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Role of pulse shape in cell membrane electropermeabilization

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Abstract

The role of the amplitude, number, and duration of unipolar rectangular electric pulses in cell membrane electropermeabilization in vitro has been the subject of several studies. With respect to unipolar rectangular pulses, an improved efficiency has been reported for several modifications of the pulse shape: separate bipolar pulses, continuous bipolar waveforms, and sine-modulated pulses. In this paper, we present the results of a systematic study of the role of pulse shape in permeabilization, cell death, and molecular uptake. We have first compared the efficiency of 1-ms unipolar pulses with rise- and falltimes ranging from 2 to 100 μs, observing no statistically significant difference. We then compared the efficiency of triangular, sine, and rectangular bipolar pulses, and finally the efficiency of sine-modulated unipolar pulses with different percentages of modulation. We show that the results of these experiments can be explained on the basis of the time during which the pulse amplitude exceeds a certain critical value.

Keywords: Electropermeabilization; Electroporation; Pulse shape; Cell survival; Cell membrane permeability; Molecular uptake

1. Introduction

Electropermeabilization (also termed electroporation) is an effective method of internalization of various molecules into biological cells, with an increasing number of applications in oncology [1,2], genetics [3], immunology [4], and cell biology [5,6]. In parallel with the practical use of the method continues the quest for understanding the underlying mechanisms of the phenomenon [3,7–12].

The efficiency of electropermeabilization in vitro depends on various physical and chemical parameters, such as the molecular composition of the membrane [13,14] and osmotic pressure [15], but above all, on the parameters of electric pulses. Investigations of the role of the amplitude, number, and duration of unipolar rectangular pulses have been the subject of several comprehensive studies [16–20]. In addition, at least two studies have focused on a comparison of the efficiency of unipolar and bipolar rectangular pulses in vitro. Tekle et al. [21] compared continuous unipolar and bipolar 60-kHz rectangular waves of 400 μs total duration, and obtained a significantly better DNA transfection with a bipolar wave. Because electropermeabilization is usually not performed with continuous waves, but with sequences (trains) of separate pulses [18,22–25], our group compared the efficiency of unipolar and symmetrical bipolar rectangular pulses, in both cases delivering eight 1-ms pulses at 1-s intervals [26]. With bipolar pulses, permeabilization was achieved at lower pulse amplitudes and molecular uptake was significantly higher, while the pulse amplitude leading to cell death was practically unaltered. We also demonstrated that electrolytic contamination caused by the release of metal ions from the electrodes can be reduced considerably by the use of bipolar instead of unipolar pulses [27]. Even before these studies were published, bipolar pulses were applied successfully in electrochemotherapy [28], as well as for DNA transfection in vivo in mice [29,30].

Unlike the role of the amplitude, number, duration, and polarity of pulses, a hypothetical role of pulse dynamics, or the "pulse shape," has not yet been a subject to a broader systematic investigation.[1] One of the reasons for this is the absence of commercially available programmable genera-

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[1] Unipolar and bipolar pulses should not be conceived as having different "shapes," since a bipolar pulse is a sequence of two consecutive, oppositely polarized unipolar pulses.

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tors of high-voltage waveforms, which confines these studies to the laboratories with access to custom-designed electronic equipment. Kinosita and Tsong [31] used linearly increasing electric fields with rates of increase from 12.5 V/cm per microsecond up to 50 V/cm per microsecond for permeabilization of erythrocytes, and concluded that field intensity at which permeabilization occurs is not affected by this rate of increase. Chang et al. reported an improved efficiency of cell permeabilization and fusion [32], as well as gene transfection [33] when a sine wave (40 kHz–1 MHz) was superimposed to a rectangular pulse. Xie and Tsong [34] compared rectangular, sine, and triangular waves with extremely long durations (1–100 s) and relatively low amplitudes (voltage-to-distance ratio 50–200 V/cm), obtaining the most efficient transfection for rectangular, less for sine, and the least for triangular waves. It is, however, uncertain (as the authors themselves agree) whether the transfection was achieved due to permeabilization itself, or rather due to subsequent electrophoresis and electroosmosis caused by the very long pulse duration. Moreover, Šatkauskas et al. [35] recently clearly demonstrated that, even though prior electropermeabilization is necessary, efficiency of DNA-electrotransfer in skeletal muscle depends critically on the electrophoretic component of the pulses.

In this paper, we present the results of a systematic study of the role of pulse shape in cell permeabilization, cell death, and uptake of exogenous molecules. Using custom-designed electronic equipment developed in our laboratory, we compared the efficiency of: (i) unipolar rectangular (trapezoidal) pulses with different rise- and falltimes, (ii) bipolar rectangular, sinusoidal, and triangular pulses, and (iii) unipolar unmodulated and sine-modulated rectangular pulses. Part (i) of the study aimed at an investigation of possible role of the first derivative of the pulse amplitude in electropermeabilization. Parts (ii) and (iii) then focused on the influence of other parameters of the pulse shape, and on the search of a plausible explanation of the obtained results, as well as of the related results published in previous studies [31–34].

2. Materials and methods

2.1. Cells

DC3F cells, a line of spontaneously transformed Chinese hamster fibroblasts [36], were grown in monolayers at 37 °C and 5% CO_2 (Universal Water Jacketed Incubator, Forma Scientific, Marietta, OH, USA). One hundred fifty-square centimeter flasks were used for general cultivation, and 60-mm petri dishes were used for cloning efficiency assays (both from TPP, Trasadingen, Switzerland). The culture medium consisted of Eagle minimum essential medium EMEM 41090 supplemented with 10% fetal bovine serum (both from Life Technologies, Rockville, MD, USA), 100 U/ml of penicillin and 125 μg/ml of streptomycin (both from Sarbach/Solvay Pharma, Brussels, Belgium).

2.2. Exposure to electric pulses

To allow for comparison with experiments using different electrode distances, the intensity of the pulses was characterized by the voltage-to-distance ratio (VDR), which was calculated as the voltage delivered to the electrodes divided by the distance between them. Since for stainless steel electrodes, the voltage drop at the electrode–electrolyte interface is very small [37], and the dimensions of the droplet in the plane parallel to the electrode plates were several times larger than the distance between the electrodes, the VDR is also a good estimate of the electric field within the droplet.

After trypsination with trypsin–EDTA (Life Technologies), cells were centrifuged for 5 min at 1000 rpm in a C312 centrifuge (Jouan, St. Herblain, France) and resuspended at 2×10^7 cells/ml in the electropermeabilization medium. Spinner minimum essential medium SMEM 21385 (Life Technologies) with electrical conductivity of 1.6 S/m was used for trapezoidal (unipolar rectangular with adjustable rise- and falltimes), bipolar rectangular, bipolar sine, and bipolar triangular pulses. These pulses were generated by an AFG 310 programmable function generator (Tektronix, Wilsonville, OR, USA), amplified with a bipolar amplifier built in the Laboratory of Biocybernetics at the Faculty of Electrical Engineering of the University of Ljubljana (the device is described in detail in Ref. [38]). The medium consisting of 250 mM sucrose, 10 mM phosphate buffer, and 1 mM $MgCl_2$ (made according to Ref. [39]) with electrical conductivity of 0.15 S/m was used for the sine-modulated pulses, which were generated by a custom-designed high-voltage waveform generator built in the same laboratory. In all experiments, the pulses were delivered through a pair of flat stainless steel electrodes 2 mm apart, between which a 50-μl droplet of the cell suspension was placed.

2.3. Determination of cell survival

The percentage of surviving cells was determined by their cloning efficiency after pulsation in the electropermeabilization medium. Subsequent to pulsation, the cells were incubated for 10 min at room temperature and then diluted by the addition of 950 μl of SMEM to prevent drying. After additional 30 min, cells were diluted in the culture medium to 100 cells/ml, and 4 ml of suspension was transferred into each 60-mm petri dish where the cells were grown for 5 days. Cells were then fixed for 15 min with 100% ethanol (Carlo Erba Reagenti, Milan, Italy) and consecutively stained for 15 min with 1% crystal violet (Sigma, St. Louis, MO, USA). Clone colonies were counted under a light microscope (Leica, Wetzlar, Germany) and normalized to the control (cells not exposed to electric pulses) to obtain the percentage of surviving cells.

2.4. Determination of cell electropermeabilization

The percentage of electropermeabilized cells was determined by their cloning efficiency after pulsation in the electropermeabilization medium containing 5 nM bleomycin (Laboratoires Roger Bellon, Neuilly-Sur-Seine, France). An intact membrane is impermeable to bleomycin, and while at 5 nM external concentration bleomycin has no effect on nonpermeabilized cells, it causes the death of electropermeabilized cells [40]. This method is highly selective and accurate, as well as affordable.

Subsequent to pulsation, the cells were incubated for 10 min at room temperature and then diluted by the addition of 950 μl of SMEM. After additional 30 min, cells were diluted in the culture medium, grown for 5 days and then fixed and stained as described above. Clone colonies were counted and normalized to the control (unpulsed cells, 5 nM bleomycin) to obtain the percentage of cells surviving the exposure to electric pulses in suspension with 5 nM bleomycin. By subtracting this percentage from 100%, the percentage of permeabilized cells was obtained.

2.5. Determination of uptake of exogenous molecules

Uptake of exogenous molecules was determined by the cell fluorescence after pulsation in the electropermeabilization medium containing 1 mM lucifer yellow (Sigma), a nonpermeant fluorescent dye. Subsequent to pulsation, cells were incubated for 10 min at room temperature and then diluted by the addition of 950 μl of SMEM. After additional 30 min, cells were diluted in 5 ml of phosphate buffer saline (PBS, Life Technologies), and extracellular lucifer yellow was then washed away by two consecutive centrifugations and resuspensions in PBS. Each centrifugation was performed for 5 min at 1000 rpm, which causes no loss of cell viability. Cells were then broken down by ultrasonication (Sonifier 250, Branson Ultrasonics, Danbury, CT, USA) and fluorescence was measured in arbitrary units on a spectrofluorometer (SFM 25, BioTek, Winooski, VT, USA). Excitation was set at 418 nm wavelength and emission was detected at 525 nm.

At pulse amplitudes which lead to the death of practically all the cells in the population, the detected uptake of lucifer yellow remains somewhat above the background (control) level. This is due to the fact that dead cells are not only those that burst or decompose due to the intense permeabilization, but also those that initially reseal, yet due to the loss of their internal constituents have no ability to form clones. Such "cellular ghosts" retain some lucifer yellow, and this results in an apparent discrepancy between the survival and uptake at high pulse amplitudes (see Figs. 2, 4, and 7).

2.6. Treatment of experimental data

All experiments were repeated three times at intervals of several days or more. For each experimental point, mean and standard deviation were determined. Voltage-to-distance ratio was used as an estimate of electric field strength of the pulses. The percentages of surviving and permeabilized cells as functions of the applied voltage-to-distance ratio (VDR) were each fitted by a two-parameter sigmoidal curve,

$$y(x) = \frac{100\%}{1 + \exp[(x_c - x)/b]},$$

where x is the VDR, y is the percentage of cells, x_c is the x-value corresponding to $y = 50\%$, and b determines the slope of the sigmoid curve. The VDR leading to the permeabilization of 50% cells was denoted as $P_{50\%}$, and the VDR causing the death of 50% cells was denoted by $D_{50\%}$.

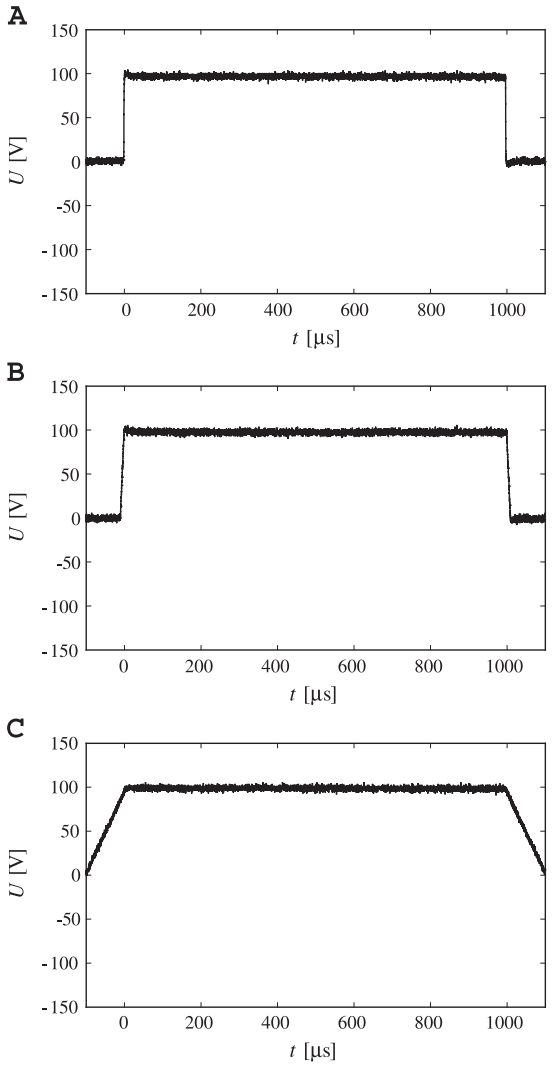

Fig. 1. The time course of a generated unipolar trapezoidal pulse of 1 ms duration, with rise- and falltimes of 2 μs (A), 10 μs (B), and 100 μs (C), and 100 V peak amplitude.

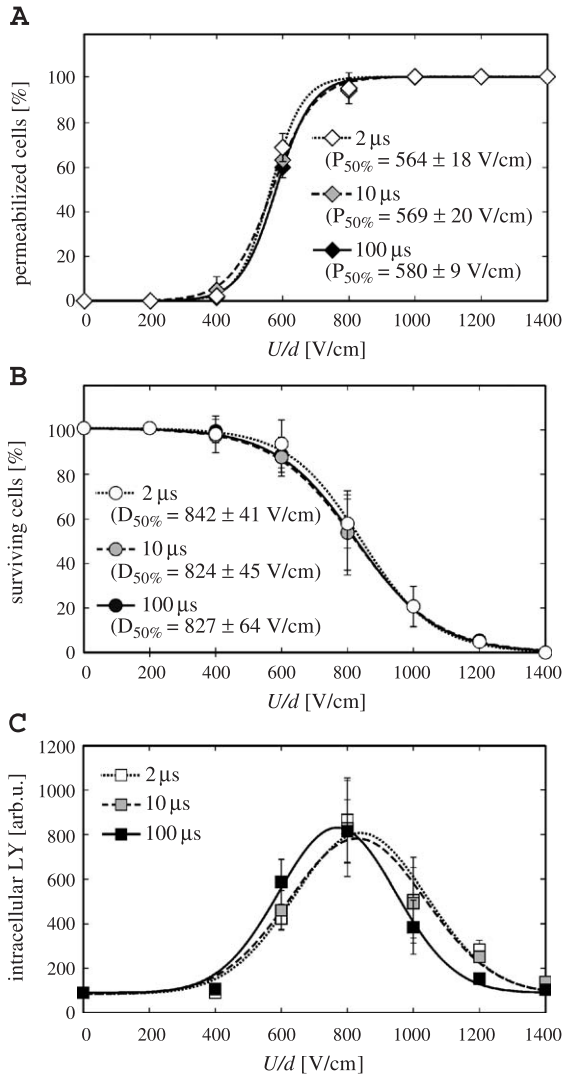

Fig. 2. The efficiency of eight unipolar trapezoidal pulses, 1 ms each, delivered in 1-s intervals, with rise- and falltimes of 2, 10, and 100 μs.

The fitting of sigmoidal curves to permeabilization and survival data is widely used, mostly on an empirical ground. However, in a recent theoretical paper [41], we showed that the two-parameter sigmoidal curves as given above are also in good agreement with the permeabilization and survival curves that would correspond to a Gaussian distribution of cell radii.

For the uptake of lucifer yellow, the intensity of fluorescence was fitted by a three-parameter Gaussian peak,

$$y(x) = y_{\max}\exp(-(x_c - x)^2/2b^2),$$

where x is again the VDR, y is the intracellular concentration of lucifer yellow, $y_{\max}$ is the maximum intracellular concentration of lucifer yellow in a given experiment, x_c is the x-value corresponding to $y = y_{\max}$, and b determines the width of the peak.

All fits were obtained by least-squares nonlinear regression using Sigma Plot 5.05 (SPSS, Richmond, CA, USA).

3. Results and discussion

3.1. Unipolar pulses with different rise- and falltimes

Fig. 1 shows the unipolar pulses with rise- and falltimes of 2, 10, and 100 μs generated by our setup, and Fig. 2 shows the percentage of electropermeabilized cells (panel A), percentage of surviving cells (panel B), and the internalized lucifer yellow (panel C) obtained with these pulses, each given as a function of voltage-to-distance ratio (VDR). In each case, eight pulses were delivered at intervals of 1 s, each pulse with the maximum VDR lasting for 1 ms (i.e., total pulse duration = risetime + 1 ms + falltime). The results shown in Fig. 2 imply that at least in the investigated range, the risetime and the falltime of the pulse do not play a detectable role in cell membrane permeabilization, which is in agreement with the results of Kinosita and Tsong [31] on erythrocytes.

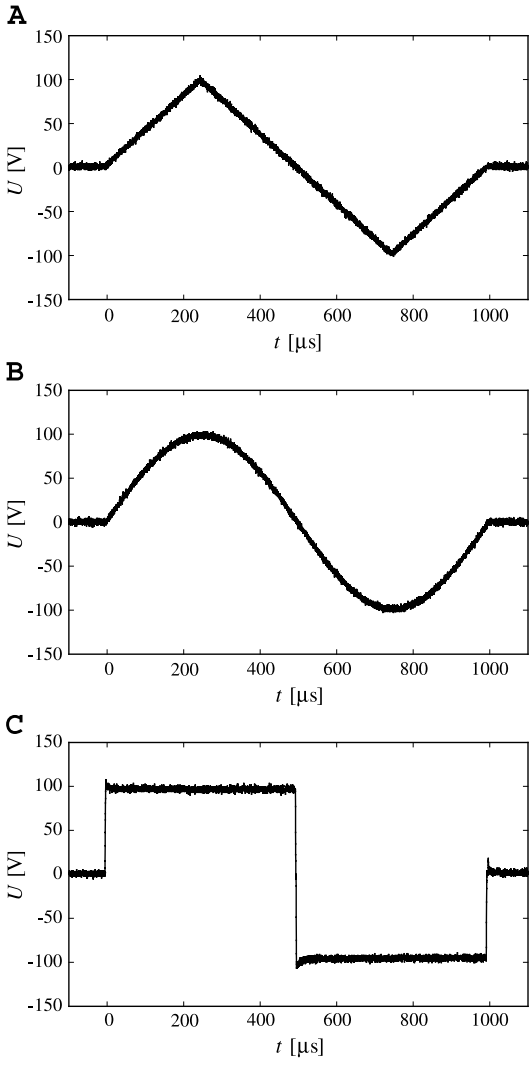

Fig. 3. The time course of a generated bipolar triangular (A), sine (B), and rectangular (C) pulse of 1 ms duration and 100 V peak amplitude.

3.2. Bipolar triangular, sine, and rectangular pulses

We previously reported on a significant difference between the efficiency of unipolar and symmetrical bipolar rectangular pulses of the same VDR and total duration [26]. In the present study, we compared the efficiency of bipolar pulses of different shapes: triangular, sine, and rectangular (Fig. 3). The results of this comparison are shown in Fig. 4, which reveals that electropermeabilization, cell death, and the peak of the uptake all occur at the lowest VDR amplitude for rectangular, and at the highest VDR amplitude for triangular pulses. In principle, these results alone could be explained by either different durations of above-critical VDR (i.e., different durations of VDR exceeding a certain critical value), or by different pulse dynamics (i.e., different values of the first derivative of the VDR with respect to time). However, the time

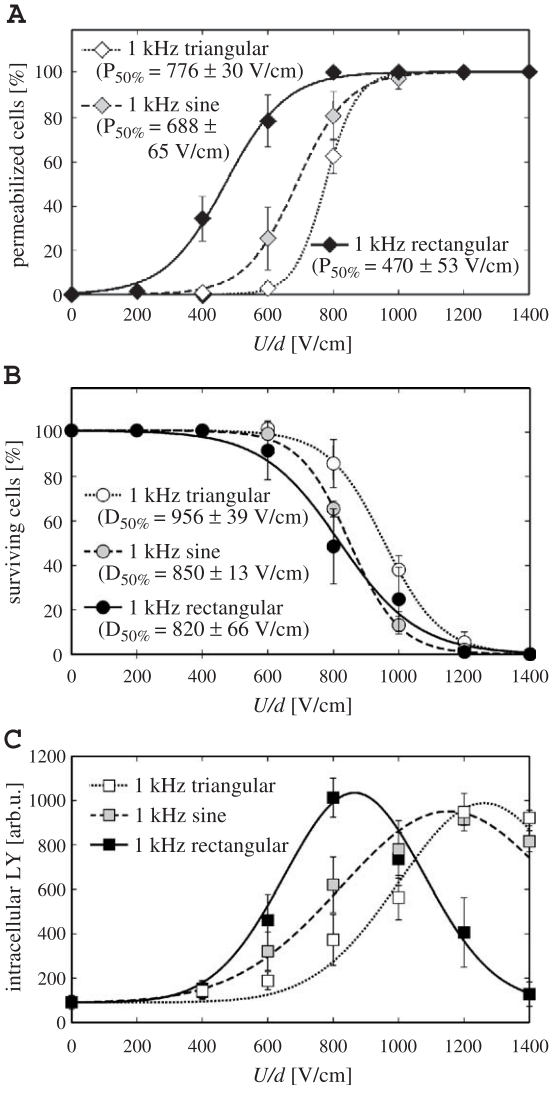

Fig. 4. The efficiency of eight symmetrical bipolar pulses, 1 ms each, delivered in 1-s intervals, for triangular, sine, and rectangular pulse shape.

Table 1
The peak VDR of 1 ms bipolar pulses of various shapes required for VDR of 470 V/cm to be exceeded without interruption for 200 and for 250 μs, compared to the experimentally determined values of $P_{50\%}$

	470 V/cm exceeded for 200 μs	470 V/cm exceeded for 250 μs	$P_{50\%}$
triangular pulse	783 V/cm	940 V/cm	776 V/cm
sine pulse	581 V/cm	665 V/cm	688 V/cm
rectangular pulse	470 V/cm	470 V/cm	470 V/cm

derivative of VDR was the only variable in the comparison of pulses with different rise- and falltimes (see Unipolar pulses with different rise- and falltimes subsection and Fig. 2), and this comparison shows no statistically significant differences. Consequently, the explanation based on pulse dynamics is unlikely, and it is plausible to attribute the results with different pulse shapes to differences in the duration of above-critical VDR. To an extent, this argument can also be supported quantitatively. In Table 1, the measured values of $P_{50\%}$ for triangular, sine, and rectangular bipolar pulses are compared to the theoretical values of the peak VDR required for the critical VDR of 470 V/cm to be exceeded without interruption for 200 μs and for 250 μs (depicted in Fig. 5). Applying durations other than these two does not provide a better agreement between the $P_{50\%}$ values and the peak VDR values, and this suggests that while the duration of above-critical VDR is indeed important for the efficiency of permeabilization, other parameters also contribute to this efficiency.

The importance of the duration of above-critical VDR is also compatible with the theory of electroporation, which attributes the increase of plasma membrane permeability to the formation of hydrophilic structures (“aqueous pores”) traversing the lipid bilayer and lined with the lipid headgroups [9]. According to this theory, considered by many as a plausible explanation of electropermeabilization, there is a threshold value of transmembrane voltage above which formation of aqueous pores becomes energetically favorable. Since the induced transmembrane voltage is proportional to the electric field [10], and thus practically proportional to the VDR (see Materials and methods section, Exposure to electric pulses subsection), this explains

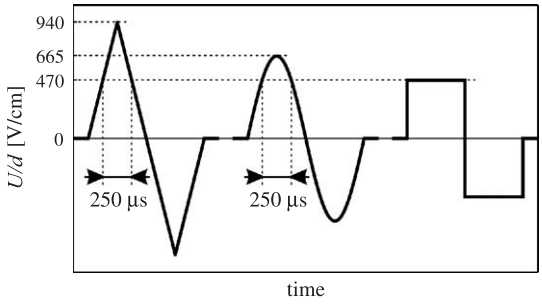

Fig. 5. The peak VDR required for a given value of VDR to be exceeded for a given duration varies with pulse shape. The figure shows the case of symmetric bipolar pulses of 1 ms duration, with VDR of 470 V/cm exceeded during 250 μs.

why an above-critical VDR is required for electropermeabilization. In addition, because the formation of aqueous pores is governed by statistical thermodynamics, the probability of formation of individual pores increases with the duration of the above-threshold transmembrane voltage, and thus with the duration of electric pulses.

3.3. Unipolar sine-modulated pulses

In the final part of this study, we compared the efficiency of unipolar unmodulated and sine-modulated rectangular pulses, the modulation at 50 kHz representing 10% or 90% of the peak VDR. The modulated pulses are shown in Fig. 6 (the unmodulated pulse is identical to the one shown in Fig. 1A), and the results of this comparison are given in Fig. 7. No statistically significant difference could be detected between an unmodulated and a 10%-modulated pulse, while with the 90%-modulated pulse, both $P_{50\%}$ and

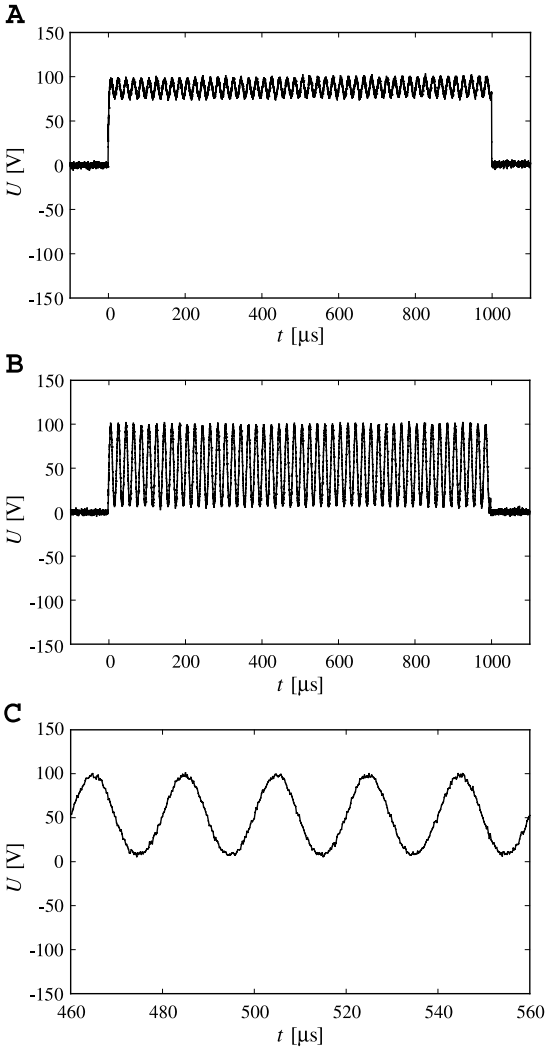

Fig. 6. The time course of a generated unipolar sine-modulated pulse of 1 ms duration and 100 V peak amplitude, with 10% modulation (A) and 90% modulation (B). The modulation frequency was 50 kHz. Panel C shows a part of the 90%-modulated signal in more detail.

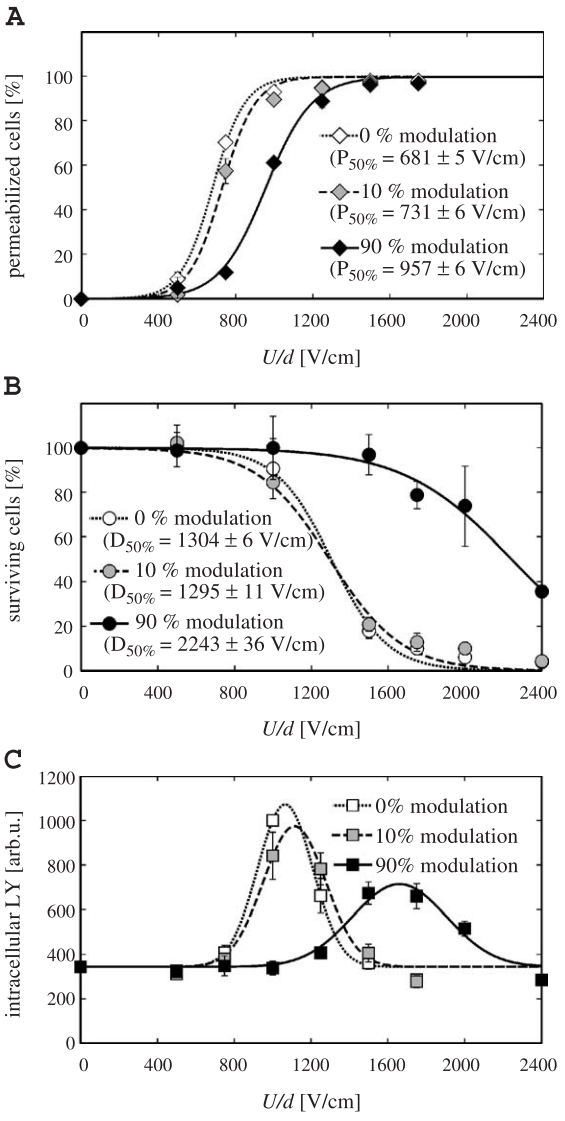

Fig. 7. The efficiency of one unipolar sine-modulated pulse of 1 ms duration, for 0% modulation (unmodulated), 10% modulation, and 90% modulation.

$D_{50\%}$ correspond to significantly higher VDRs than with either unmodulated or 10%-modulated pulse. The peak of the uptake is also rather similar for an unmodulated and a 10%-modulated pulse, while for a 90%-modulated pulse, this peak is much lower, and shifted towards much higher VDRs. These results can be explained by the same argument as the results with different waveforms (see Bipolar triangular, sine, and rectangular pulses)—namely, that the duration of the above-critical VDR has a major role in the efficiency of electropermeabilization. Despite the increase in pulse amplitude, this duration remains short for a 90%-modulated pulse, and consequently the peak of the uptake is rather low. Even at pulse amplitudes where practically all cells are permeabilized, the level of permeabilization is much lower with 90%-modulated pulses than with unmodulated or 10%-modulated pulses, which can also explain why

the value of $D_{50\%}$ is much higher with 90%-modulated pulses.

4. Conclusions

The results of this study show that among the parameters that describe the pulse shape, the time during which the pulse amplitude exceeds a certain critical value has a major role in the efficiency of electropermeabilization. As described above, this conclusion is in agreement with the theory of electroporation, considered by many as a plausible explanation of electropermeabilization. The differences in the duration of above-critical pulse amplitude can to a large extent explain the differences between various shapes of bipolar pulses, as well as the differences between pulses with different magnitude of modulation. In contrast, the time derivative of the pulse—at least in the range from several volts per microsecond up to several hundred volts per microsecond—has no detectable influence on the efficiency of electropermeabilization. This suggests that pulse generators with sub-microsecond risetimes are not a necessity for successful electropermeabilization.

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