the different factors that could affect the appearance of ROS such as the presence of metal ions, light, oxygen or antioxidants. Considering the molecular complexity of cells that can greatly hamper the understanding of the results, this study has been conducted on giant unilamellar vesicles (GUVs). GUVs are cell membrane models that have been widely used to study the properties of biological membranes [25,26]. In particular, GUVs have already proven to be an effective model to analyze membrane electropulsation [13]. GUVs are advantageous compared to small unilamellar vesicles since their diameter is close to that of a biological cell, they contain phospholipids that constitute cell membranes and the curvature of GUVs, which can greatly impact experimental results, is similar to that of biological cells [27,28].

2. Materials and methods

2.1. Materials

1,2-Dilinoleoyl-*sn*-glycero-3-phosphocholine (DLPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) ammonium salt (DOPE-Rhodamine) were purchased from Avanti Polar Lipids. Their purity was checked by mass spectrometry before use to avoid any oxidation of the source material. All other chemicals and solvents were purchased from Sigma Aldrich. All reagents were used without further purification.

2.2. Giant Unilamellar Vesicles (GUVs) preparation

DLPC or DOPC were dissolved in chloroform at 0.5 mg/mL. The fluorescent dye DOPE-Rhodamine was added at a 0.1% molar concentration. The vesicles were prepared at 4 °C using a previously described electroformation protocol [29]. Briefly, 15 μL of the lipid solution were deposited on the conducting side of two glass slides coated with indium tin oxide (Sigma). The slides were then kept under vacuum for 2 h in a desiccator to remove all traces of organic solvent. A chamber was assembled with the slides spaced by a 1.5 mm silicone isolator (Sigma). The chamber was filled with a solution containing 240 mM sucrose, 1 mM NaCl, buffered at pH 7.4 with 1 mM K_2HPO_4/KH_2PO_4 (sucrose buffer). The slides were then connected to a function/arbitrary waveform generator (HP Agilent 33120A). A sinusoidal voltage of 0.006 V peak to peak and 8 Hz was applied. The voltage was increased by 25 mV steps every 5 min, up to a value of 0.6 V and maintained under these conditions 3 h for DLPC and 18 h for DOPC. Finally, a square-wave AC field of the same amplitude was applied at 4 Hz for 1 h in order to detach the GUVs from the slides.

DLPC vesicles containing α-tocopherol in their membranes were prepared with the same protocol by dissolving α-tocopherol at 15 μg/mL in the initial solution of DLPC in chloroform. This corresponds to a ratio DLPC:α-tocopherol of 100:3 w/w in the final vesicle solution.

Solutions of DLPC vesicles containing ascorbic acid were prepared with the same protocol by including ascorbic acid at 1.1 μg/mL in the sucrose buffer used for preparation. This corresponds to a ratio DLPC:ascorbic acid of 100:3 w/w in the final vesicle solution.

2.3. Pulse delivery

For pulse delivery, 200 μL of vesicle solution were placed in aluminium electropulsation cuvettes (STD, Dutcher). The distance between the two planar electrodes of the cuvettes was d = 1 mm. Nanopulses of 12 ns were applied with a commercial generator purchased from FID (FID GmbH, Model FPG 10-ISM10) with output impedance of 50 Ω. It generates trapezoidal monopolar pulses with a full-width at half maximum of 12 ns. This set-up and the pulse traces were previously described [30,31]. Micropulses of 20, 25, 50, 100, 200, 250 and 600 μs and millipulses of 1 and 1.5 ms were applied with the commercial generator Cliniporator™ (Igea), which generates square-wave electric pulses. The pulse traces are provided in Supplementary information (SI Fig. 1).

2.4. Lipid oxidation measurement by electrospray mass spectrometry on DOPC and DLPC vesicles

50 μL of the vesicle solution were added to 250 μL of a mixture of chloroform/methanol (2/1, v/v). Solutions were then vortexed and centrifuged. The inorganic phase was removed and the organic phase was evaporated under a stream of nitrogen. 50 μL of methanol were added and the solutions were vortexed. The Quattro LC mass spectrometer (Micromass) controlled by the MassLynx software system (version 3.4) and equipped with an electrospray ionizer and a quadrupole analyzer was operated in the positive ion mode with capillary voltage of 3 kV, cone voltage of 30 V, source block temperature set to 90 °C and desolvation temperature set to 200 °C. The elution solvent was acetonitrile:5 mM ammonium acetate (90:10, v/v). Multiple Reaction Monitoring (MRM) spectra of organic extracts of DOPC and DLPC vesicles solution were recorded. The MRM spectrum allows quantifying peaks corresponding to chosen compounds which are characteristic of the phospholipid oxidation or the phospholipid oxidation followed by chain breaks. We considered additions of up to six oxygen (corresponding to increases in mass of 16, 32, 48, 64, 80 and 96) and the major peaks corresponding to chain breaks (and therefore to a decrease in mass). An example of the mass spectrometry spectra obtained for DLPC is presented in SI Fig. 2. The spectrum of standard DLPC only displays the peak at m/z = 782 which is the molecular ion of DLPC. The spectra of the organic extract of DLPC vesicles displays new peaks at higher and lower masses. The MRM spectrum allows obtaining and integrating the ion current as a function of the elution time for each ion peak of interest. Finally, the percentage of non-oxidized lipids of each sample was calculated by dividing the value of the integration of the ion current corresponding to the intact lipid over the integration of the ion current corresponding to all the masses of interest, both intact and oxidized lipids.

2.5. Fluorescence-Activated Cell Sorting (FACS) cytometry of vesicles

Solutions of GUVs were analyzed by flow cytometry immediately after pulsing using the C6 flow cytometer (BD Accuri). For each sample, 10,000 data points were recorded. The flow cytometer simultaneously recorded the Forward laser light SCattering (FSC), the Side laser light SCattering (SSC), and the level of fluorescence of each event as well as the number of events. The fluorescence excitation wavelength was 488 nm, and the emission was detected through a 585 ± 40 nm band pass filter in order to detect the rhodamine-labeled vesicles. The graph of the SSC as a function of the fluorescence on all events was drawn (SI Fig. 3A). The vesicle population was selected to obtain the number of events per microliter and the mean fluorescence. Then, the graph of the FSC as a function of the fluorescence for the vesicle population only was drawn to obtain the mean FSC value (SI Fig. 3B).

2.6. Lipid oxidation measurement by the ferrous oxidation xylenol orange (FOX) assay on DLPC vesicles

DLPC vesicles were prepared in the sucrose buffer. This buffer alone or the vesicle solution received no treatment or 500 nanopulses of 12 ns and 1.5 GV/m at 10 Hz. The electric pulses were delivered either in classical electroporation cuvettes or in isolated cuvettes. Isolated electrodes