Size of the Pores Created by an Electric Pulse: 
Microsecond vs Millisecond Pulses

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ABSTRACT

Modeling studies have revealed that the pulse of nanosecond-duration should create smaller pores than the pulse of micro-millisecond duration. It can be expected, that similar differences may also exist between the pore populations created by the pulses of micro- and millisecond-durations. Here, the size of the pores created by a single square-wave electric pulse with different durations has been compared. In the large portion (30-60 %) of electroporated mouse hepatoma MH-22A and Chinese hamster ovary cells the pores created by an electric pulse with the duration of 100 µs are smaller than the molecule of bleomycin. In the case of 2 ms-duration pulse, irrespective of the amplitude of an electric pulse, in almost all cells, which were electroporated, the pores created were larger than the size of the molecules of bleomycin. The pulses with the duration of 100 µs and 2 ms were used. For the cells studied, short 100 µs-duration pulse created smaller pores than longer 2 ms-duration pulse.

Keywords: electroporation, electroporemeabilization, mouse hepatoma, CHO, bleomycin

1 INTRODUCTION

For many applications of cell electroporation, it is desirable to know the size of the pores created in the cell plasma membrane. Although, the size of the pores created in the cell membrane by electric pulses has been estimated numerous times [1-4], there is a lack in the studies in which the size of the pores corresponding to the threshold of electroporation would be estimated for different pulse durations. Meanwhile, modeling studies have revealed that the pulse of nanosecond-duration should create smaller pores than the pulse of micro-millisecond duration [5]. However, the number of pores created by nanosecond-duration pulses should be by 2-3 orders of magnitude more than in the case of longer pulse [5]. It can be expected, that similar differences should also exist between the pore populations created by the pulses of micro- and millisecond-durations. Here, the sizes of the pores, which were created by a square-wave electric pulse with the duration of 100 µs and 2 ms, have been compared. This has been done by determining the fraction of electroporated cells (permeable to potassium ions the hydrated radius of which is 0.16–0.22 nm [6]) and the cells permeable to bleomycin (M_r ~ 1500 Da, r ~ 0.8 nm). Also, the fraction of the cells that were killed by the electric treatment was estimated. Experiments were carried out with mouse hepatoma MH-22A and Chinese hamster ovary (CHO) cells.

2 MATERIALS AND METHODS

The culture medium consisted of Dulbecco’s modified Eagle’s medium (cat. no. D5546, Sigma-Aldrich Chemie, Steinheim, Germany) supplemented with 10 % fetal bovine serum (cat. no. F7524, Sigma-Aldrich Chemie), 1% L-glutamine (cat. no. G7513, Sigma-Aldrich Chemie), 100 U/ml penicillin, and 100 µg/ml streptomycin (cat. no. P0781, Sigma-Aldrich Chemie). As an electroporation medium, either the culture medium (for the determination of cell electroporation) or minimum essential medium Eagle (Sigma-Aldrich Chemie) was used. Bleomycin hydrochloride (Bleocin, Nippon Kayaku, Tokyo, Japan) was obtained as a crystalline powder and dissolved in sterile 0.9 % NaCl solution (Balkanpharma-Troyan, Troyan, Bulgaria) at a concentration of 1 mM. Further dilutions were also made in sterile 0.9 % NaCl solution.

The mouse hepatoma MH-22A and Chinese hamster ovary cells were grown in monolayer cultures in 75-cm² flasks at 37 °C and 5 % CO₂ in a water-jacketed incubator. When cells reached confluence they were trypsinized, suspended in the culture medium at approximately 2-5×10⁶ cells/ml, and kept for 60–70 min at room temperature (20–21 °C) [7].

For electroporation, single square-wave pulses with the duration of 100 µs and 2 ms and the amplitude ranging from 0.2 to 2.4 kV/cm were used. A 50-µl droplet of cell suspension was placed between a pair of flat stainless-steel electrodes and was subjected to a single square-wave electric pulse.

When determining the fraction of electroporated cells, after the exposure to an electric pulse, the cell suspension was pipetted out of the chamber and immediately transferred to a chilled Eppendorf tube. To prevent pores from closing and to allow equilibration between intracellular and extracellular K⁺ concentrations, the cells were kept on ice for 5-10 min and then kept for 30-40 min at 10-11 °C. Then, the extracellular potassium concentration
was measured by means of a mini K⁺-selective electrode [7,8].

Mini K⁺-selective and reference electrodes (cat. nos. 601 and 401, respectively, Diamond Electro-Tech) manufactured by Diamond Micro Sensors (Ann Arbor, MI, USA) were utilized. The reference electrode was of the silver/silver chloride type with an internal salt bridge. Filling electrolyte was 3 M NaCl, so contamination of the solution with K⁺ ions did not occur. Potential measurements were made with pH-meter-millivoltmeter pH-150M (Gomel Factory of Measurement Instruments, Gomel, Byelorussia). All measurements were made at a temperature of 10-11 °C [7,8].

The fraction of electroporated cells was determined from [7,8]:

\[
F = \frac{[K^+]_x - [K^+]_{100\%}}{[K^+]_{100\%} - [K^+]_{50\%}},
\]

where [K⁺]₀, [K⁺]ᵢ, and [K⁺]₁₀₀% are the extracellular concentrations of potassium ions in the sample and solutions with intact and 100% electroporated cells, respectively.

When determining the fraction of cells the membranes of which have become permeable to bleomycin, 45 µl of the cell suspension (prepared in a minimum essential medium Eagle) was mixed with 5 µl of 200 mM bleomycin solution, placed between two stainless-steel electrodes 2 mm apart, and subjected to a square-wave electric pulse [7].

After a 50-µl droplet of cell suspension was subjected to a single square-wave electric pulse, the fraction of the cells whose membrane has become permeable to bleomycin and the fraction the dead cells were determined from the reduction of the cell viability [9].

Cell viability was determined by means of a colony-forming assay [10]. Although this assay is time consuming and labor intensive, particularly when many samples are being processed, but it is the most consistent, relevant, and reproducible viability assay in vitro [11].

The cells were seeded in Petri dishes in duplicate and were grown at 37 °C and 5% CO₂ in water-jacketed incubator IR AutoFlow NU-2500E (NuAire, Plymouth, MN, USA) for 6-7 (CHO cells) or 9-10 (mouse hepatoma MH-22A cells) days. Then, the formed colonies were fixed with 96% ethanol (Stumbrau, Kaunas, Lithuania), stained with a gram’s crystal violet solution (Fluka Chemie, Buchs, Germany), and counted under a binocular light microscope (MBS-9, LOMO, St. Petersburg, Russia). The survival of the cells treated with electric pulses was calculated as the percentage of the colonies obtained from the untreated control cells.

The dependences of the fraction of electroporated cells, the cells permeable to bleomycin or killed by an electric pulse, \( F(E₀) \), on the pulse amplitude were fitted by a three-parameter sigmoid curve [7],

\[
F(E₀) = \frac{F_{max}}{1 + \exp((E_c - E₀)/b)},
\]

where \( F_{max} \) is the maximal value of the fraction of electroporated cells, the cells permeable to bleomycin or killed by an electric pulse, \( E_c \) is the amplitude of the electric pulse at which \( F(E_c) = F_{max}/2 \), and \( b \) controls how steeply the curve rises.

### 3 RESULTS AND DISCUSSION

Theoretical modeling shows that while electric pulse is on, the pore population evolves by changing both the number and sizes of pores [12–14]. For example, modeling studies have revealed that the pulse of nanosecond-duration should create smaller pores than the pulse of micro-millisecond duration. At the same time, the number of pores created by nanosecond-duration pulses should be by 2–3 orders of magnitude higher than in the case of longer pulse [5]. Theoretical analysis of electroporation of a single cell showed that similar differences should also exist between the pore populations created by the pulses of 30–100 µs and 1 ms-durations [14,15].

The experimental relationships between various parameters, such as, the amplitude, duration, and/or number of pulses as well as other ones, of the electric treatment required for electroporation (increase of the membrane permeability to the particular test molecules or ions) have also been obtained [2,3,16–19]. However, there is a lack in the studies, in which the size of the pores corresponding to the electroporation threshold would be estimated for pulses of different durations. Here, the size of the pores, which were created by a square-wave electric pulse with the duration of 100 µs and 2 ms and the amplitude just over the electroporation threshold, has been compared.

The fraction of electroporated cells can be estimated by determining the extent of the release of intracellular K⁺ ions from the cells exposed to an electric pulse [7,8]. In this study, this approach was used to obtain the dependences of the fraction of electroporated cells on the pulse intensity. The cells were exposed to a single square-wave electric pulse with the duration of 100 µs or 2 ms, the amplitude of which was varied from 0.2 to 1.2 kV/cm.

In addition, the dependences of the fraction of the cells the plasma membrane of which has become permeable to bleomycin on the pulse intensity were obtained for the cells exposed to a single square-wave electric pulse with the duration of 100 µs and 2 ms. The amplitude of the pulse was varied from 0.2 to 2.4 kV/cm.

Bleomycin is a membrane-impermeable cytotoxic drug with a molecular weight of approximately 1450 Da [20]. The smallest radius of the molecule of bleomycin is approximately 0.8 nm. At the concentrations lower than 50 nM, bleomycin does not influence the survival of intact mouse hepatoma MH-A22 and Chinese hamster ovary cells [7]. However, if, under the action of an electric pulse, the cell membrane becomes permeable to bleomycin, it enters the cell cytosol and causes cell death through apoptosis or necrosis, depending on the number of molecules that reach...
the cytosol [21]. So, the fraction of the cells whose membrane has become permeable to bleomycin can be estimated from the reduction of the cell viability [9].

The results obtained for mouse hepatoma MH-22A and CHO cells exposed by a single square-wave electric pulse with the duration of 100 µs are shown in Fig. 1. It can be seen, that in the large portion of electroporated cells the pores created by an electric pulse with the duration of 100 µs are smaller than the molecule of bleomycin. For example, exposure of cells by an electric pulse with the amplitude of 0.8 kV/cm leads to electroporation of 68-75 % of cells. However, the pores permeable to the molecules of bleomycin were created only in 24-38 % of electroporated cells. This means that the pores created in about 30-60 % of cells were large enough to allow potassium ions to leave the cells but small enough to prevent bleomycin from reaching the cell cytosol.

Figure 1: The dependences of electroporated, permeabilized to bleomycin, and dead cells on the amplitude of a square-wave electric pulse with the duration of 100 µs obtained for (A) mouse hepatoma MH-22A and (B) Chinese hamster ovary cells.

Figure 2: The dependences of electroporated, permeabilized to bleomycin, and dead cells on the amplitude of a square-wave electric pulse with the duration of 2 ms obtained for (A) mouse hepatoma MH-22A and (B) Chinese hamster ovary cells.

In the case of a 2 ms-duration pulse, the curves showing the dependence of the fraction of the cells that have become permeable to bleomycin are close to the ones showing the release of intracellular potassium ions (Fig. 2). That is, irrespective of the amplitude of an electric pulse, in almost all cells, which were electroporated, the created pores were larger than the size of the molecules of bleomycin. This is strong evidence that the pores created by the pulse with the duration of 2 ms are larger than the pores created with a 100 µs-duration pulse.

So, the experimental results on mouse hepatoma and CHO cells presented here as well as obtained in different laboratories on other cell lines [16,18] confirm the conclusions obtained in theoretical studies [5,12–14] that increasing the amplitude and/or the duration of the electric pulse, increases the size of pores. Usually, to create larger pores longer pulses are needed [3,5,7,16,18].
4 CONCLUSION

The size of the pores created by a square-wave electric pulse depends on the pulse duration. A short 100 µs-duration pulse creates smaller pores than the longer 2 ms-duration pulse.

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REFERENCES