Equivalent Pulse Parameters for Electroporation

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***Abstract*—Electroporation-based applications require the use of specific pulse parameters for a successful outcome. When recommended values of pulse parameters cannot be set, similar outcomes can be obtained by using equivalent pulse parameters. We determined the relations between the amplitude and duration/number of pulses resulting in the same fraction of electroporated cells. Pulse duration was varied from 150 ns to 100 ms, and the number of pulses from 1 to 128. Fura 2-AM was used to determine electroporation of cells to Ca^{2+}. With longer pulses or higher number of pulses, lower amplitudes are needed for the same fraction of electroporated cells. The expression derived from the model of electroporation could describe the measured data on the whole interval of pulse durations. In a narrower range (0.1–100 ms), less complex, logarithmic or power functions could be used instead. The relation between amplitude and number of pulses could best be described with a power function or an exponential function. We show that relatively simple two-parameter power or logarithmic functions are useful when equivalent pulse parameters for electroporation are sought. Such mathematical relations between pulse parameters can be important in planning of electroporation-based treatments, such as electrochemotherapy and nonthermal irreversible electroporation.**

***Index Terms*—CHO cells, electropermeabilization, Fura 2-AM, strength–duration relationship.**

I. Introduction

ELECTROPORATION, as a method for increasing cell membrane permeability to molecules that are otherwise poorly membrane permeant, is used in various biotechnological and biomedical applications, such as the introduction of molecules into cells [1], [2], cell fusion [3], [4], tissue ablation [5]–[7], and sterilization of water and liquid food [8]–[10]. In experimental settings, electroporation is normally performed by placing a biological sample (e.g., cell suspension or a small part of a tissue) between the electrodes and delivering a single electric pulse or a train of such pulses to the electrodes, thus creating the electric field between them. The efficiency of electroporation can be interpreted differently in different applications of electroporation. For example, in electroporation-mediated uptake of molecules, efficient electroporation is associated with a high number of cells loaded with exogenous molecules that also survive the treatment, while electroporation efficiency in tissue ablation and sterilization is related to killing the largest amount of target cells or microorganisms. However, efficient electroporation is obtained only after careful adjustment of the pulse parameters, among which the pulse amplitude, pulse duration, and number of pulses have the largest impact on the outcome of the experiment.

Each specific application of electroporation requires somewhat different settings of pulse parameters. In addition, pulse parameters need to be adjusted for a particular cell type, cell size, orientation and density of cells, and other experimental conditions, meaning that they can differ substantially even within a given application of electroporation. To date, there have been a vast number of different pulse protocols reported for various applications of electroporation. For example, for the introduction of small molecules, pulses with amplitudes in the range of 1 kV/cm and durations extending from hundred μs to ms are used [11]–[17]. Larger molecules can be introduced using three different combinations of pulse parameters: 1) with pulse amplitudes up to few kV/cm, lasting from few μs to hundred μs [18], [19]; 2) with low pulse amplitudes of few hundred V/cm but durations ranging into tens of ms [20]; 3) with a combination of short high-amplitude pulses and long low-amplitude pulses (mostly for the uptake of DNA) [21]–[25]. For sterilization in food and drink industry, the pulse amplitudes should be larger than 15 kV/cm in order to electroporate the membranes of microorganisms, which are smaller than eukaryotic cells, while pulse durations range from μs to ms [9], [26]. For electroporation of the cell organelle membranes, pulses with durations of several tens of ns and amplitudes of tens of kV/cm or more are used [27]–[30].

Sometimes, the specific pulse parameters required for efficient electroporation are difficult to obtain. This might be due to experimental setup (e.g., large samples of cells, high conductivity of the medium, electrode configurations, etc.) and limitations of the available pulse generators in terms of the maximum output current or voltage (see, e.g., a review of pulse generators in [31]). For these reasons, the pulse parameters can be usually adjusted within a confined range of values. But, as illustrated in the aforementioned paragraph, similar outcomes of the experiment can also be obtained by using equivalent pulse parameters. For example, instead of using a number of short, high-voltage pulses, one can either use longer pulses with lower voltage or adjust the number of pulses by keeping the amplitude or duration of the pulses unchanged. However, finding a suitable combination of pulse parameters proved to be a difficult task, since simple relations, such as keeping the same energy of the pulses, turned out to be inefficient [1], [32]. Therefore, more specific functional relations between the pulse parameters should be identified in order to avoid the excessive amount of experiments and time needed to determine the suitable parameters.

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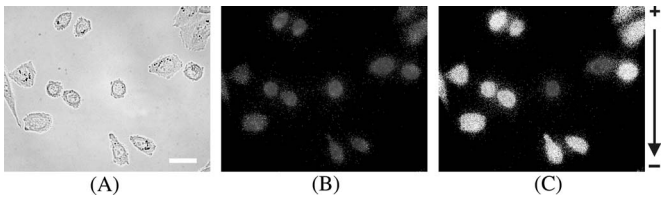

Fig. 1. Monitoring electroporation. (A) CHO cells—bright field. (B) Ratio of fluorescence (F_{345}/F_{385}) for cells in control (nonporated cells). (C) Cells 1 min after electroporation with a 250 V/cm, 10 ms pulse. Brighter cells were electroporated. Arrow denotes the field direction. Bar represents 20 μm.

The role of pulse parameters in the efficiency of electroporation was already systematically addressed in several studies [1], [12], [15], [32]–[37]. In some of these studies, the authors also tried to determine the mathematical relations between pulse parameters that lead to the same efficiency of electroporation [33]–[35], [38]–[40]. We describe them in more detail in Section II. In general, different functional dependences between pulse parameters were reported and they were mostly given for the relation between the pulse amplitude and the pulse duration. Besides, the relations were obtained from relatively narrow ranges of parameter values, and the parameters were taken from different intervals. In our present study, we measured the relation between amplitude and duration, and between amplitude and number of pulses that result in the same fraction of electroporated cells. Pulse duration was varied in the range from 150 ns to 100 ms and the number of pulses from 1 to 128 pulses (for 100 μs pulse duration). Mathematical relations from the literature were then fitted to the measured data in order to investigate whether these relations could be used to determine equivalent pulse parameters on a wide range of their values. The relations between pulse parameters obtained in this manner are needed in specific electroporation-based applications, such as treatment planning in electrochemotherapy, where equivalent pulse parameters can be used if predicted parameters are unavailable with the particular pulse generator.

II. Materials and Methods

A. Cells

Chinese hamster ovary cells (CHO-K1) were plated in Lab-Tek II chambers (Nalge Nunc Int., NY) or on cover glasses at 2×10^5 cells/mL in the culture medium HAM-F12 supplemented with 8% fetal calf serum, 0.15 mg/mL L-glutamine (all three from Sigma-Aldrich, Germany), 200 units/mL benzylpenicillin (penicillin G), and 16 mg/mL gentamicin and incubated in 5% CO_2 at 37 °C. The experiments were performed 12–18 h after plating, when most cells firmly attached to the surface of the chamber or the cover glass and most of them did not yet divide [see Fig. 1(A)].

B. Detection of Electroporation

To determine which cells were electroporated, a fluorescent calcium indicator Fura 2-AM (Molecular probes, The Netherlands) was used. Fura 2-AM enters the cell through an intact membrane, and is transformed in the cytosol into Fura 2, a membrane-impermeant ratiometric dye. Electroporation results in the entry of Ca^{2+} ions into the cells, where their binding to Fura 2 causes the change in the fluorescence of the dye. With moderate pulse parameters, the cell membrane recovers after electroporation (see Section II-D), and the cell stores the excess Ca^{2+} into its intracellular reservoirs or excludes it from the cytoplasm. The fluorescence thus returns gradually to the initial value, allowing for another repetition of the experiment on the same cells.

Prior to experiments, the culture medium was replaced by a staining solution, which was a mixture of fresh medium and 2 μM of Fura 2-AM. After 25 min of incubation at room temperature, the staining mixture was washed with a fresh culture medium to remove the excess dye. The culture medium contains approximately 1 mM of Ca^{2+}, meaning that Ca^{2+} ions were present in the extracellular medium but were nearly absent from the cytosol, as Ca^{2+} do not readily cross the nonporated cell membrane.

Cells were observed under a fluorescence microscope ($\times$40 objective, AxioVert 200, Zeiss, Germany) equipped with a charge-coupled device camera and a monochromator (both Visitron, Germany). The changes in intracellular concentration of calcium, resulting from electroporation, were determined ratiometrically using MetaFluor 7.1 software (Molecular Devices, GB), with the excitation wavelengths set at 345 and 385 nm, and the emission measured at 540 nm for both excitation wavelengths. The ratio images were obtained by dividing the fluorescence image of cells excited at 345 nm with the image excited at 385 nm (ratio $= F_{345}/F_{385}$) [see Fig. 1(B) and (C)]. The fraction of cells electroporated for Ca^{2+} ions (% electroporated) were determined by counting the number of fluorescent cells, n_F, and dividing their number with the whole number of cells, n_C, in the field of view (% electroporated $= 100(n_F/n_C)$). The concentration of intracellular Ca^{2+} was determined qualitatively by measuring the ratio values for each electroporated cell for a period of 1 min after electroporation. These values were determined by encircling the cells with regions of interest in MetaFluor and integrating the ratio values within these regions. The maximum ratios measured in each electroporated cell were averaged and then presented on a graph.

C. Electroporation

Laboratory prototype of a Cliniporator device (IGEA, Italy), a prototype of a microsecond square wave pulse generator with a fast switch [41], and a prototype of a nanosecond pulse generator [42] were used to generate rectangular electric pulses needed to electroporate the cells. Different generators had to be used because all parameters could not be generated by a single device. Either a single pulse with duration of 150 ns, 1 μs, 3 μs, 10 μs, 30 μs, 100 μs, 1 ms, 10 ms, 50 ms, or 100 ms or a train of 2, 4, 8, 16, 32, 64, or 128 pulses (1 Hz repetition frequency) with duration of 100 μs was delivered to cells. The pulse amplitude and the current flowing through the cells were monitored with an oscilloscope. The duration or the number of pulses in each experiment was chosen randomly from the given set of parameters. Attention was paid not to apply too many pulses to cells in

one experiment, especially when a train of pulses was delivered (e.g., 64 and 128 pulses were never applied to the same cells). For a given pulse duration or number of pulses, the pulse amplitude was increased stepwise until ∼70% of cells (an arbitrarily chosen value) were electroporated. These pulse amplitudes were then transformed into equivalent voltage-to-electrode-distance ratios (or electric field intensities E_{70}) and plotted on a graph.

Between two successive increments of pulse amplitude we waited for at least 5 min for cell recovery (verified in a separate experiment, see later), except if cells already became electroporated. In this case, we first waited until the fluorescence of cells returned to the initial value, and then, after additional 5 min, delivered the pulses with higher amplitude. During the experiments, cells were kept at 37 °C by means of a heated microscope stage to facilitate cell recovery.

Pulses longer than 1 μs were delivered to a pair of parallel Pt/Ir wire electrodes with 0.8 mm diameter and 4 mm distance between them, which were positioned at the bottom of the Lab-Tek chamber. The field distribution between the electrodes was homogenous in the central region between the electrodes, where the calculated field was equal to the applied voltage-to-electrode-distance ratio [43]. Different electrodes, with smaller interelectrode distance, had to be used for pulses of 150 ns duration, because of the high intensity of the field, required to electroporate the cells with such pulses. The electrodes were made of two adjacent 30 μm thick flat gold layers mounted on a microscope glass slide, and separated by 100 μm [30]. The cover glass with cells was placed on top of the electrodes with cells facing down.

D. Cell Recovery After Electroporation

To test if cells recovered during the 5 min delay between two successive pulses, we performed an additional experiment. Cells were prepared and incubated with the dye as described previously. However, they were not electroporated in the culture medium but in a calcium-depleted modification of minimum essential medium (MEM) (SMEM, Gibco) supplemented with 5 μM of ethylene glycol tetraacetic acid (EGTA) to remove the remaining Ca^{2+} from the medium. The pulse with the amplitude leading to 70% of electroporated cells was delivered, and 5 min later the medium was replaced with fresh SMEM supplemented with 1 mM $CaCl_2$.

The ratio of fluorescence from cells did not change significantly after electroporation in Ca^{2+} depleted medium and remained at the same level even after the addition of Ca^{2+}, 5 min after electroporation. This shows that the fraction of electroporated cells (% electroporated) were not influenced by the possible intracellular release of Ca^{2+} (Ca^{2+} induced Ca^{2+} release) and that 5 min interval was sufficient for cell membrane recovery to Ca^{2+} ions. The same pulse applied again resulted in an increase of the ratio of fluorescence, confirming that the previous pulse indeed electroporated the cells. Cell recovery after exposure to all investigated pulse parameters was verified in the same manner.

E. Fitting the Relations Between Pulse Parameters to the Measured Data

Experimentally determined pulse amplitudes leading to the same fraction of electroporated cells at different pulse durations or number of pulses were plotted on graphs using Sigma Plot 8.0 (Systat, IL). To these data, various expressions were fitted using SigmaPlot 8.0 and Matlab 7.5 (Mathworks, MA). These expressions were taken from the literature and are described later, together with the parameter range from which they were determined. Expressions (1)–(4) were obtained empirically, while those given by (5) and (6) have a theoretical basis in models describing electroporation.

Rols and Teissié [33] investigated the threshold value of the electric field E needed for electroporation of cells with pulses lasting from $t_P = 2$ to 100 μs and obtained a hyperbolic relation between E and t_P:

$$E = a + \frac{b}{t_P}. \tag{1}$$

Vernhes with coauthors [39] investigated the effects of electric fields on the inactivation of amoebae. They determined the electric field E required to kill at least 95% of amoebae. For pulse durations t_P in the range from 50 μs to 100 ms, they obtained a logarithmic relation between E and t_P: $E = a - b \log(t_P)$. In a slightly modified form, this expression can also be written as

$$E = a - b \log\left(\frac{t_P}{t_0}\right). \tag{2}$$

In (2), the term t_P/t_0 presents the duration of the pulse t_P (ms), normalized to unit of pulse duration $t_0 = 1$ ms in order to obtain the dimensionless argument of the logarithmic function.

Krassowska and coworkers [34] exposed the cells to pulses with durations t_P ranging from 50 μs to 16 ms and determined the amplitude of the field E required to kill 50% of the cells. They proposed a relation of the form $E = a\, t_P^b$, which can be rearranged in a similar manner as (2), to obtain the dimensionless argument of the power function

$$E = a\left(\frac{t_P}{t_0}\right)^b. \tag{3}$$

In (3), the term t_P^b was replaced with $(t_P/t_0)^b$, where t_P is given in (ms) and $t_0 = 1$ ms.

The same relation was obtained by Abram and coworkers for inactivation of *Lactobacillus plantarum* [40] in the pulse duration range from 0.85 to 5.1 μs.

Huiqi He with coworkers [35] performed single-cell measurements of electroporation-mediated uptake of molecules of different sizes using pulses with durations from 400 μs to 15 ms. For each investigated molecule, the relation between the threshold field E needed to load molecules into cells and pulse duration t_P was determined. These relations formed three-parameter exponential functions:

$$E = a + b\,\exp(-c\,t_P). \tag{4}$$

Saulis derived the expression for the fraction of electroporated cells F_P using equations for kinetics of pore formation,

originating from the theory of electroporation [38]. The slightly modified expression for F_P yields

$$F_P(E, t_P) = 1 - \exp(-k_f(E)t_P) \tag{5a}$$

where the rate of pore formation $k_f(E)$ is given by

$$k_f(E) = \frac{2\pi v R^2}{a} \exp\left(-\frac{\Delta W_0}{k_B T}\right) \cdot \int_{-\pi}^{\pi} \exp\left(\pi C_m r_*^2 \frac{(\varepsilon_w/\varepsilon_m - 1)}{2k_B T}\right) \cdot \left(K_1 ER\cos\varphi\left(1 - \exp\left(\frac{-t}{K_2\tau}\right)\right) + \Delta\Phi\right)^2 d\varphi \tag{5b}$$

and τ is the time constant of membrane charging [44]

$$\tau = \frac{RC_m}{(2\lambda_o\lambda_i)/(2\lambda_o + \lambda_i) + (R\lambda_m)/h}. \tag{5c}$$

In (5a)–(5c), ν is the frequency of lateral fluctuations of lipid molecules, R is the cell radius, a is the area per lipid molecule, ΔW_0 is the energy barrier for pore formation at zero membrane voltage, k_B is Boltzmann's constant, T is the absolute temperature, C_m is the capacitance of the membrane, r_* is the radius of the pore, ε_m and ε_w are the relative permittivities of the membrane and the water inside the pore, respectively, $\Delta\Phi$ is the resting membrane voltage, λ_o, λ_i, and λ_m are the conductivities of the extracellular medium, cytoplasm, and membrane, respectively, and h is the membrane thickness. For a fixed value of F_P (in our case $F_P = 0.7$), the relation between E and t_P can be determined by numerically solving (5). The values of these parameters together with their descriptions are given in Table I. The parameters marked with "#" were changed during the fitting of (5) to the measured data. For spherical cells, the fitted parameters were ΔW_0 and r_*, as suggested in [38]. For attached cells, the fitted parameters were ΔW_0, r_*, R, K_1, and K_2, with R, K_1, and K_2 reflecting the size of the attached cell, the shape of the cell, and the influence of the shape on the membrane charging, respectively (for a spherical cell $R = 6.5\,\mu$m, $K_1 = 1.5$, and $K_2 = 1$).

Neumann [45] derived the relation between the electric field E needed to electroporate 50% of green algae cells *Chlamydomonas reinhardtii* and the pulse duration t_P from the interfacial polarization model. Originating from the energy of the pulse $W_P : W_P = \text{const} \cdot E^2 t_P$, he derived the following relation:

$$E^2 = \frac{a}{t_{\text{Peff}}} \tag{6a}$$

where t_{Peff} is the effective pulse duration given by [45]

$$t_{\text{Peff}} = t_p - \frac{1}{2}\tau\left[3 + \exp\left(-\frac{2t_P}{\tau}\right) - 4\exp\left(-\frac{t_P}{\tau}\right)\right]. \tag{6b}$$

For pulses with $t_P >> \tau$, where τ is given by (5c), t_{Peff} equals t_P. When experimental data were plotted in terms of E^2 versus $1/t_{\text{Peff}}$, it turned out that (6) was piecewise linear: in the range from 100 to ~500 μs and in the range from 500 μs to 16 ms [45]. Since the purpose of this study was to find continuous expressions, which would describe the data in the whole range of t_P, (6) was not included in the fitting. Instead, the expression was used in the analysis of the data in Section IV.

TABLE I
DESCRIPTION OF THE PARAMETERS USED IN (5) AND THEIR VALUES

Symbol	Value	Description
ν	1×10^{11} s^{-1}	Frequency of lateral fluctuations of lipids
$R^{\#}$	6.5 μm	Radius of CHO cell
a	0.6 nm^2	Area per lipid molecule
$\Delta W_0{}^{\#}$	45 k_BT	Energy barrier for pore formation at 0 membrane voltage
k_B	1.38×10^{-23} J/K	Boltzmann's constant
T	297 K	Absolute temperature
C_m	1 μF/cm^2	Capacity of the membrane
$r_*{}^{\#}$	0.3 nm	Radius of the pore
ε_w	78	Relative permittivity of the water in the pore
ε_m	4	Relative permittivity of the membrane
τ	0.36 μs	Time constant of membrane charging
$K_1{}^{\#}$	1.5	Parameter affecting the shape of the cell
$K_2{}^{\#}$	1	Parameter affecting τ
$\Delta\Phi$	-25 mV	Resting membrane voltage
λ_o	1 S/m	Medium conductivity
λ_i	0.2 S/m [44]	Cytoplasmic conductivity
λ_m	5×10^{-7} S/m [44]	Membrane conductivity
h	5 nm	Thickness of the membrane
F_P	0.7	Fraction of electroporated cells

Most of the values were taken from [38] except if noted otherwise. The parameters marked with # were varied during the fitting of (5) to the data.

III. RESULTS

A. Relation Between the Pulse Amplitude and Pulse Duration

The pulse amplitudes leading to electroporation of roughly 70% of cells at pulse durations ranging from 150 ns to 100 ms are presented in Fig. 2(A) (black circles). The results show that with longer pulses, lower amplitudes are needed to maintain roughly the same fraction of electroporated cells [see Fig. 2(B)]. The relation is strongly nonlinear since pulses shorter than 1 ms require progressively higher amplitudes for the same effect. For example, if 137 V/cm was sufficient to electroporate cells with a 100 ms pulse, the field had to be increased to 575 V/cm to electroporate cells with a 100 μs pulse, and up to 10 kV/cm to obtain the same effect with a 150 ns pulse. To display the nonlinear relation between the pulse amplitude and the pulse duration, the same results are plotted also on a linear scale [see the inset in Fig. 2(A)]. The ratio of the fluorescence (F_{345}/F_{385}), which reflects the change in intracellular Ca^{2+} concentration, varies with pulse duration. While it remains at rather constant value in the range from 30 μs to 10 ms, the ratio decreases for pulses, which are out of this range [see Fig. 2(C)].

To determine if functional relations given with (1)–(5) could describe the measured results, we fitted each of these equations to our data [see Fig. 2(A)]. As the figure shows, most of the equations could not be adequately fitted to the data. The only curve that could at least qualitatively describe the data is the one given by (5). The remaining four equations either could not reproduce the increase in pulse amplitudes at shorter pulse durations, (2) and (3), or fail to describe the moderate decrease of pulse amplitudes at longer pulse durations, (1) and (4). However, in a narrower range of pulse durations, from 10 μs to 100 ms, we

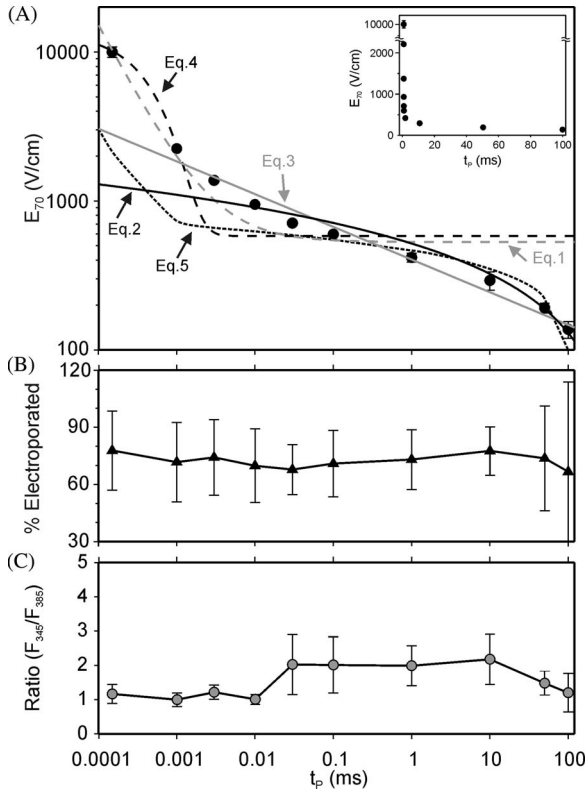

Fig. 2. Relation between the field amplitude E_{70} and the pulse duration t_P for electroporation with a single pulse. (A) Field amplitudes leading to electroporation of roughly 70% of cells at pulse durations ranging from 150 ns to 100 ms. Black circles are the measured data presented as means ± SD ($N = 9$). The inset shows the same data on a linear scale of t_P. The curves are the fitted expressions describing the relations between E (V·cm^{-1}) and t_P (ms). Equation (1) (dashed gray): $E_{70\%} = 520.9\ \mathrm{V{\cdot}cm^{-1}} + 1.43\ \mathrm{V{\cdot}cm^{-1}\ ms}/t_P$, Equation (2) (solid black): $E_{70\%} = 508.9\ \mathrm{V{\cdot}cm^{-1}} - 190.8\ \mathrm{V{\cdot}cm^{-1}{\cdot}log}\,(t_P/t_0)$. Equation (3) (solid gray): $E_{70\%} = 397.0\ \mathrm{V{\cdot}cm^{-1}}(t_P/t_0)^{-0.22}$. Equation (4) (dashed black): $E_{70\%} = 573.0\ \mathrm{V{\cdot}cm^{-1}} + 12\,760\ \mathrm{V{\cdot}cm^{-1}}\exp(-2024\ \mathrm{ms^{-1}}\cdot t_P)$. Equation (5) (dotted black): $r_* = 0.64$ nm, $\Delta W_0 = 44.8\ k_B T$. (B) Corresponding fraction of cells electroporated to Ca^{2+}. (C) Ratio of fluorescence (F_{345}/F_{385}) due to intracellular change in Ca^{2+}.

found that (2), (3), and (5) could be fitted to the data reasonably well (see Fig. 3), with the best fit obtained for a two-parameter logarithmic function (2) and a two-parameter power function (3).

We also estimated the maximum increase of temperature of the medium during the pulse. Temperature change ΔT was determined from the assumption that electrical energy is transformed into heat completely

$$\Delta T = \frac{U I t_P N_P}{(\rho V c_P)}. \tag{7}$$

Here, U is the amplitude of the pulse, I is the current through cell suspension, t_P is the pulse duration, N_P is the number of pulses, ρ is the specific density of the medium ($\rho = 1000\ \mathrm{kg/m^3}$), V is the volume of the medium ($V = 1$ mL), and c_p is the specific heat capacity of the medium ($c_p = 4186\ \mathrm{J{\cdot}kg^{-1}{\cdot}K^{-1}}$). The maximum temperature increase of 1.5 K (from the initial 293 K) is generated by the longest, 100 ms pulse, and is well below the temperature rise that could harm the cells.

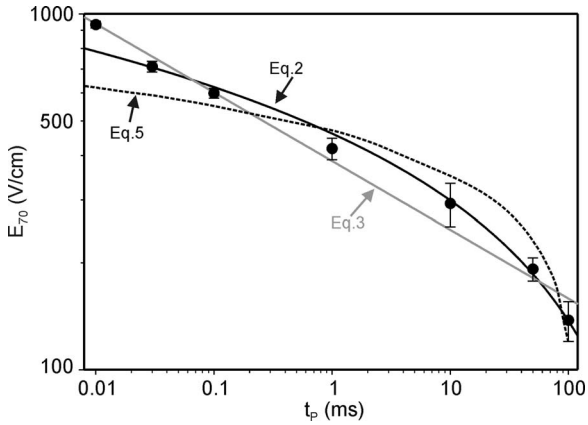

Fig. 3. Relation between the field amplitude E_{70} and the pulse duration t_P in the range of pulse durations from 10 μs to 100 ms. The black circles present the measured data (same as in Fig. 2), while the curves are the fits of (2) (solid black), (3) (solid gray), and (5) (dotted black) to the data in the given range. For E in (V·cm^{-1}) and t_P in (ms), the parameters yield Equation (2): $E_{70\%} = 460.1\ \mathrm{V{\cdot}cm^{-1}} - 161.6\ \mathrm{V{\cdot}cm^{-1}{\cdot}log}\,(t_P/t_0)$; (3): $E_{70\%} = 385.1\ \mathrm{V{\cdot}cm^{-1}}{\cdot}(t_P/t_0)^{-0.19}$; (5): $r_* = 0.65$ nm, $\Delta W_0 = 44.8\ k_B T$.

B. Relation Between the Pulse Amplitude and the Number of Pulses

The pulse amplitudes leading to electroporation of approximately 70% of cells after the exposure to a train of 1, 2, 4, 8, 16, 32, 64, or 128 pulses, with each pulse lasting 100 μs, are shown in Fig. 4(A) and (B). With increasing number of pulses, the pulse amplitude needed to obtain the same fraction of electroporated cells decreases. Trains with less than 16 pulses require increasingly higher pulse amplitudes to maintain the same fraction of electroporated cells. At the same time, with higher number of pulses, the ratio of the fluorescence of cells (F_{345}/F_{385}) decreases [see Fig. 4(C)]. The results show that the decrease of pulse amplitude can be compensated by increasing the number of pulses.

Functional relations given by (1)–(4) were again fitted to the measured results. Despite the fact that these equations were primarily used to describe the relation between the pulse amplitude and the pulse duration, they can also be used to represent the relation between the amplitude and the number of pulses. Namely, except for (5) and (6), these equations were obtained empirically and do not reflect any physical process related to electroporation. The curves obtained after fitting (1)–(4) to the data are shown in Fig. 4(A). In this case, the relation between amplitude and number of pulses could be best described by a two-parameter power function (3) or a three-parameter exponential function (4).

Again, we calculated the change in the temperature ΔT of the medium using (7). In this case, the maximum increase in the temperature was 0.7 K and was generated by a train of 128 pulses. This increase does not significantly affect the viability of cells and is in practice probably even lower, due to the relatively low pulse repetition frequency of 1 Hz, which allows for the cooling of the medium between successive pulses.

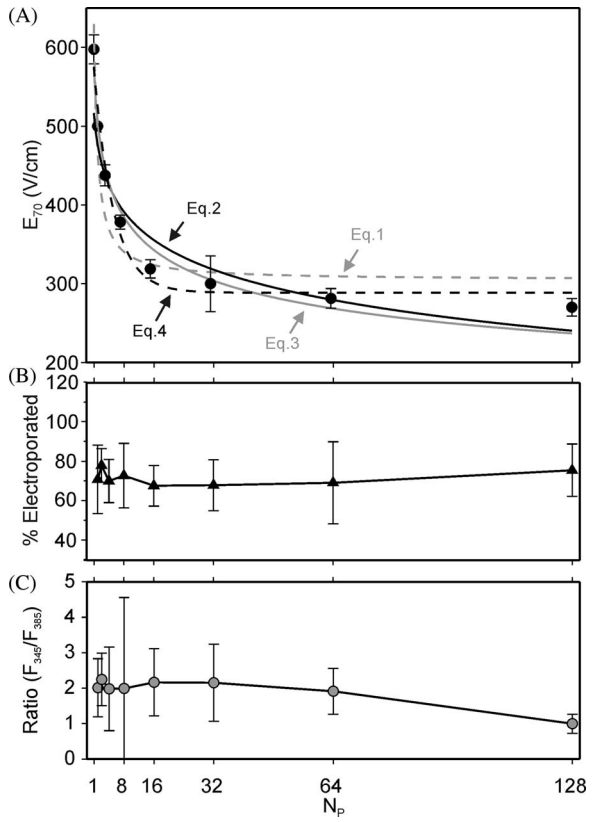

Fig. 4. Relation between the field amplitude E_{70} and the number of pulses N_P at pulse duration $t_P = 100\ \mu$s and pulse frequency of 1 Hz. (A) Field amplitudes leading to electroporation of roughly 70% of cells at N_P ranging from 1 to 128. Black circles are the measured data presented as means $\pm$ SD ($N = 12$). The curves are the fitted expressions describing the relations between E (V·cm^{-1}) and N_P. Equation (1) (dashed gray): $E_{70\%} = 304.4$ V·cm^{-1} + 325.1 V·cm^{-1}/N_P. Equation (2) (solid black): $E_{70\%} = 516.4$ V·cm^{-1} − 131 V·cm^{-1}·log (N_P). Equation (3) (solid gray): $E_{70\%} = 574.8$ V·cm^{-1}·$N_P{}^{-0.18}$. Equation (4) (dashed black): $E_{70\%} = 288.5$ V·cm^{-1} + 347.4 V·cm^{-1}·exp ($-0.19N_P$). (B) Corresponding fraction of cells electroporated to Ca^{2+}. (C) Ratio of fluorescence (F_{345}/F_{385}) due to intracellular change in Ca^{2+}.

IV. Discussion

In this paper, we evaluated different reported functional relations between amplitude and pulse duration, and amplitude and number of pulses that result in the same fraction of electroporated cells and also investigated the level of cell electroporation. Such relations could help researchers finding equivalent pulse parameters for electroporation.

From our results, it follows that the change in the value of one parameter can be compensated by carefully adjusting the value of another parameter. For example, the decrease in the pulse amplitude can be compensated by increasing the pulse duration or increasing the number of pulses. This can be useful in cases where experimental settings or pulse generator limitations confine the pulse parameters within a given range of values. In such cases, adjusted, i.e., equivalent pulse parameters could be used to maintain the efficiency of electroporation.

Usually, the efficiency of electroporation is expressed either as the fraction of electroporated cells, cell viability, or the uptake of molecules into electroporated cells. In this paper, the efficiency of electroporation was characterized by the fraction of electroporated cells. Alternatively, we could choose the uptake of molecules criterion, but in this case, fine-tuning of the amplitude, required to maintain the constant uptake, would be a more difficult task. Besides, Fura 2-AM might not be the most suitable dye for measuring the uptake. Namely, the dye responds to changes in intracellular Ca^{2+}, which can occur either due to the inflow of extracellular Ca^{2+} through the electroporated membrane or due to the release of Ca^{2+} from intracellular reservoirs. The latter is difficult to estimate, especially because the Ca^{2+} release can be triggered by elevated cytosolic Ca^{2+} after electroporation and/or by nanosecond electric pulses [27].

Several studies have investigated the influence of pulse parameters on the efficiency of electroporation [1], [12], [15], [33]–[35], [37], [46]. In general, these studies demonstrated that the same efficiency can be obtained with different combinations of pulse parameters. Our results are qualitatively similar to these findings and further show that the same conclusions are valid on a wider interval of parameter values, i.e., pulse durations from 150 ns to 100 ms, and the pulse number from 1 to 128.

A wide variety of mathematical expressions, describing the relation between pulse parameters, can be found in the literature [33]–[35], [38]–[40], [45]. These expressions range from basic mathematical functions to more complicated mathematical expressions, (1)–(6). The relatively large collection of different mathematical expressions might be due to the fact that the relations between parameters were determined within different intervals of parameters and that the approaches used to derive these expressions were different. For example, (1)–(4) were all obtained empirically, (5) was derived from kinetics of pore formation, originating from the theory of electroporation [47]–[49], and (6) was derived from the interfacial polarization model [45]. When (1)–(5) were fitted to our results, we found that they could not describe the measured data reliably on the whole interval of pulse durations. The largest disagreement between calculated curves and measured data was observed for pulse durations shorter than 10 μs, where pulse amplitudes sharply increased. This increase can be attributed mainly to the membrane charging time, which, at short pulses, becomes comparable or even longer than the pulse duration, see (5c). Since (1)–(4) were determined empirically, they are not physically related to the processes of membrane charging and electroporation. Besides, (2)–(4) were obtained for pulses longer than few tens of μs, where charging time of the membrane is insignificant with respect to pulse durations considered, meaning that the extrapolation of these expressions to shorter pulses essentially led to errors. However, for longer pulses, (2), (3), and (5) could be fitted to the data with better accuracy (see Fig. 3).

Equation (5) was the only equation that could, at least qualitatively, describe the measured data in the whole range of pulse durations. The observed difference between theoretical predictions of (5) and experiments at short pulse durations could be partly explained by the fact that (5) was originally derived for a spherical cell. Cells in our study were, for the purposes of reproducible experiments, attached to the bottom of the chamber, meaning that they were flat and had different shapes and sizes (see Fig. 1). Under the same experimental conditions, attached

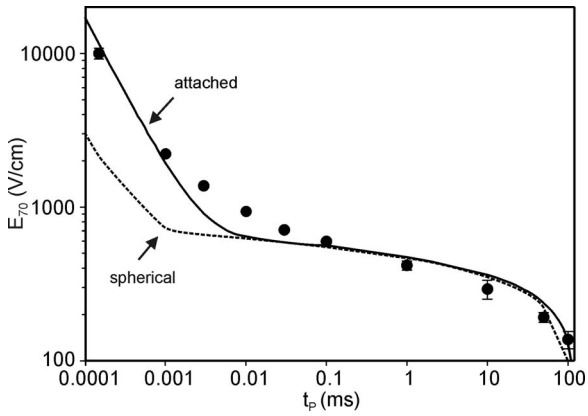

Fig. 5. Comparison of the calculated curves obtained by fitting (5) to the measured data for the case of a spherical cell (dotted curve) and an attached cell (solid curve). Black circles are the measured data presented as means ± SD ($N = 9$). Spherical cell: $r_* = 0.64$ nm, $\Delta W_0 = 44.8\ k_B T$. Attached cell: $r_* = 0.32$ nm, $\Delta W_0 = 46.6\ k_B T$, $R = 15.9\ \mu$m, $K_1 = 1.24$, $K_2 = 2.56$.

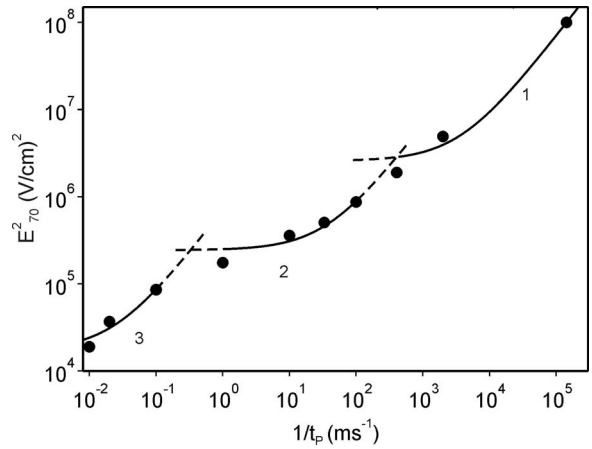

Fig. 6. Relation between the E^2_{70} and $1/t_P$. The curves are the fits of (6) to the measured data. The fitted parameter a in $E^2 = a/t_P$ is 1) $a = 681.4$ V^2·cm^{-2} ms; 2) $a = 6446.6$ V^2·cm^{-2} ms; 3) $a = 0.69$ kV^2·cm^{-2} ms. Note that t_P at short pulses was replaced with $t_{P\,\mathrm{eff}}$ according to (6b).

cells present less of an "obstacle" to the electric field than spherical cells, meaning that the transmembrane voltage induced on their membranes is generally lower, and the charging time of the membrane can be higher. Together, both of these effects could explain the disagreement between the curve calculated for spherical cells and the data measured on attached cells. To partially account for the effect of cell shape, we modified (5) by also varying the parameters R, K_1, and K_2 to reflect the change in the size of the attached cell, the decrease of the voltage on the membrane, and the increase in the time constant of membrane charging, respectively. Equation (5) modified for an attached cell was then again fitted to the measured data (see Fig. 5, solid curve). Compared to the spherical cell, a better agreement with the data was now obtained (cf., solid and dotted curve in Fig. 5). The parameters R, K_1, and K_2 in (5) have changed from $R = 6.5\ \mu$m, $K_1 = 1.5$, and $K_2 = 1$ (spherical cell) to $R = 15.9\ \mu$m, $K_1 = 1.24$, and $K_2 = 2.56$ (attached cell), implying that the voltage on larger but thinner attached cells is lower compared to the voltage on smaller spherical cells, while the time constant of membrane charging is higher. From our previously reported calculations for attached cells, we estimate that these values are reasonable [50]. The fitted value for r^* (0.32 nm) is within the reported values obtained for lipid bilayers and erythrocytes ($r^* = 0.3$–0.5 nm, [48], [51], [52]), while the value of ΔW_0 (46.6 $k_B T$) is slightly larger ($\Delta W_0 = 40$–$45\ k_B T$ [48], [51], [52]).

Despite the fact that (5) is based on the theory of electroporation, and could also describe the dependence between pulse amplitude and duration on the whole interval of pulse parameters, the logarithmic and power functions, given by (2) and (3), might be more practical in determining equivalent pulse parameters. Namely, these two equations contain only two parameters to be fitted, and are also relatively easy to evaluate computationally, but they can be applied only for pulses lasting at least several microseconds.

Neumann suggested that the relation between pulse amplitude and pulse duration given by (6), when plotted as E^2 versus $1/t_P$, should be piecewise linear [45]. For his relatively short interval of pulse durations ($t_P = 100\ \mu$s–16 ms), he could discern two domains of pulse durations in which the data could be described with (6). When our results in Fig. 2(A) were transformed to comply with (6a) and (6b), we were able to discern three such domains, which are shown in Fig. 6. The first domain contains pulse durations up to few μs, the second domain pulses in the range of few ms, and the third domain pulses longer than 10 ms. Due to the wide interval of pulse durations, the results are presented in logarithmic scale. For the same reason, the linear fits appear bent. Compared to the results of Neumann, we obtained somewhat different domains of pulse durations, which can be attributed to the fact that our data were taken from a considerably wider range of pulse durations (150 ns to 100 ms) and also due to the fact that our cell population was heterogeneous. The existence of the third domain, for the longest pulse durations used in our study, might indicate the effects associated with long electric pulses, such as electrophoresis or electroendocytosis.

We should mention that theoretical expressions (1)–(6), which were fitted to our experimental data, were initially derived to describe different experimental assessments. They can be divided into three groups: 1) electroporation of cells: (1), (5), (6) [33], [38], [45]; 2) inactivation of amoebae/killing of cells: (2), (3) [39], [34]; and 3) uptake of molecules: (4) [35]. Among the investigated expressions, our data were in agreement with theoretical expression (5) from group 1 and expressions (2) and (3) from group 2. This is an interesting observation, since our investigated parameter, the fraction of electroporated cells, was used only in the models from group 1.

A similar mathematical fitting of expressions was also performed for the dependence of pulse amplitude on the number of pulses. In this case, a theoretical expression based on the model of electroporation is not explicitly stated. However, it might be possible to derive such an expression by using the equations for distribution functions for cell forming and resealing times, given in recent papers of Saulis [53] [54], and following the directions in the same papers. The four expressions (1)–(4) do not have any physical background that would relate them

to electroporation meaning that they can also be used to describe the relation between amplitude and number of pulses. The two-parameter power function (3) and the three-parameter exponential function (4) seem to best describe the dependence between the amplitude and the number of pulses. One drawback of using a larger number of pulses is that such protocol requires more time to perform the experiment. For example, delivering 128 pulses with 1 Hz pulse repetition frequency takes 128 s. The time to perform an experiment increases even further if an array of electrodes is used, where pairs of electrodes are sequentially activated. Increasing the pulse repetition frequency would solve the problem of excessive duration, but might at the same time result in increased heating of the sample [55]. Our experiments do not show these relations since they were performed with 100 μs pulse duration and at 1 Hz pulse repetition frequency only. It is, therefore, possible that different functional relations would be obtained with other combinations of pulse durations and pulse repetition frequencies.

Another interesting finding originates from the functional relations between pulse amplitude and pulse duration. Namely, (2), (3), and (5) suggest that electroporation can be obtained at any pulse amplitude provided that pulse duration is sufficiently long. There have been many opposing reports in the literature on the existence of the threshold pulse amplitude for electroporation of cells. While experimental studies mostly reported that electroporation could be obtained only with amplitudes above a certain value [56]–[59], theoretical studies, especially those based on the theory of pore formation [18], [49], predicted that electroporation is not a threshold phenomenon. Our results obtained with small divalent Ca^{2+} ions suggest that there might be no threshold (see Fig. 2). On one hand, this might be explained by the fact that at longest pulse durations the uptake of Ca^{2+} was more related to processes such as electrophoresis or electrically stimulated endocytosis, rather than to electroporation. On the other hand, Ca^{2+} ions are small compared to molecules used in many experimental studies (e.g., tripan blue, lucifer yellow, propidium iodide, etc.). Larger molecules need more time and more intense electroporation to cross the electroporated membrane, which might be the reason for the observed threshold [60]. This was demonstrated by He and coworkers who investigated the threshold values of the electric field for molecules of different sizes and obtained higher thresholds for larger molecules [35].

In the last decade, electroporation with nanosecond electric pulses has become increasingly widespread. Pulses in these applications can last less than 1 ns, while the number of pulses can exceed several thousand. In this study, only pulses with durations longer than 150 ns were considered. For even shorter pulses, the readers should refer to a recent study from Schoenbach and coauthors [61], where the authors derived the mathematical relations between the pulse parameters and bioelectric effects of nanosecond pulses.

V. Conclusion

Electroporation-based technologies and medical applications have already shown their laboratory and clinical relevance. Electroporation of cells is becoming a standard tool in biotechnology and biomedicine [16], [23], [62]–[65]. Although the number of successful applications is increasing, several questions concerning the optimization of pulse protocols for specific application are still open. Among them is determination of appropriate amplitude, duration and number of electric pulses that assure successful application, or treatment with minimum possible side effects. A review of the studies related to pulse parameters used in different electroporation-based applications shows a number of efficient combinations of pulse parameters. In our present study, we demonstrated that the change in the value of a specific pulse parameter can be compensated by carefully selected value of the other parameter. In addition, we showed that the relation between pulse parameters can be described by relatively simple mathematical expressions, such as power, logarithmic, or exponential functions. On the basis of these functions, equivalent pulse parameters that assure similar effectiveness of electroporation can be selected. Such parameters can be extremely useful in the process of electroporation-based treatment planning [66], where limitations of the electrical devices and position of the electrodes have to be taken into account.

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