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Cell membrane electropermeabilization by symmetrical bipolar rectangular pulses ☆
Part I. Increased efficiency of permeabilization

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Abstract

The paper presents a comparative study of electropermeabilization of cells in suspension by unipolar and symmetrical bipolar rectangular electric pulses. While the parameters of electropermeabilization by unipolar pulses have been investigated extensively both in cell suspensions and in tissues, studies using bipolar pulses have been rare, partly due to the lack of commercially available bipolar pulse generators with pulse parameters suitable for electropermeabilization. We have developed a high-frequency amplifier and coupled it to a function generator to deliver high-voltage pulses of programmable shapes. With symmetrical bipolar pulses, the pulse amplitude required for the permeabilization of 50% of the cells was found to be approximately 20% lower than with unipolar pulses, while no statistically significant difference was detected between the pulse amplitudes causing the death of 50% of the cells. Bipolar pulses also led to more than 20% increase in the uptake of lucifer yellow. We show that these results have a theoretical background, because bipolar pulses (i) counterbalance the asymmetry of the permeabilized areas at the poles of the cell which is introduced by the resting transmembrane voltage, and (ii) increase the odds of permeabilization of cells having a nonspherical shape or a nonhomogeneous membrane. If similar results are also obtained in tissues, bipolar pulse generators could in due course gain a wide, or even a predominant use in cell membrane electropermeabilization. © 2001 Published by Elsevier Science B.V.

Keywords: Electropermeabilization; Electroporation; Bipolar pulses; Cell survival; Cell membrane permeability; Molecular uptake

1. Introduction

Electropermeabilization, also referred to as 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 of 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. Several studies have addressed the roles of the amplitude, number and duration of unipolar rectangular pulses [16–19]. A systematic comparison of the efficiency of unipolar and bipolar rectangular pulses in electropermeabilization in vitro has been, to date, a subject of a single study, in which the authors have shown that the efficiency of DNA transfection in vitro was significantly higher with a bipolar wave than with a unipolar square wave. In their study, Tekle et al. [20] applied 60 kHz square waves (i.e. 8.33 μs duration of a single polarity) of 400 μs total duration. However, electropermeabilization is usually performed using sequences (trains) of rectangular pulses, with typical durations from hundreds of microseconds to tens of milliseconds, and separated by intervals from several milliseconds to several seconds [18,21–24]. Thus, an increased efficiency of separate bipolar rectangular pulses in comparison to unipolar pulses of same amplitude and duration remained to be demonstrated.

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The efficiency of separate unipolar and bipolar pulses has been compared in planar and patch-clamped membranes, where it was found that electropermeabilization occurs above the same threshold voltage for both types of pulses [25]. This is an anticipated result, since in these experiments, the transmembrane voltage is delivered directly and uniformly to the whole membrane. In contrast in cells, the induced transmembrane voltage varies with the position in the membrane with respect to the direction of the field (see, e.g. Ref. [26]). Moreover, because of the resting transmembrane voltage which is inherently present in cells, the total transmembrane voltage is asymmetric; for the usual case of a negative potential of the cell interior with respect to the cell exterior, the absolute value of the transmembrane voltage is higher at the pole facing the positive electrode than at the pole facing the negative electrode. This implies that the cell membrane electropermeabilization produced by unipolar pulses is also necessarily asymmetrical, which was confirmed by experiments [27]. This asymmetry can be counterbalanced by applying a symmetrical bipolar pulse instead of a unipolar one, i.e. by juxtaposing to the unipolar pulse, another pulse of the same duration, amplitude, and reversed polarity. While it would thus be plausible to investigate the efficiency of symmetrical bipolar pulses in cell electropermeabilization both in vitro and in vivo, according to the knowledge of the authors, no systematic investigation on this subject was reported so far. One of the conceivable reasons for this lies in the absence of commercially available generators of short, high-voltage rectangular bipolar pulses. To this date, the only application of high-voltage alternating (though not rectangular) pulses is found in external (transthoracic) cardiac defibrillators, which use a commuted exponential discharge, consecutive discharges of two oppositely polarized capacitors, or a damped sine wave [28,29]. For all these approaches, an improved defibrillation was reported with respect to the effect of customary unipolar waveforms.

A possible alternative (though, as we argue in Results and discussion, not entirely equivalent) to applying bipolar pulses is to alternate the polarity of consecutive unipolar pulses [30]. In tissues, another possibility is to reorient the electrodes between consecutive unipolar pulses in the train, which was shown to significantly improve the efficiency of electropermeabilization with respect to the protocol in which the electrode orientation was fixed [31].

In this paper, we describe the results of a comparative evaluation of cell permeabilization, cell death, and uptake of small exogenous molecules (lucifer yellow) achieved by trains of unipolar and bipolar rectangular pulses of the same amplitude and total duration. For the purpose of this study, we have developed a high-frequency bipolar amplifier and used it to amplify unipolar and bipolar rectangular pulses produced by a programmable function generator. With bipolar pulses, a noteworthy improvement was observed with respect to unipolar pulses: permeabilization was achieved at lower pulse amplitudes, molecular uptake was higher, while the pulse amplitude leading to cell death was practically unaltered. We show that these effects are in agreement with the established model according to which electropermeabilization only occurs at the locations with an above-critical transmembrane voltage. In addition, as we demonstrate in Part II of this study [32], the concentrations of ions released by bipolar pulses are more than an order of magnitude lower than those released by unipolar pulses of the same amplitude and duration, and thus, detrimental effects of electrolytic contamination on cells can be largely reduced by the use of bipolar instead of unipolar pulses. The improved efficiency of electropermeabilization with bipolar pulses, especially if also confirmed in tissues, suggests that the development of bipolar pulse generators is another step toward the establishment of electropermeabilization as a standard tool in biology and medicine.

2. Theory

For a spherical cell of radius R, the transmembrane voltage induced by the exposure to an electric field E is given by [10,33]:

$$U_{TI} = f_S ER\cos\varphi,$$

where f_s is a function reflecting the electrical and geometrical properties of the cell, and φ is the polar angle measured from the center of the cell with respect to the direction of the field. Thus, at the pole facing the negative electrode (at $\varphi = 0°$), the induced transmembrane voltage is positive, while at the pole facing the positive electrode (at $\varphi = 180°$), it is negative, with the same absolute value. The induced component is superimposed to the resting transmembrane voltage, U_{TR}, which is inherently present in biological cells, typically between -90 and -40 mV [34]. Denoting the maximum value of U_{TI} by $U_{TI0} = f_s ER$, the total transmembrane voltage is:

$$U = U_{TI} + U_{TR} = U_{TI0}\cos\varphi + U_{TR},$$

which shows that the absolute values of the transmembrane voltage at the two poles differ by $2U_{TR}$.

According to the established theory, electropermeabilization only occurs in those regions of the membrane where the transmembrane voltage exceeds the critical value U_c, for which different authors give values between 150 mV and 1 V [7–9,35,36]. While electropermeabilization occurs irrespective of the polarity of U, the lowest voltage at which it occurs depends on experimental conditions [15,37], as well as on the specific molecular constitution of the membrane [13,14]. For the usual case of $U_{TR} < 0$, electropermeabilization occurs at the pole facing the positive electrode if $(U_{TI0} + |U_{TR}|) \geq U_c$, and at the pole facing

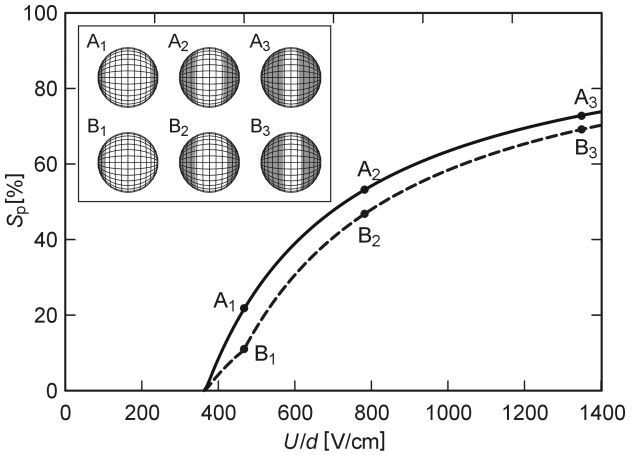

Fig. 1. The electropermeabilized membrane area (in percent of the total area) as a function of pulse amplitude for symmetrical bipolar (solid) and unipolar (dashed) pulses, for cells with a radius of 8 μm, resting transmembrane voltage of $U_{TR} = -60$ mV, and electropermeabilization occurring at transmembrane voltages $|U| \geq 500$ mV.

the negative electrode if $(U_{TI0} - |U_{TR}|) \geq U_c$; for the less common case of $U_{TR} > 0$, the situation is reversed. The fraction of the membrane area that is electropermeabilized can be written as (see Appendix A for derivation):

$$S_p/S = \begin{cases} 0 & U_{TI0} + |U_{TR}| < U_c \\ \dfrac{U_{TI0} - |U_{TR}| - U_c}{2U_{TI0}} & U_{TI0} + |U_{TR}| \geq U_c > U_{TI0} - |U_{TR}| \\ \dfrac{U_{TI0} - U_c}{U_{TI0}} & U_{TI0} - |U_{TR}| \geq U_c \end{cases}$$

If, however, a symmetrical bipolar rectangular pulse is delivered instead of the unipolar one, the asymmetry of electropermeabilization described above is compensated for, and the permeabilized area[1] of the membrane is described by:

$$S_p/S = \begin{cases} 0 & U_{TI0} + |U_{TR}| < U_c \\ \dfrac{U_{TI0} + |U_{TR}| - U_c}{U_{TI0}} & U_{TI0} + |U_{TR}| \geq U_c \end{cases}$$

Fig. 1 shows an example of the described differences between the permeabilized areas obtained by a unipolar (dashed) and a bipolar pulse (solid), for $U_c = 500$ mV, $U_{TR} = -60$ mV, and cell radius of 8 μm.

3. Materials and methods

3.1. Cells

DC3F cells, a line of spontaneously transformed Chinese hamster fibroblasts [38], were grown in monolayers at 37 °C and 5% CO_2 (Universal Water Jacketed Incubator, Forma Scientific, Marietta, OH, USA). 150-cm^3 flasks were used for general cultivation, while 60-mm petri dishes were used for determination of cloning efficiency (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 units/ml of penicillin and 125 μg/ml of streptomycin (both from Sarbach/Solvay Pharma, Brussels, Belgium).

3.2. Exposure to electric pulses

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 Spinner minimum essential medium SMEM 21385 (Life Technologies), which is a calcium-depleted modification of EMEM. Electric pulses were generated by an AFG 310 programmable function generator (Tektronix, Wilsonville, OR, USA) and amplified to the required voltages in the range from 0 to 280 V with a bipolar amplifier built in the Laboratory of Biocybernetics at the Faculty of Electrical Engineering of the University of Ljubljana. The time needed for complete inversion of the pulse (i.e. from the peak value in one polarity to the peak value in the opposite polarity) was below 1.8 μs for all the pulse amplitudes used. In the frequency range from 500 Hz to 35 kHz, the total shape distortion of the signal introduced by the amplifier was below 5%, and up to 55 kHz, it was below 15%. The pulses were delivered to a pair of flat stainless steel electrodes 2 mm apart, between which a 50-μl droplet of the cell suspension was placed.

3.3. Determination of cell survival

The percentage of surviving cells was determined by their cloning efficiency after pulsation in SMEM. 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

[1] Throughout the paper, the term "permeabilized area" refers to the area of the membrane where transmembrane voltage exceeds the critical voltage for electropermeabilization and thus, transmembrane transport *can* occur, though it does not necessarily proceed in this entire region. For example, if one assumes that electropermeabilization is caused by formation of aqueous pores (electroporation), then these pores represent only a small fraction of the whole area where the transmembrane voltage is above the critical value—in other words, the "porated area" is much smaller than what we term "permeabilized area". This does not, however, impair the validity of the presented arguments, since for the net uptake of exogenous molecules per cell, it is irrelevant whether this uptake is concentrated in very small pores having a very high permeability, or proceeds through the whole "permeabilized area" having an average permeability of the pores and the intact membrane between them.

were then fixed by a 15-min exposure to 100% ethanol (Carlo Erba Reagenti, Milan, Italy) and 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.

3.4. Determination of cell electropermeabilization

The percentage of electropermeabilized cells was determined by their cloning efficiency after pulsation in SMEM 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 [39]. 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.

3.5. Determination of uptake of exogenous molecules

Uptake of exogenous molecules was determined by the cell fluorescence after pulsation in SMEM 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 (Life Technologies), and extracellular lucifer yellow was washed by two consecutive centrifugations and resuspensions in phosphate buffer saline. Cells were then broken down by ultrasonication (Sonifier 250, Branson Ultrasonics, Danbury, CT, USA) and fluorescence was measured on a spectro-fluorometer (SFM 25, BioTek, Winooski, VT, USA). Excitation was set at 418-nm wavelength and emission was detected at 525 nm. The background fluorescence was subtracted, and the fluorescence was converted into and the molarity of LY in the solution through a calibration using 10 nM, 100 nM and 1 μM lucifer yellow solutions.

3.6. 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 electropermeabilized cells as functions of the applied voltage-to-distance ratio were each fitted to a two-parameter sigmoidal curve,

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

where x is the pulse amplitude, 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 pulse amplitude leading to the permeabilization of 50% cells was denoted as $P_{50\%}$, and the amplitude causing the death of 50% cells was denoted by $D_{50\%}$.

For the uptake of lucifer yellow, the intracellular concentration of LY was estimated by multiplying the measured molarity in the solution by 465—the ratio of the total volume of solution (1 ml) and the approximate volume of the cells in the sample (2.15 μl, for 10^6 spherical cells with a radius of 8 μm). These data were then fitted to a three-parameter Gaussian peak,

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

where x is again the pulse amplitude, 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. The maximum intracellular concentration of LY was denoted as LY_{max}.

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

4. Results and discussion

In our study, we compared the efficiency of three trains of rectangular pulses, sketched in Fig. 2: (i) a train of eight 1-ms unipolar rectangular pulses delivered in intervals of 1 s, (ii) a train of eight 1-ms symmetrical bipolar rectangular pulses delivered in intervals of 1 s, and (iii) a train of four 2-ms symmetrical bipolar rectangular pulses delivered in intervals of 1 s. The parameters of the unipolar train (i) were chosen after an optimization of the number and duration of unipolar rectangular pulses (data not shown), and the parameters of the two bipolar trains, (ii) and (iii), were then set to give these trains the same total duration of all the pulses as for the train (i). The train (ii) delivered the same number of pulses as the train (i), while in the train (iii) the duration of a single polarity was the same as in the train (i).

Fig. 3 shows the percentage of electropermeabilized cells (panel A), percentage of surviving cells (panel B), and the internalized lucifer yellow (panel C) as functions of pulse amplitude, obtained with these pulse trains.

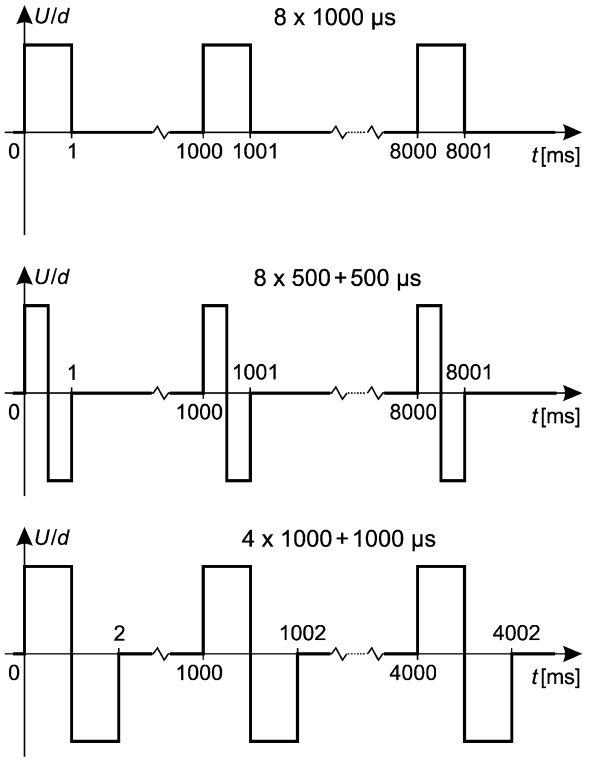

Fig. 2. The three trains of rectangular pulses compared in the experiments: (i) a train of eight 1-ms unipolar rectangular pulses delivered in intervals of 1 s, (ii) a train of eight 1-ms symmetrical bipolar rectangular pulses delivered in intervals of 1 s, and (iii) a train of four 2-ms symmetrical bipolar rectangular pulses delivered in intervals of 1 s.

As Fig. 3 demonstrates, with bipolar pulses, the pulse amplitude required for the permeabilization of 50% of the cells was approximately 20% lower than with unipolar pulses, while no statistically significant difference was detected between the pulse amplitudes causing the death of 50% of the cells. Bipolar pulses also led to more than 20% increase in the uptake of lucifer yellow. Because the rate of diffusion, which is the predominant mechanism of transport for small molecules [18], intensifies with the increase of the permeabilized area of the membrane, the increased uptake obtained by bipolar pulses is in a good agreement with the theoretical arguments presented in the previous section (see Fig. 1). These arguments can also explain the similarity of the results of cell survival, because the difference between the permeabilized areas created by unipolar and bipolar pulses decreases with the increase of pulse amplitude, and cell death occurs at higher pulse amplitudes than permeabilization.

On the other hand, the difference between the permeabilized areas alone cannot account for the increased cell permeabilization obtained with bipolar pulses. Namely, as Fig. 1 shows, the theoretically predicted threshold of permeabilization is the same for all pulse shapes. Due to the high sensitivity of the bleomycin method we have used, cell permeabilization was detected very close to the threshold amplitude, yet we have observed different thresholds for bipolar pulses with respect to unipolar ones (Fig. 3, panel A). A plausible argument for the observed difference is provided by the fact that at least some of the cells in suspension are neither entirely spherical, nor are their membranes entirely homogeneous. Thus, cell permeabilization is not only dependent on the amplitude of the pulses, but also on the orientation of the pulses with respect to the cell, i.e. on the site within the cell membrane where the largest hyperpolarization is induced by the pulses. We sketch this concept in Fig. 4: for nonspherical

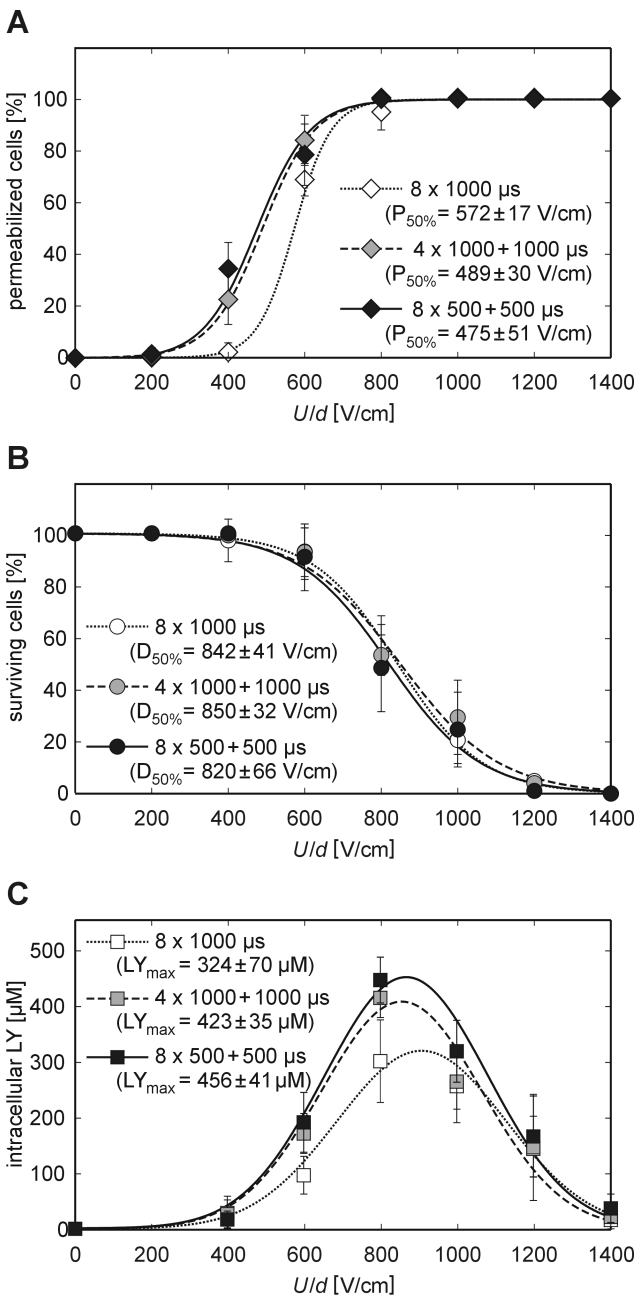

Fig. 3. Experimental results. (A) The percentage of electropermeabilized cells, (B) the percentage of surviving cells, and (C) the average intracellular concentration of lucifer yellow, all plotted as functions of the pulse amplitude, which is given by the ratio between the voltage applied to the electrodes (U) and the distance between them (d).

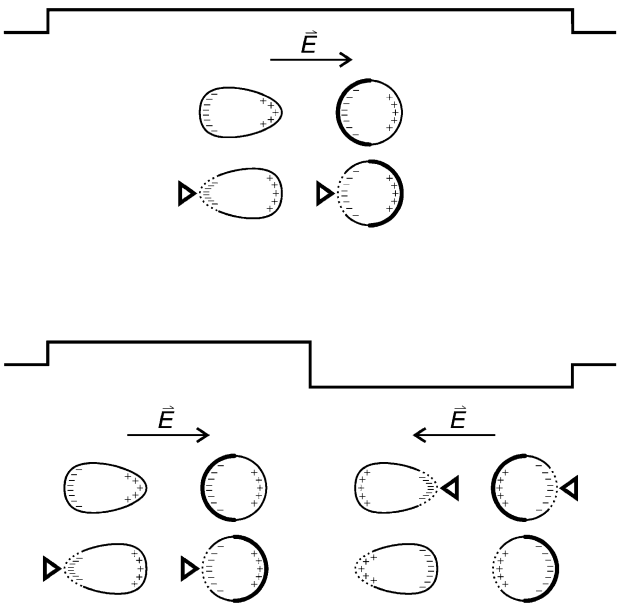

Fig. 4. Exposure of nonspherical cells and cells with a nonhomogeneous membrane to a unipolar and a bipolar pulse. For nonspherical cells, with a given pulse amplitude, the induced transmembrane voltage is higher on the sharper-pointed pole than on the flatter one, and at sufficiently low pulse amplitudes, electropermeabilization occurs only if the sharper-pointed pole is hyperpolarized. For cells with a nonhomogeneous membrane, the critical transmembrane voltage is different for each of the two regions (e.g. two domains with different lipid composition), and at sufficiently low pulse amplitudes, electropermeabilization occurs only if the region with the lower critical voltage (schematically shown as the thinner region) is hyperpolarized. The triangles indicate the occurrence of electropermeabilization.

cells, electropermeabilization occurs at lower voltages if the sharper-pointed regions or processes of the cell are subject to hyperpolarization [40], while with a nonhomogeneous membrane, the critical voltage differs between various regions of the membrane.

There is an additional, electrochemical argument for increased efficiency of bipolar pulses with respect to unipolar ones. In Part II of this study [32], we show that electrolytic contamination of the cell suspension by metal ions released from the electrodes is more than an order of magnitude lower with bipolar pulses than with unipolar pulses of the same amplitude and duration. This is the case for both aluminum electroporation cuvettes (released Al^{3+}) and for stainless steel electrodes (released Fe^{2+}/Fe^{3+}), and could explain why, despite the larger permeabilized area, no decrease in cell survival was observed when bipolar pulses were applied. Importantly, if unipolar pulses with alternating polarity were used instead of bipolar pulses, delivered again in intervals of 1 s, electrolytic contamination was not reduced at all; the concentration of released metal ions was even slightly higher than with an analogous train of unipolar pulses with constant polarity. This shows that applying unipolar pulses with alternating polarity is not entirely equivalent to applying bipolar pulses.

5. Conclusions

The aim of our study was to verify the theoretical prediction of an increased efficiency of symmetrical bipolar pulses in achievement of cell membrane electropermeabilization with respect to unipolar pulses of the same duration and amplitude. As we show, there are at least three theoretical arguments that support these results. Due to the resting transmembrane voltage, the areas of the two poles of the cell permeabilized by unipolar pulses are different, while bipolar pulses, yielding a larger total permeabilized area for the latter case, compensate for this asymmetry. It is also plausible to expect that the bipolar pulses increase the odds of permeabilization of cells having a nonspherical shape or a nonhomogeneous membrane. In addition, bipolar pulses decrease the electrolytic contamination of the suspension by metal ions released from the electrodes [32].

Since bipolar pulses decrease the minimum pulse amplitude leading to electropermeabilization and increase the uptake obtained with the same pulse amplitude, while the cell survival remains practically identical to that obtained with unipolar pulses of the same amplitude and duration, the importance and potential applicability of these findings exceed the academical interest. Besides its numerous applications in biomedical and pharmaceutical research, electropermeabilization today is also gaining an increasing importance in clinical applications, such as electrochemotherapy and electrogenetherapy [1,41–43]. In practically all of these applications, a high percentage of permeabilized cells and a sufficient uptake per cell are of utmost importance for a successful treatment. Thus, the next logical step is the comparison between the efficiency of unipolar and bipolar rectangular pulses in tissues and organs. Recently, bipolar pulses have been used for electrochemotherapy [44], as well as for DNA transfection in vivo in mice [45,46]. These studies demonstrate that also in tissues, electropermeabilization with bipolar pulses can be efficient, but a systematic comparison of the efficiency of unipolar and bipolar pulses in tissues and organs has not yet been published. If an improved efficiency is also consistently obtained in these environments, this will offer a strong motivation for development of bipolar pulse generators, which could in due course gain a wide, or even a predominant use in electropermeabilization.

Acknowledgements

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European Commission. T.K. was also a recipient of a part-time PhD scholarship of the French Government (CNOUS).

Appendix A. Membrane area permeabilized by unipolar and bipolar pulses

The area of a spherical cap is given by:

$$S_{cap} = 2\pi R^2\left(1 - \cos\varphi_{cap}\right), \quad \text{(A1)}$$

where R denotes the radius of the sphere, and φ_{cap} is the angle formed by the axis of rotational symmetry of the cap and the radius vector from the center of the sphere to the edge of the cap.

The transmembrane voltage on a spherical cell has the form:

$$U_T = U_{TR} + U_{TI} = U_{TR} + U_{TI0}\cos\varphi,$$

with U_{TR} denoting the resting transmembrane voltage, U_{TI} the induced transmembrane voltage, and U_{TI0} the maximum value of the latter (i.e. the value of U_{TI} at $\varphi = 0$).

Because of the resting transmembrane voltage, the total transmembrane voltage is higher at one pole of the cell than on the other, and an above-critical transmembrane voltage, $|U_T| \geq U_c$, would occur at:

$$|\cos\varphi| \geq \cos\varphi_c = \left(U_c - |U_{TR}|\right)/U_{TI0},$$

$$\text{for } U_{TI0} + |U_{TR}| > U_c,$$

at the hyperpolarized pole, and at:

$$|\cos\varphi| \geq \cos\varphi_c = \left(U_c + |U_{TR}|\right)/U_{TI0},$$

$$\text{for } U_{TI0} - |U_{TR}| > U_c,$$

at the depolarized pole.

Applying Eq. (A1), the total electropermeabilized area of the membrane, S_p, is then given by:

$$S_p = 0, \quad \text{for } U_{TI0} + |U_{TR}| < U_c,$$

$$S_p = 2\pi R^2\left(1 - \left(U_c + |U_{TR}|\right)/U_{TI0}\right)$$

$$= 2\pi R^2\left(U_{TI0} - |U_{TR}| - U_c\right)/U_{TI0},$$

$$\text{for } U_{TI0} + |U_{TR}| \geq U_c > U_{TI0} - |U_{TR}|, \quad \text{and}$$

$$S_p = 2\pi R^2\left(1 - \left(U_c - |U_{TR}|\right)/U_{TI0}\right)$$

$$+ 2\pi R^2\left(1 - \left(U_c + |U_{TR}|\right)/U_{TI0}\right)$$

$$= 4\pi R^2\left(U_{TI0} - U_c\right)/U_{TI0},$$

$$\text{for } U_{TI0} - |U_{TR}| \geq U_c.$$

If within the duration of the electric pulse, its polarity is reversed, the same maximum transmembrane voltage is reached at both poles of the cell, and this leads to a simpler situation,

$$S_p = 0, \quad \text{for } U_{TI0} + |U_{TR}| < U_c,$$

$$S_p = 4\pi R^2\left(1 - \left(U_c - |U_{TR}|\right)/U_{TI0}\right)$$

$$= 4\pi R^2\left(U_{TI0} + |U_{TR}| - U_c\right)/U_{TI0},$$

$$\text{for } U_{TI0} + |U_{TR}| \geq U_c.$$

Dividing these expressions by the total membrane area, $S = 4\pi R^2$, we obtain the electropermeabilized fraction of the membrane area, S_p/S.

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