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Electroporation in dense cell suspension—Theoretical and experimental analysis of ion diffusion and cell permeabilization

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Abstract

Electroporation is a process where increased permeability of cells exposed to an electric field is observed. It is used in many biomedical applications including electrogene transfection and electrochemotherapy. Although the increased permeability of the membrane is believed to be the result of pores due to an induced transmembrane voltage U_m, the exact molecular mechanisms are not fully explained.

In this study we analyze transient conductivity changes during the electric pulses and increased membrane permeability for ions and molecules after the pulses in order to determine which parameters affect stabilization of pores, and to analyze the relation between transient pores and long-lived transport pores. By quantifying ion diffusion, fraction of transport pores f_{per} was obtained. A simple model, which assumes a quadratic dependence of f_{per} on E in the area where $U_m > U_c$ very accurately describes experimental values, suggesting that f_{per} increases with higher electric field due to larger permeabilized area and due to higher energy available for pore formation. The fraction of transport pores increases also with the number of pulses N, which suggest that each pulse contributes to formation of more and/or larger stable transport pores, whereas the number of transient pores does not depend on N.

Keywords: Electroporation; Ion diffusion; Conductivity; Cell suspension; Electrochemotherapy

1. Introduction

Electroporation is usually described as the formation of transient pores which are formed in the cell membrane in presence of a strong external electric field. The permeable state of the cell membrane lasts up to minutes after the application of electric pulses, which enables transport of molecules and ions that otherwise cannot pass across the cell membrane. Electroporation, known also as electropermeabilization, is used in many important biological and medical applications, the most promising of these being electrochemotherapy of tumors [1–3] and electrogene transfection [4–7]. Yet in spite of successful use of electroporation in biomedical applications the molecular mechanisms of the involved processes are still not fully explained and there is lack of connection between experimental data and theoretical descriptions of pore formation [8–12].

Electroporation has been observed in many different systems, i.e. lipid bilayers, vesicles, cells in vitro and in vivo. The extensive in vitro studies of electroporation [13–16] examining the effect of different parameters (electric field strength, number of pulses, duration) on the extent of permeabilization—uptake of exogenous molecules, cell survival and resealing, have shown that the critical parameter for electroporation is the electric field strength. Permeabilization occurs only above a certain (phenomenological) E_c which is governed by both duration t_E and number of pulses N [10]. It was also shown [14,16] that neither electrical energy, nor charge of the electric pulses alone determine the extent of permeabilization and that the dependency on E, N and t_E is more complex. Post-electroporation membrane resealing lasts for minutes, is strongly temperature dependant and is governed by slow ATP dependant biological processes.

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Previous more theoretical studies [8–12,17–20] focused on the biophysical aspects of the mechanisms of pore formation. The authors describe formation of hydrophilic pores in the cell membrane, where the change in free energy also depends on the applied electric field strength. For an experimental validation usually measurement of conductivity was made in vitro or with patch-clamp method [17,19,21–26]. The observed transient increase in conductivity during the pulse was explained by the formation of transient pores in the cell membrane. However, this short-lived transient pores cannot explain increased long-lasting permeability of the membrane, which is observed after the pulses.

Based on all these studies it became clear that the relation between transient conductivity changes (transient pores) and transport after the pulses (long-lived pores) is more complex and cannot be explained with theories which only analyze the transient pores during the electric pulses. Only few studies, directly observed and quantified the actual transport across the membrane and analyze the relation between transient changes and long-lived increased permeability of the cell membrane [21–23,26–29]. There is also no general agreement of how the long-lived transport pores become stable and enable transport of molecules as large as DNA.

The main focus of our study is exactly in trying to connect the findings and theoretical description on the level of pore formation and increased membrane conductivity during the electric pulses with direct observation and quantification of increased transport for ions and molecules after the pulses. In order to achieve this, we analyze in parallel the transient pores and the transport pores, how they depend on the electric field and the number of pulses, and the relation between short-lived transient pores and the transport pores. We measured together the observable quantities, which indicate increased membrane permeability: (i) the transient conductivity changes during the pulses (related to transient pores), and (ii) the ion efflux and (iii) the transport of molecules after the pulses (both related to long-lived pores). We performed experiments where the conductivity of a dense cell suspension was measured during and after the application of electrical pulses in a low-conductive medium. We analyzed how electric field strength and the number of pulses affect transient conductivity changes and the ion efflux, and compare both to transport of molecules. We further used diffusion equation to quantify the time dependent ion efflux and present a simple model, which describes the permeability of the membrane (the fraction of long-lived pores) for applied electric field strength and for a given number of pulses.

2. Materials and methods

2.1. Electroporation and current–voltage measurements

The experimental setup consisted of a generator that delivered square pulses, an oscilloscope and a current probe. Two high-voltage generators were used; for protocol where 8×100 μs pulses were used (Fig. 1a) a prototype developed at the University of Ljubljana, Faculty of Electrical Engineering, was used and for the second protocol to deliver N×100 μs+8×1 ms pulses Cliniporator™ (IGEA s.r.l., Carpi, Modena, Italy) device was used, which allowed us to deliver two sets of pulses with a given delay in between (Fig. 1b).

During the pulses the electric current was measured with a current probe (LeCroy AP015, New York, USA) and the applied voltage with the high-voltage probe (Tektronix P5100, Beaverton, USA). Both current and voltage were measured and stored on the oscilloscope (LeCroy 9310 C Dual 400 MHz, New York, USA). In the first experimental protocol we used a train of eight square pulses of 100 μs duration with 1 Hz repetition frequency (8×100 μs protocol). Pulse amplitudes were varied to produce applied electric fields $E_0=U/d$, between 0.4 and 1.8 kV/cm. In the second experimental protocol first a given number N (1, 2, 4 and 8) of 100 μs pulses with repetition frequency 10 Hz were delivered, and after a delay of 4200 ms after the first pulse eight 1 ms test pulses of the same amplitude with the repetition frequency 1 Hz were delivered (N×100 μs+8×1 ms pulsing protocol). The frequency should not effect permeabilization neither the diffusion of ions, since it was shown that the relaxation of conductivity after the pulse is few ms [26] and that changing the pulse repetition frequency between 1 Hz and 1 kHz does not influence significantly the transport of small molecules [30]. The 8×1 ms test pulses were used to determine post-pulses conductivity changes after 4200 ms and the maximum increase (1 ms long pulses were used due to the limitation of the pulse generator, but since only the initial level of the conductivity of the first pulse was analyzed and other pulses were used to determine the maximum conductivity increase, this had no effect on the results).

Each type of experiments was repeated twice. The amplitudes of N pulses in the train and the test pulses were set to voltage which gave applied electric field E_0=1 kV/cm. The memory segmentation function of the oscilloscope was used in order to obtain high time resolution during the pulses and only 100 μs after the pulses were recorded. Parallel aluminum plate electrodes (Eppendorf cuvettes) with 2 mm distances between the electrodes were used. For every set of parameters a reference measurement on medium with no cells was also performed.

2.2. Cells and medium

Mouse melanoma cell line, B16F1, was used in experiments. Cells were grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) at 37 °C in a humidified 5% CO_2 atmosphere in the incubator (WTB Binder, Labortechnik GmbH, Germany). For all experiments the cell suspension was prepared from confluent cultures with 0.05% trypsin solution containing 0.02% EDTA (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany). From the

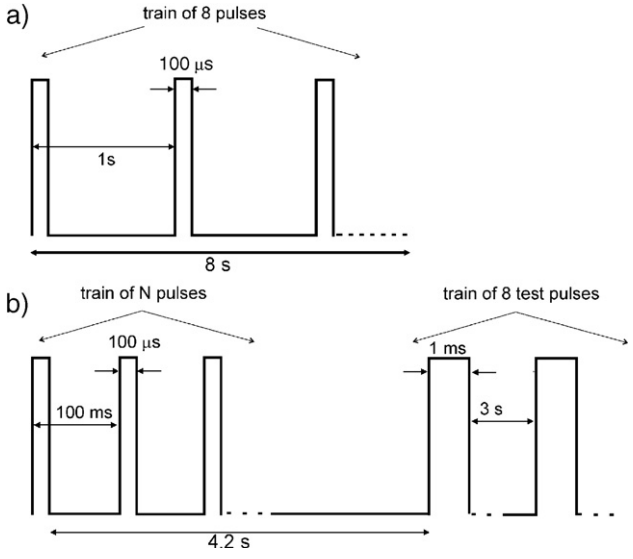

Fig. 1. The two pulsing protocols. In one set of experiments a train of eight pulses 8×100 μs with repetition frequency 1 Hz was delivered (a). In the second set of experiments we used a sequence of N=1, 2, 4 and 8 pulses with repetition frequency 10 Hz and after a delay (between the first pulse and first test pulse), which was set to be 4200 ms, a train of eight test pulses of the same amplitude as of the first pulses was delivered—a N×100 μs+8×1 ms protocol (b).