Dependence of the Energy of Electric Pulse Required to Electroporate the Cell on the Pulse Duration for Pulses from 50 ns to 500 ms

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ABSTRACT

The aim of this study was to investigate the relationships between the energy of the PEF treatment required to electroporate the cell and pulse duration and to determine whether there exists the electric pulse with the minimum energy required to electroporate the cell.

Experiments were performed *in vitro* with human erythrocytes and mouse hepatoma cell line MH-22A. Cell electroporation was determined from the extent of the release of intra-cellular K^+ and from the extent of erythrocyte lysis after long incubation at low temperature.

Energy of an electric pulse required to electroporate the cell was a function of the pulse duration and there existed a minimum of the pulse energy required to porate the cell for both theoretical and experimental dependencies. It was situated in the range of 0.1–10 μs. It depended on the cell size and was about 0.13 and 0.07 J/g for human erythrocytes (radius 3.0 μm) and mouse hepatoma MH-22A cells (radius 7.7 μm), respectively.

Keywords: PEF, pore energy, mouse hepatoma, human erythrocytes, electropermeabilization

1 INTRODUCTION

Treatment with pulsed electric fields (PEF) is a promising technology with an increasing number of applications in biology, oncology, genetics, immunology, and biotechnology [1]. However, to become competitive with other alternative treatments of food, cells, tissue, etc., PEF-processing costs (estimated within the range of 0.01–0.2 US$/L [2]) have to be reduced. For practical applications of pulsed electric fields, it is important to know whether there is any particular electric treatment (pulse duration, field strength, etc.), which would require the minimum energy. In PEF treatment, single or multiple (up to tens of thousands) pulses of various durations (from 5 ns to 100 ms – more than 6 orders of magnitude!) are used. However, the detailed relationships between treatment energy and pulse parameters have not examined in details yet.

The aim of this study was to investigate the relationships between the energy of the PEF treatment required to electroporate the cell and pulse duration both theoretically and experimentally and to determine whether there exists the electric pulse with the minimum energy required to electroporate the cell.

2 MATERIALS AND METHODS

2.1 Theoretical Calculations

The energy of an ideal square-wave electric pulse per unit volume W (absorbed dose, in J/g) was calculated from

$$W = \left(\frac{\kappa}{V}\right)\tau_i E_0^2 \tag{1}$$

where κ is the specific conductivity of the medium, V is the volume of the medium (50 μl) subjected to an electric pulse, τ_i is the pulse length, and E_0 is the electric field strength.

Relationship between the parameters of the electric pulse as a result of the exposure to which a single pore has appeared in the cell was obtained from [3]:

$$\int_0^\infty k_{f0}(E,t)dt = 1 \tag{2}$$

where $k_{f0}(E,t)$ is the rate of pore formation at electric field strength E.

For a spherical cell, this rate is can be calculated from [4]:

$$k_{f0}(E_0) = D\int_{-1}^{1} \exp\left[G\left(1.5E_0 ay - \Delta\Phi_0\right)^2\right]dy, \tag{3}$$

where

$$D = \frac{2\pi\nu a^2}{a_l}\exp\left[-\frac{\Delta W_f(0)}{k_B T}\right],$$
$$G = \pi C_m \frac{\varepsilon_w/\varepsilon_m - 1}{2k_B T} r_*^2, \tag{4}$$
$$y = \cos\theta.$$

Here a is the cell radius, $\Delta\Phi_0$ is the resting potential, ν is the frequency of lateral fluctuations of the lipid molecules, a is the cell radius, a_l is the area per lipid molecule, k_B is Boltzmann's constant, T is the absolute temperature, $\Delta W_f(0)$ is the energy barrier to pore formation at zero

transmembrane potential and r_* is the radius of the pore corresponding to the top of this barrier, C_m is the specific membrane capacitance, ε_m and ε_w are the specific permittivities of the membrane and water inside the pore respectively, and θ is the angle between the normal to the membrane surface and the field direction.

The transmembrane potential generated by a square-wave electric pulse across the non-conducting membrane of a spherical cell increases over time according to [5,6]:

$$\Delta\Phi_g(t) = 1.5 E_0 a\left[1 - \exp\left(-\frac{t}{\tau_c}\right)\right], \text{ for } 0 \le t \le \tau_i. \quad (5)$$

$$\Delta\Phi_g(t) = \frac{3}{2} E_0 a \exp\left(-\frac{t-\tau_i}{\tau_c}\right), \text{ for } t > \tau_i. \quad (6)$$

Here τ_c is a time constant of the cell plasma membrane charging process. For a spherical cell with the radius a = 1–50 μm, this time constant is within the range of 100 ns – 10 μs [7,8].

Theoretical relationships between the parameters of the electric treatment, required to porate the cell were obtained from (1)-(6). The definite integral in equation (2) was calculated numerically by using Gaussian quadrature technique.

For calculations, the following set of parametersparameters (we called this set a "standard cell"): $\nu = 10^{11}$ s^{-1} [9], a_l = 0.6 nm^2 [9], $\Delta W_f(0)$ = 45 k_BT [9], r_* = 0.3 nm [9], C_m = 1 μF/cm^2 [10], ε_m = 2 [11], ε_w = 81, a = 3.5 μm, T = 295 K, $\Delta\Phi_0$ = 25 mV, τ_c = 0.3 μs, and κ = 1.0 S/m.

2.2 Media and Chemicals

A a medium for electroporation of human erythrocytes, a 9:1 mixture of 154 mM sodium chloride or sodium phosphate buffer (138 mM NaCl, 15 mM Na_2HPO_4/NaH_2PO_4, pH 7.4) and isotonic (272 mM) sucrose solution was used. The sucrose solution was a 9:1 mixture of phosphate-buffered and isotonic (272 mM) sucrose solutions.

The cell culture medium was Dulbecco's modified Eagle's medium (cat. no. D5546, Sigma-Aldrich Chemie, Steinheim, Germany) supplemented with 10% fetal bovine serum (F7524, Sigma-Aldrich Chemie), 1% L-glutamine (G7513, Sigma-Aldrich Chemie), 100 U/ml penicillin, and 90 μg/ml streptomycin (P0781, Sigma-Aldrich Chemie).

Calibration solutions containing 0.2-100 mM KCl were prepared by diluting a stock solution of 100 mM KCl and adding 150 mM sodium chloride and 8 mM sodium benzoate [12].

2.3 Cells

Experiments were performed *in vitro* with human erythrocytes and mouse hepatoma cell line MH-22A. Blood was collected on heparin and the erythrocytes were isolated by centrifugation. The plasma and buffer coat comprising the white blood cells and platelets was carefully removed by aspiration. The cells were then washed three times with isotonic sodium chloride solution (0.9% NaCl) and suspended in the electroporation medium at a volume concentration of 1 or 9%. Erythrocytes were electroporated within the day of preparation.

The MH-22A cells were grown in monolayer cultures in 75-cm^2 (250 ml) flasks (Greiner Bio-One, Frickenhausen, Germany) at 37 °C in a humidified 5 % CO_2/95 % O_2 atmosphere in incubator IR AutoFlow NU-2500E (NuAire, Plymouth, MN, USA). Manipulations under sterile conditions were done in vertical laminar flow cabinet Aura Vertical SD4 (BIOAIR Instruments, Siziano, Italy).

When cells reached confluence, they were trypsinazed for 2–10 min with 2 ml of 0.25% trypsin-0.02% ethylenediaminetetraacetic acid (EDTA) solution (cat. no. T4049, Sigma-Aldrich Chemie). When cells detached from the flask bottom, cell suspension was supplemented with 2 ml culture medium to protect cells from further action of trypsin. After centrifugation of the suspension for 5 min at 1000 rpm at room temperature, cells were resuspended in the culture medium at approximately 2–5×10^7 cells/ml and kept for 60–70 min at room temperature (20–21 °C). During this time, the cells restored the normal level of the intracellular concentration of potassium ions [12]. Then the cells were electroporated within 15–20 min.

2.4 Cell Electroporation

A 50-μl droplet of cell suspension was placed between a pair of flat stainless-steel electrodes and subjected to a single square-wave electric pulse. The distance between the electrodes was 2 mm. Single square-wave pulses with duration ranging from 95 ns to 2 ms and the amplitude ranging from 0.25 to 12.5 kV/cm were used.

2.5 Determination of Cell Electroporation

Two methods were employed to determine the fraction of electroporated cells: i) from the extent of the release of intracellular potassium ions [12] and ii) from the extent of the hemolysis of electroporated erythrocytes after long (20–24 h) incubation in 0.9 or 0.63% NaCl solution at 4 °C [4].

i) After the electric pulse, cells were immediately transferred to a chilled Eppendorf tube, kept on ice for 5-10 min, and then kept for 30–40 min at 10–11 °C to prevent pores from closing and to allow equilibration between intracellular and extracellular K^+ concentrations. The extracellular potassium concentration was measured by means of a mini K^+-selective and reference electrodes (Diamond Micro Sensors, Ann Arbor, MI, USA) [12]. Potential measurements were made with a pH-meter-millivoltmeter pH-150M (Gomel Factory of Measurement

Instruments, Gomel, Belorussia).

ii) After the electric pulse, 12 or 200 µl of the erythrocyte suspension was mixed with 5 ml of 0.63 or 0.9% NaCl at 4 °C and incubated at the same temperature. After 20–24 h, the erythrocytes were spun down and the concentration of haemoglobin in the supernatant was measured photometrically at λ = 410 nm. The value corresponding to 100% haemolysis was obtained by hypotonic lysis in distilled water. The extent of haemolysis shows the fraction of cells with pores permeable to sodium ions (electroporated cells) [4].

3 RESULTS

First, the relationship between the electric field strength required to porate a spherical cell, E_p, and the length of the square-wave pulse, τ_i, was calculated for the "standard cell" according to Eqns. (2)-(6). Results are shown in Fig. 1A.

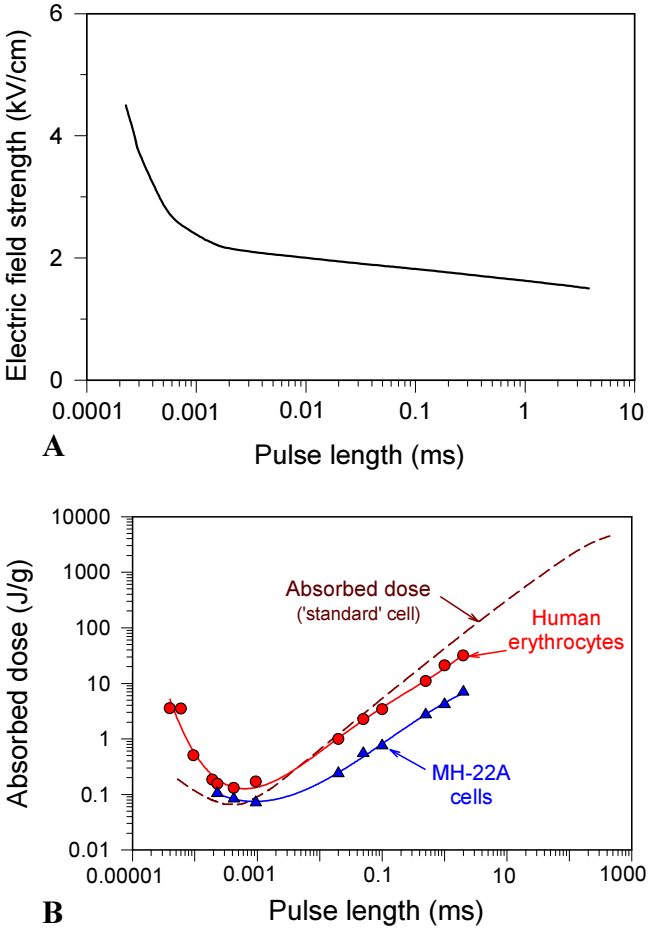

Figure 1: **(A)** Theoretical dependence of the electric field strength necessary to create one pore in the cell (electroporation), E_p, on the pulse duration calculated from (1)-(7) for the "standard cell" for the square-wave electric pulse. **(B)** The theoretical (dashed line) and experimental dependencies of the energy of an electric pulse (absorbed dose) required to porate the cell on the duration of the square wave electric pulse.

It has been obtained that the cell poration time depends on the pulse intensity: the shorter the pulse length, the higher the field strength should be (Fig. 1A). This dependence is much more pronounced for short pulses (τ_i <10 τ_m). For long pulses (τ_i >10 τ_m) the electric field strength required for the poration of cell membranes increases significantly slower with decreasing the pulse length (Fig. 1A).

Then the theoretical dependence of the energy of an electric pulse (absorbed dose) required to porate the 'standard' cell on the duration of the square-wave was calculated according to Eqs. (1)–(6) for pulses with the duration from 50 ns to 500 ms (dashed line in Fig. 1). It can be seen that the dependence has a minumum at the pulse duration equal to 0.3–0.5 µs. The value of the mimimal energy pulse which is enough to porate the 'standard' cell is equal to 0.066 J/g (the radius of our "standard cell" is 3.5 µm).

Theoretical analysis was compared with experimental data by determining the experimental dependencies of the energy (absorbed dose) of a single square-wave electric pulse required to electroporate 50% of human erythrocytes and mouse hepatoma MH-22A cells as a function of the pulse duration. The experiments were carried out for pulses with the durations from 40 to 2 ms (Fig. 1B). Their shape is similar to the theoretical dependence.

Both experimental dependences have a minimum as well, which is situated at τ_i = 0.3–0.5 and 0.7–1.0 µs for human erythrocytes and mouse hepatoma MH-22A cells, respectively. The minimum energy required to electroporate the cells is equal to 0.13 J/g for human erythrocytes (radius 3 µm) and 0.07 J/g for mouse hepatoma MH-22A cells (radius 7.7 µm).

The values are by a few orders of magnitude lower than typical values of the energy of electric treatment required for microorganism inactivation (in the range of hundreds of J/g [2,13]). However, we recall that the transmembrane potential generated by the external electric field is directly proportional to the cell radius [5] and due to this it is more difficult to electroporate smaller cells, e.g. bacteria. In addition, to kill the cell by the electric treatment, stronger electric pulses are needed than those, which are enough to just electroporate the cell [14,15]. One of the main reasons of this is pore resealing [16] – under appropriate conditions, pores disappear completely [17,18] and cells retain their viability [19,20].

4 CONCLUSION

Energy of an electric pulse required to electroporate the cell is a function of the pulse duration and there exists a minimum of the pulse energy required to porate the cell situated in the range of 0.1–10 µs. It depends on the cell size and is about 0.13 and 0.07 J/g for human erythrocytes (radius 3 µm) and mouse hepatoma MH-22A cells (radius 7.7 µm).

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