Cell electropermeabilization to small molecules *in vitro*: control by pulse parameters

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A systematic study concerning the role of the different electric field parameters (pulse number, duration and amplitude) on electropermeabilization of DC3F cells to small molecules (propidium iodide) and on cell viability is presented. Cell permeabilization and viability dependence on the pulse amplitude was determined by twenty different sets of electrical parameters. The number of pulses varied between 1 and 64 and pulse duration between 20 µs and 1 ms. The most important parameter was the pulse amplitude because it triggered the electropermeabilization process and the process of cell death. Either in the case of electropermeabilization as well as in the case of cell viability experiments, the parameter U_{50} (the pulse amplitude leading to permeabilization or to the death of 50% of cell population) was not changed if the set of electrical parameters consisted of more than 16 pulses. This was independent of the pulse duration. The efficiency of permeabilization was enhanced by using of longer pulses. Such a systematic study of the influence of different electric field parameters on electropermeabilization and cell viability may serve as a base for optimization of the electropermeabilization conditions for different applications.

Key words: electroporation; electromagnetic fields; cell survival; propidium iodide

Introduction

The phenomenon of cell membrane electropermeabilization can be described as a dramatic increase in the transmembrane permeability induced by an externally applied electric field. This transient state has important

practical applications like the fusion of cells [1] and the introduction of the biologically active substances like drugs [2] and genetic material [3] into cells. Electropermeabilization is nowadays widely used to manipulate biological cells, organelles, cell aggregates and tissue. The clinical applications gain increasing importance, particularly in oncology.[4,5] Electropermeabilization can be achieved with different sets of pulse parameters, *i.e.* the strength of applied field (voltage of applied pulses), the number and the shape of pulses, their duration and repetition frequency. An identical set of electrical parameters is not necessarily efficient for all applications. While eight

square-wave electric pulses of 100 µs delivered at 1 Hz is the most common used set of electrical parameters in electrochemotherapy, the pulses must be much longer (eight square-wave electric pulses of 20 ms delivered at 1 Hz or 2 Hz) for DNA electrotransfer *in vivo*.[5] Likewise, it has been shown in vitro that only pulse duration equal to or longer than 1 ms was associated with the detection of macromolecules in pulsed and viable cells, while the permeabilization of cells to small molecules was already detected for the microsecond range.[6] Therefore it is important to know how the pulse parameters affect the electropermeabilization process.

The role of pulse parameters in obtaining a higher efficiency of electropermeabilization *in vitro* was investigated in a number of studies.[6-9] The permeabilization as a function of the parameters of applied electric field was quantified in two ways. Either the fraction of electropermeabilized cells in suspension was measured using fluorescence optical microscopy [10], or the permeability of each cell was integrated over the whole cell population by measuring radioactive incorporation [11] or ATP leakage.[6] Both types of information were gathered in some studies using flow cytometry.[12] Independently of the method, it was shown that the electric field intensity is the crucial parameter for inducing membrane permeabilization. The permeabilization occurs only if the electric field intensity is higher than a certain threshold value. This threshold value is a function of the pulse number and the pulse duration. It decreases by increasing either the pulse duration or the number of pulses until it reaches a "real" threshold value below which no permeabilization occurs even if using longer pulses or higher number of them. The "real" threshold value was obtained using 10 pulses or more with the duration longer than 100 µs.[13] The permeability threshold depends on the molecular size of the probe used for its measurement: the larger the test molecule, the higher

the apparent threshold.[14] It also depends on the cell line because of their differences in size [15] and membrane properties.[16,17]

In almost all studies, the effect of pulse duration was studied at a given field intensity and pulse number. Similarly, the effect of pulse number was studied at a given field intensity and pulse duration, and vice-versa the effect of field intensity was studied at a given pulse duration and pulse number.[6-8] However, the fraction of electropermeabilized cells as a function of pulse duration at a given number of pulses is strongly dependent on selected field intensity (Fig. 1). Namely, a plateau is reached at shorter pulses if higher field intensity is used.

In this paper, we present a systematic study concerning the role of different electric field parameters (field intensity, pulse number, and duration) on electropermeabilization of DC3F cells to small molecules and on cell viability. DC3F cells have been chosen because considerable information about the electropermeabilization of that strain is available.[2,18,19] The fraction of electropermeabilized cells was quantified by the penetration of propidium iodide and the viability of the cells by their cloning efficiency. The cells were pulsed with twenty different sets of electrical parameters (Table 1). The number of pulses varied between 1 and 64, the pulse duration between 20 µs and 1 ms and the pulse amplitude from 40 to 600 V.

Materials and methods

Chemicals

Eagle's minimal essential medium (EMEM), trypsin and propidium iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal calf serum (FCS) and L-glutamine were obtained from Gibco BRL (Galthersburg, MD), penicillin, streptomycin, gentamicin from Lek (Ljubljana, Slovenia), and Crystal violet from Kemika (Zagreb, Croatia).
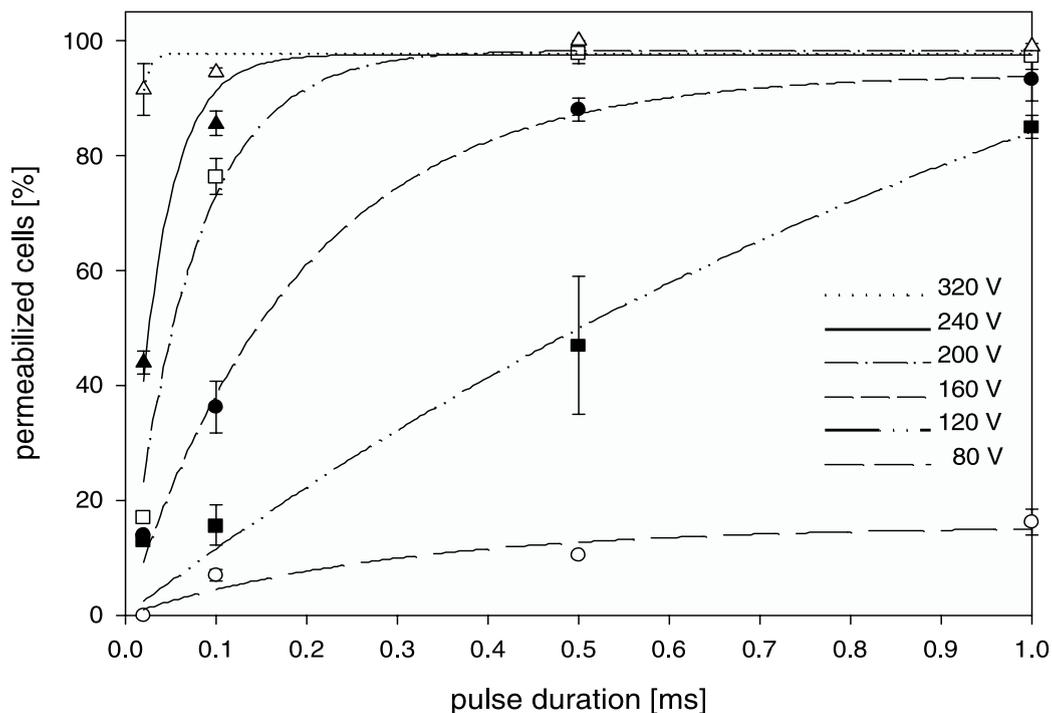
Figure 1. Fraction of electropermeabilized cells as a function of pulse duration. Cells were pulsed eight times at different pulse amplitudes. The error bars in the fraction of electropermeabilized cells represent standard deviations of the data.

PI was dissolved in sterile H_2O at a concentration of 100 μM.

Cell culture

DC3F cells, a line of spontaneously transformed Chinese hamster lung fibroblasts, were grown in monolayers in the culture medium consisting of EMEM supplemented with 10% heat-inactivated FCS, 10mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 11 μg/ml gentamicin. The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2, and were routinely subcultured every 4 days.

Cell exposure to electric field

Cells from the exponential growth phase were trypsinized and centrifuged for 5 min at

4°C and 1500 rpm in the culture medium. They were then resuspended in the serum-free medium supplemented with 0.5 mM $CaCl_2$ at a concentration of 2.2×10^7 cells/ml. 90 μl cell suspension was mixed with 10 μl PI for the determination of electropermeabilization, or with medium supplemented with 0.5 mM $CaCl_2$ for determination of electropulsed cell viability. A 50 μl droplet of the cell suspension was placed between two flat, parallel, stainless steel electrodes (length = 6 mm, width = 6mm, interelectrode distance = 2 mm). The electrodes were connected to a voltage generator (Jouan GHT 1287 B, France) generating monophasic square-wave electric pulses with independently adjustable electric parameters (voltage, number of pulses and duration). The cells were pulsed at 1 Hz frequency. The pulse parameters were monitored by an oscilloscope (Hameg HM 205-3,

Table 1. Sets of electrical parameters. Repeated pulses were delivered at 1 Hz frequency

Set of electric field parameters	Number of pulses	Pulse duration [μs]
1.20	1	20
1.100	1	100
1.500	1	500
1.1000	1	1000
4.20	4	20
4.100	4	100
4.500	4	500
4.1000	4	1000
8.20	8	20
8.100	8	100
8.500	8	500
8.1000	8	1000
16.20	16	20
16.100	16	100
16.500	16	500
16.1000	16	1000
64.20	64	20
64.100	64	100
64.500	64	500
64.1000	64	1000

Germany). All experiments were performed under sterile conditions in a laminar flow hood at room temperature.

Determination of electropermeabilization

Electropermeabilization of cells was quantified by the penetration of impermeant dye PI. When the membrane is permeable, PI binds to nucleic acids and becomes highly fluorescent. Therefore, it is not necessary to wash the cells to eliminate nonincorporated PI as in case of other fluorescent dyes. A selected evaluation method avoids the negative consequences of pipetting and centrifuging the cells that have been already pulsed.[20] The cells were pulsed and incubated 5 min at room temperature. Thereafter, 25 μl of cell suspension was resuspended in 1 ml of 0.01 M phosphate-buffered saline (PBS, pH 7.4) and kept at 4°C till being analyzed by flow cy-

tometry (FACSort, Becton Dickinson, CA). The flow cytometer was used to measure the number of fluorescent and therefore permeabilized cells. Excitation was set at the wavelength 488 nm and emission was detected at 640 nm. Fluorescence was recorded for 5000 particles. Only particles large enough to qualify as cells were taken into consideration. The number of stained cells was determined and normalized to the number of all cells to get the percentage of permeabilized cells.

Determination of electropulsed cell viability

Cell viability was determined by means of colony-forming assay. After the exposure to electric pulses, the cells were incubated for 5 min at room temperature. They were then diluted in the culture medium and seeded in triplicate (300 cells per 60 mm diameter petri dish). After five days, the colonies were fixed with 96% ethanol, stained with Crystal violet and counted. The survival of the cells treated with electric pulses was calculated as the percentage of the colonies obtained from the untreated control cells.

Statistical analysis

All experiments were repeated at least three times on different days. For each experimental point, mean and standard deviation were calculated. Using nonlinear regression, a two-parameter sigmoid curve was fitted to the data

$$f(U) = \frac{100}{1 + \exp\left[(U_{50} - U)/b\right]}$$

where U is the pulse amplitude, f is the percentage of permeabilized or alive cells, and U_{50} and b are the two parameters of the sigmoid curve. Parameter U_{50} is the pulse amplitude leading to permeabilization of 50% of cell population in the case of electropermeabilization and the pulse amplitude leading to the death of 50% of cell population in the case
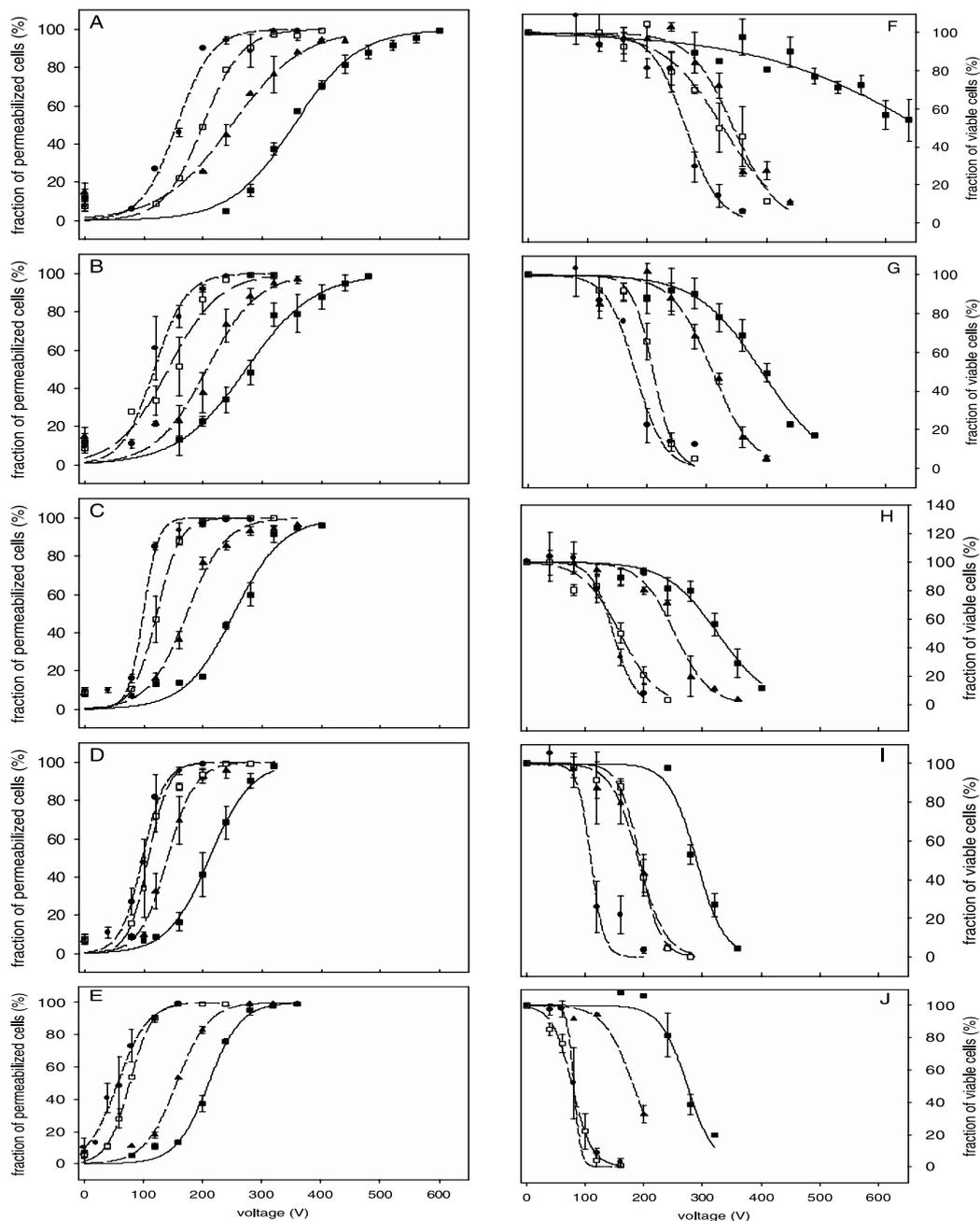
Figure 2. Effect of pulse amplitude on electropermeabilization of cells and cell viability. DC3F cells were pulsed at 1 Hz one time (A - electropermeabilization, F - cell viability), four times (B - electropermeabilization, G - cell viability), eight times (C - electropermeabilization, H - cell viability), sixteen times (D - electropermeabilization, I - cell viability) and sixtyfour times (E - electropermeabilization, J - cell viability). Pulse duration was equal to (■) 20 μs, (▲) 100 μs, (□) 500 μs and (●) 1000 μs. The symbols denote the means and the error bars are the standard deviations.

of cell viability experiments. Parameter b governs the inclination of the sigmoid curve. Smaller absolute value of parameter b means that the process reaches its plateau at smaller interval of pulse amplitudes, while its larger value means that the process reaches its plateau at larger interval of pulse amplitudes. In the literature, parameter b was paralleled with the efficiency of permeabilization.[13]

Results

In this study we focused on the effect of pulse amplitude, number of pulses and pulse duration on the cell permeabilization and viability. Repeated pulses were delivered at 1 Hz frequency. The cell permeabilization and viability dependence on the pulse amplitude was determined using twenty different sets of electrical parameters (Table 1). Figure 2 shows the results of these measurements. The symbols denote the means and the error bars are standard deviations. A two-parameter sigmoid curve is fitted to the data of each set of electrical parameters. For easier comparison of different sets of electrical parameters, the parameter U_{50} and b of all electropermeabilization curves and curves presenting cell viability are collected in Table 2 and presented in Figures 3 and 4.

Electropermeabilization

The fraction of electropermeabilized cells in population is under control of the field intensity, the pulse duration and the number of pulses. Electropermeabilization occurs only for pulse amplitudes higher than a certain threshold value. This value is lower if longer pulses are used, or if the number of pulses is higher. Permeabilization curves are completely shifted to the lower pulse amplitudes (Fig. 2A-E). If the set of electrical parameters consisting of 16 and 64 pulses, 0.5 ms electropermeabilization curve more or less coincides

with 1 ms electropermeabilization curve (Fig. 2D, E). It means that, in the sets of electrical parameters that consist of 16 pulses or more, the usage of the pulses longer than 0.5 ms does not change the fraction of electropermeabilized cells in population at certain pulse amplitude.

Parameter U_{50} is not changed if the set of electrical parameters consists of more than 16 pulses (Fig. 3A). This is independent of the pulse duration. The relation between the pulse duration and the number of pulses at a given parameter U_{50} is linear on logarithmic scale, if less than 16 pulses are used.

In the literature, parameter b was paralleled with the efficiency of permeabiliza-

Table 2. Parameters U_{50} and b at different sets of electrical parameters. Parameters are determined by sigmoid curves which are fitted to the experimental data of electropermeabilization and cell viability using nonlinear regression.

Set of electric field parameters	Electropermeabilization		Cell viability	
	U_{50} [V]	$\lvert b \rvert$ [V]	U_{50} [V]	$\lvert b \rvert$ [V]
1.20	354	53	664	143
1.100	249	59	345	36
1.500	200	33	328	50
1.1000	156	29	265	28
4.20	273	58	392	54
4.100	206	43	309	34
4.500	141	43	210	18
4.1000	118	27	180	24
8.20	252	41	326	43
8.100	173	32	253	30
8.500	122	20	159	32
8.1000	99	13	148	18
16.20	211	35	290	24
16.100	140	23	191	23
16.500	108	17	193	17
16.1000	98	19	110	11
64.20	211	27	274	23
64.100	157	27	182	25
64.500	77	19	77	17
64.1000	57	25	80	6

tion.[13] Parameter b of the sigmoid curve is smaller, if the duration of the pulses is longer (Fig. 3B). Therefore, the efficiency of permeabilization is enhanced by the usage of longer pulses. The enhancement is less pronounced if the set of electrical parameters consists of higher number of pulses.

Cell viability

Also the fraction of viable cells in population is under control by the field intensity, the pulse duration and the number of pulses as well. Cell death occurs at the field intensities higher than a certain threshold value. This value is lower if the pulses are longer or their number is higher. In that case cell viability curves are completely shifted to the lower pulse amplitudes (Fig. 2 F-J).

Likewise, in the case of electropermeabilization, the relation between the pulse duration and the number of pulses at a given parameter U_{50} is linear on logarithmic scale if less than 16 pulses are used. Parameter U_{50} is not changed if the set of electrical parameters

consists of more than 16 pulses (Fig. 4A). This is evident for the pulses shorter than 500 µs.

Absolute value of the parameter b of the sigmoid curve is smaller if the duration of the pulses is longer (Fig. 4B). The viability of cells is changed on smaller interval of pulse amplitudes if longer pulses are used.

Discussion

The application of electric field pulses to DC3F cells results in the permeabilization of their plasma membrane. Electrical parameters, *i.e.* pulse amplitude, pulse duration and the number of pulses, have an important role in electropermeabilization process as well as an effect on cell viability. The most important parameter is the pulse amplitude because it triggers the electropermeabilization process and the process of cell death. Both processes have their characteristic threshold values. Either pulse duration or the number of pulses can modulate these threshold values.
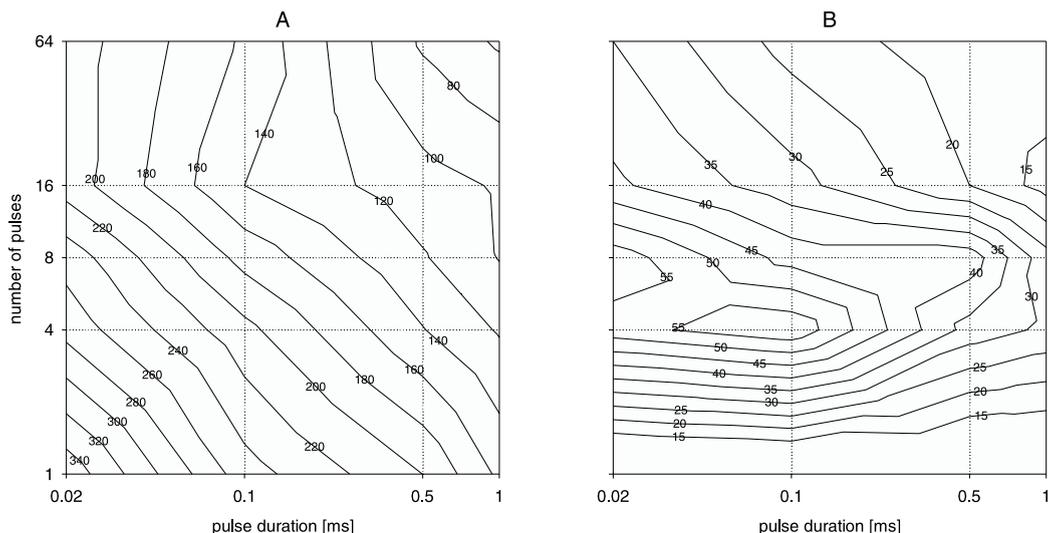
In our study, we quantified the fraction of

Figure 3. Values of parameter U_{50} and b in the field of pulse duration and number of pulses in the case of electropermeabilization experiments. Each curve represents one value of the parameter U_{50} (A) or b (B). The value is written on the curve. Scales are logarithmic.
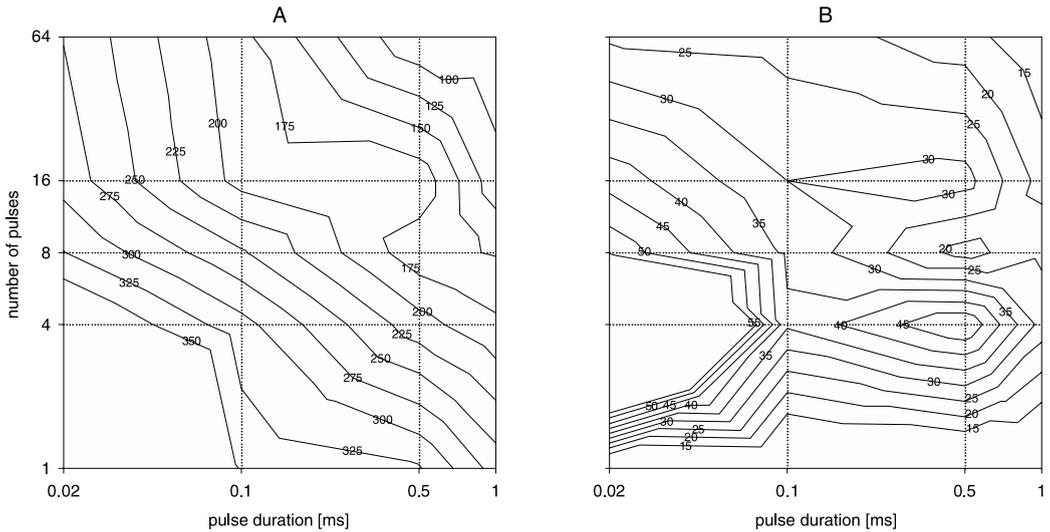
Figure 4. Values of parameter U_{50} and b in the field of pulse duration and number of pulses in the case of cell viability experiments. Each curve represents one value of the parameter U_{50} (A) or b (B). The value is written on the curve. Scales are logarithmic.

electropermeabilized cells by the penetration of PI. Because PI also leaks into healthy cells over time [21], we noticed some fluorescence cells also in the untreated control cells. Therefore, it was difficult to determine the pulse amplitude, which triggers electropermeabilization process, *i.e.* threshold value. That is why our conclusions were made by observing electropermeabilization curves, their parameters U_{50}, *i.e.* the pulse amplitude leading to permeabilization of 50% of cell population in the case of electropermeabilization and the pulse amplitude leading to the death of 50% of cell population in the case of cell viability experiments, and parameters b, *i.e.* parameter which governs the inclination of the sigmoid curve. Our conclusions can be summarized as follows: (1) In the sets of electrical parameters consisting of 16 or more pulses, the usage of pulses longer than 0.5 ms does not change the fraction of electropermeabilized cells in the population at a selected pulse amplitude. (2) Both in the electropermeabilization as well as in the cell viability experiments, parameter U_{50} is not changed if the set of electrical parameters consists of

more than 16 pulses, which is independent of the pulse duration. (3) The efficiency of permeabilization is enhanced by the usage of longer pulses. (4) The fraction of electropermabilized cells and viability of cells vary at a smaller interval of pulse amplitudes if longer pulses are used.

In spite of a variety of studies investigating the role of pulse parameters in the electropermeabilization efficiency, the studies analyzing the control of the cell viability by pulse parameters are rare.[9,18] This is probably due to the effects of pulsing media which can contain a variety of undesirable and even toxic substances. In our study, these undesirable substances are Ca2+ ions. We performed electropermeabilization experiments in EMEM medium, which is a culture medium of DC3F cells. Because of the compatibility of results the pulsation of the cells in case of the cell viability, experiments were made in EMEM like in electropermeabilization experiments. Although EMEM Ca^{2+} concentration (1.8 mM) is in the range of approximate harmless limits of the extracellular fluids concentrations for short periods (0.5 - 2.0 mM) [22],

it can be toxic due to the impairment of Ca^{2+} cellular transports during cell injury. During and some time after the cells are exposed to electrical pulses, Ca^{2+} easily diffuses through the transiently permeable membrane due to very low cytosolic Ca^{2+} free concentration (~0.1 μM). An increase in cytosolic Ca^{2+} concentration can directly lead to cell lysis by causing disruption of the cytoskeleton, DNA fragmentation or extensive damage to other cell components.[23] Therefore, the cell viability is affected at lower pulse amplitudes as in the experiments prepared in media without Ca^{2+}. However, the effects of electrical parameters have the same trends as if performed in Ca^{2+} free medium (*i. e.* SMEM) (data not shown).

Our results were obtained using DC3F cell line and small test molecules. In one of our previous studies, we showed that electropermeabilization curves, obtained by PI for a given set of electrical parameters, are comparable with the electropermeabilization curves obtained by using anticancer drug bleomycin as a marker of cell permeabilization.[19] So, we can conclude that our observations are valid also for bleomycin, which is the drug of choice for electrochemotherapy.[4,5]

A systematic study of the influence of different electric field parameters (field intensity, pulse number, and duration) on electropermeabilization and cell viability may serve as a base for the optimisation of the electropermeabilization conditions for different applications.

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