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The influence of medium conductivity on electropermeabilization and survival of cells in vitro

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Abstract

Electropermeabilization and cell death caused by the exposure to high voltage electric pulses depends on the parameters of pulses, as well as the composition of the extracellular medium. We studied the influence of extracellular conductivity on electropermeabilization and survival of cells in vitro. For this purpose, we used a physiological medium with a conductivity of 1.6 S/m and three artificial media with conductivities of 0.14, 0.005, and 0.001 S/m. Measurements of pH, osmolarity, and cell diameter were made to estimate possible side effects of the media on the cells. Our study shows that the percentage of surviving cells increases with the decreasing medium conductivity, while the percentage of electropermeabilized cells remains unaffected. Our results show that cell survival in experiments involving electropermeabilization can be improved by decreasing the medium conductivity. To provide an interpretation of experimental results, we have theoretically estimated the resting transmembrane voltage, the induced transmembrane voltage, the time constant of the voltage inducement, and heating of the cell suspension for each of the media used. These calculations imply that for accurate interpretation of experimental results, both the induced and the resting transmembrane voltage must be considered, taking into account the conductivity and the ionic composition of the extracellular medium.

Keywords: Electropermeabilization; Induced transmembrane voltage; Resting transmembrane voltage; Medium conductivity; Bleomycin; DC3F cells

1. Introduction

Application of electric pulses to cells induces a voltage across cell membrane, termed the induced transmembrane voltage. This voltage combines with the resting transmembrane voltage, which is permanently present on cell membrane. Due to the induced component, which is proportional to the pulse amplitude, the total transmembrane voltage can significantly exceed its physiological range, and above a certain critical value in the range from 200 to 1000 mV [1,2], a large increase in membrane permeability is observed. The occurrence of transient high-permeability state of the cell membrane due to high-voltage electric pulses is called electropermeabilization. The ability to influence membrane permeability by application of electric pulses has opened a variety of applications in oncology [3–7], genetics [8–10], and cell biology [11,12].

To date, only few studies have experimentally investigated the effect of medium conductivity on electropermeabilization in vitro. Rols and Teissié [13] have shown that the threshold value for permeabilization of Chinese hamster ovary cells was independent of the ionic strength of the pulsing medium. The conductivities were not given, but on the basis of the media composition, it follows that they were above 0.1 S/m. Neumann [14] has shown that the percentage of permeabilized green algae cells (*Chlamydomonas reinhardtii*) decreases if the medium conductivity decreases (the interval of conductivities was from 0.0056 to 0.035 S/m). Djuzenova et al. [15] have shown that decreasing the extracellular conductivity results in lower viability of the murine myeloma cells while the PI-uptake increases, but the investigated interval of conductivities was quite narrow (0.08–0.37 S/m). Lojewska et al. [16] confirmed the theoretical predictions that the charging time of the membrane decreases with increasing medium conductivity, but this study was performed on lipid bilayers, and only in a range of very low conductivities (up to 0.005 S/m).

In summary, each of the studies mentioned above investigated the effect of the medium conductivity on electrop-

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ermeabilization on cells and lipid bilayers at a specific, narrow interval of conductivities, with partially different observations. Therefore, we decided to investigate the influence of extracellular media on permeabilization and survival of the cells on a wider interval of conductivities ($\sim$0.001 to $\sim$1.5 S/m). In order to theoretically explain the experimental results, resting transmembrane voltage U_{TR}, time constant τ, and function f_S were calculated and their influence on the permeabilization and survival of the cells in different media was estimated. To determine the influence of the extracellular conductivity on physiological parameters of media and cells, measurements of pH, osmolarity, and cell diameter have been performed, and heating of cell suspension was estimated.

2. Materials and methods

2.1. Extracellular media

Cell line DC3F—spontaneously transformed Chinese hamster fibroblasts [17]—were grown in Eagle's Minimum Essential Medium (EMEM) with added 10% fetal bovine serum—FCS (both from Sigma, USA). Electropermeabilization was performed in four different media with conductivities in range of three orders of magnitude ($\sim$0.001 to $\sim$1 S/m). Medium 1 was prepared according to the specifications of the research group of Rols and Teissié [18]. Because mediums 2 and 3 were prepared by dilution of medium 1 with isoosmotic solution of distilled water and sucrose (medium 2: distilled water/medium 1, 100:3; medium 3: distilled water/medium 1, 100:0.45), medium 1 represented our reference point. For the last medium, we used Spinner Minimum Essential Medium (SMEM, Gibco, Life Technologies, USA), which is a Ca^{2+}-depleted version of EMEM, and has approximately 10 times higher conductivity than medium 1. The basic components of the electroporative media used are presented in Table 1.

Table 1
Composition of media used

	SMEM[a] (mg/l)	m1 (mg/l)	m2 (mg/l)	m3 (mg/l)
NaCl	6800	–	–	–
KCl	400	–	–	–
$NaHCO_3$	2200	–	–	–
NaH_2PO_4	1580	–	–	–
Na_2HPO_4	–	1125	33.75	4.95
KH_2PO_4	–	283	8.49	1.244
$MgCl_2$	–	95	2.85	0.418
$MgSO_4$	200	–	–	–
Sucrose	–	85580	85580	85580
Glucose	1000	–	–	–

m1—medium 1, m2—medium 2, m3—medium 3.

[a] Besides the components given in the table, SMEM also contains amino acids, vitamins, and Phenol Red. More detailed information on composition can be found in Gibco catalog under Catalog no. 21385.

2.2. Measurements of specific conductivity, osmolarity and pH of the media

Conductometer MA 5950 (Metrel, Slovenia) was used to determine the specific conductivity of the media. Values for osmolarity were determined with an osmometer—Vapour Pressure Osmometer 5500 (Wascor). pH was measured using pH-meter MA 5750 (Metrel). All results are values corresponding to the temperature of 25 °C. Mean values for all parameters were determined from at least three measurements.

2.3. Measurements of cell diameter

Measurements were performed with DP 10 camera fixed on CK 40 microscope (both Olympus, Germany) at 200× magnification. Cell diameters were measured on three samples of cells ($\sim$15 cells in each sample) from cell suspension not exposed to electric pulses. Measurements were performed in each of the media used and also in the presence of bleomycin.

2.4. Electropermeabilization

To generate square pulses, prototype Electroporator was used (rise time and fall time: <1 μs, pulse width: 5 μs–5 ms, pulse amplitude: 25–500 V, number of pulses: 1–128), made in our laboratory at the Faculty of Electrical Engineering, University of Ljubljana, Slovenia. A train of eight rectangular pulses, duration: 100 μs, repetition frequency: 1 Hz, was used for electropermeabilization.

2.5. Experiment

After trypsination, cells were centrifuged for 5 min at 1000 rpm ($180 \times g$) at 4 °C, resuspended in specific extracellular medium within minutes after centrifugation, and centrifuged again at the same conditions. For mediums 2 and 3, a third centrifugation was needed. Although additional centrifugation could increase the mechanical damage to the cells and reduce the survival of the cells in these two media, it was necessary in order to wash away the remains of EMEM. The high conductivity of EMEM could otherwise increase the desired low conductivity of extracellular media. However, as the absolute plating efficiency did not change significantly for any of the investigated medium, we can conclude that the cell survival in mediums 2 and 3 was not affected by the additional centrifugation (data not shown). Cells were then diluted in the specific extracellular medium to obtain 2×10^7 cells/ml and kept at 4 °C until electropermeabilization. Cells were never kept in suspension longer than 30 min, because otherwise, the viability of cells was affected (especially in medium 3). A 50-μl droplet of cell suspension

was put between two parallel plate stainless steel electrodes. Trains of eight rectangular pulses with amplitudes from 0 to 400 V (voltage kept constant for all the pulses in the train, voltage drop over the duration of the pulse $< 1\%$) were applied. All experiments were performed at 25 °C. After 10 min of incubation at this temperature, SMEM was added to prevent drying. After additional 30 min, cells were diluted with EMEM with 10% FCS to obtain 50 cells/ml. Due to its content of Ca^{2+}, EMEM was found to have a harmful effect on permeabilized cells. However, 40 min of incubation were sufficient to prevent this effect. Cell suspension was put in petri dishes (4 ml/dish) and stored in an incubator (37 °C, 5% CO_2) for plating efficiency.

After 5 days, cells were fixed with methanol (Merck, Germany) and stained with 1% crystal violet (Sigma). Colonies were then counted and normalized to the control (cells subjected to exactly the same procedures for each medium except of exposure to electric pulses) to obtain the fraction of surviving cells in this medium.

To determine the percentage of electropermeabilized cells, the cells (2×10^7 cells/ml) were exposed to electric pulses in presence of 5 nM concentration of cytotoxic agent bleomycin, as described in detail in Ref. [19]. 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 permeabilized cells. The protocol of cell handling after electropermeabilization, including fixation and staining, was the same as for survival. Colonies were counted and normalized to the control (unpulsed cells, 5 nM bleomycin) and the fraction of cells surviving the exposure of electric pulses with added bleomycin was subtracted from 100% to obtain the fraction of permeabilized cells.

2.6. Data processing

All experiments were repeated at least three times on different days. Results from different repetitions of experiments were pooled together and are presented as mean and standard error of the mean (S.E.). On the fraction of permeabilized and surviving cells, a two-parameter sigmoid was fitted,

$$y(u) = \frac{100\%}{1 + e^{\frac{u_C - u}{b}}}, \quad (1)$$

where y is the fraction of cells, u is the pulse amplitude, u_C denotes the value of pulse amplitude corresponding to electropermeabilization or survival of 50% of the cells, and b determines the slope of the sigmoid curve. All fits were obtained by least-squares nonlinear regression using SigmaPlot 5.0.

Statistical analysis was performed with Kruskal–Wallis one-way analysis of variance on ranks (ANOVA) test using SigmaStat 2.0.

3. Results

3.1. Theoretical considerations

For a spherical cell with radius r and no surface charge, the induced transmembrane voltage (U_{TI}) can be calculated using the equation [20,21]

$$U_{TI} = f_S r E \cos\varphi (1 - \exp(-t/\tau)) \quad (2)$$

where φ is the angle between the direction of the applied electric field E and the normal from the center of the cell to the point of interest on cell surface, t denotes time from the onset of the electric field, while the function f_S and the time constant τ are given by

$$f_S = \frac{3\lambda_o(3dr^2\lambda_i + (3d^2r - d^3)(\lambda_m - \lambda_i))}{2r^3(\lambda_m + 2\lambda_o)(\lambda_m + 0.5\lambda_i) - 2(r-d)^3(\lambda_o - \lambda_m)(\lambda_i - \lambda_m)} \quad (3)$$

$$\tau = \frac{rC_m}{\frac{2\lambda_o\lambda_i}{2\lambda_o + \lambda_i} + \frac{r}{d}\lambda_m}, \quad (4)$$

with C_m, the membrane capacitance, d, the membrane thickness and λ_i, λ_m, λ_o, the conductivities of the cytoplasm, cell membrane and extracellular medium, respectively. All symbols are also presented in Fig. 1, and their typical values are given in the Appendix A.

Under physiological conditions, where $\lambda_m \ll \lambda_i$, λ_o, the function f_S is reduced to a constant, $f_S = 1.5$, and the

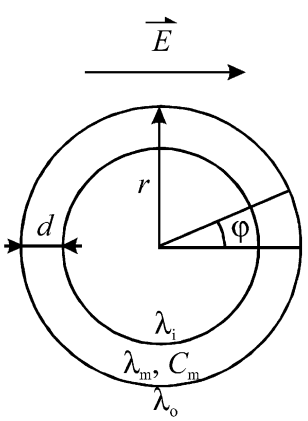

Fig. 1. Model of the cell. (E—electric field, r—cell diameter, d—membrane thickness, φ—the angle between the direction of E and a point on cell membrane, λ_i, λ_o, λ_m—intracellular, extracellular, and membrane conductivity, respectively, C_m—membrane capacitance).

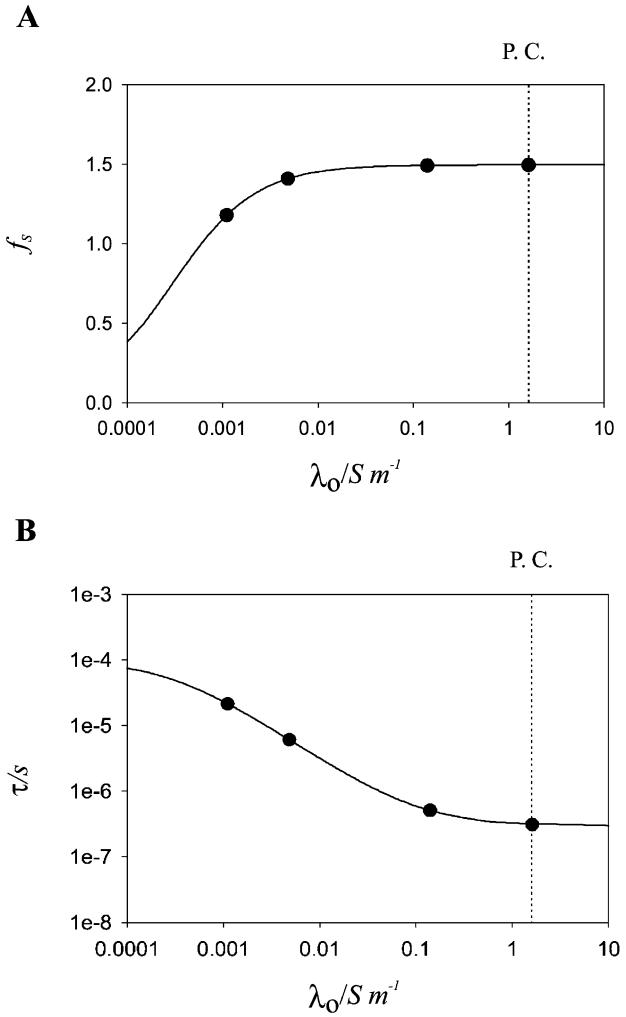

Fig. 2. The influence of extracellular conductivity on (A) the value of the function f_S, (B) the time constant τ. The symbols represent the values of conductivities of media used in our study (values of parameters used: $r = 5.94$ μm, $E = 1000$ V/cm, $\lambda_m = 5\times10^{-7}$ S/m, $\lambda_i = 0.2$ S/m, $d = 5$ nm, $C_m = 0.01$ F/m^2). P.C.—physiological conditions.

time constant τ takes values in the range of several microseconds, which is considerably shorter than pulse durations typically used in electropermeabilization. With these simplifications, Eq. (2) transforms into

$$U_{TI} = 1.5rE\cos\varphi. \quad (5)$$

If the cell suspension is placed between parallel plate electrodes with dimensions much larger than the distance between them, we can assume that the electric field is homogeneous and can be calculated as U/h, where U is the applied voltage and h is the distance between electrodes.

While Eq. (5) holds under physiological conditions, artificial extracellular media with a reduced conductivity are often used in electropermeabilization experiments, mostly to decrease the heating [22]. In addition, certain other applications of electric pulses on cells (e.g. electrofusion, electrorotation) require the conductivity of the extracellular medium to be reduced even further, e.g. by several orders of magnitude with respect to physiological conditions. A comparison of the values of the induced transmembrane voltage obtained using Eqs. (2) and (5) shows that under such conditions, the results given by the latter are incorrect. With the decrease of the medium conductivity, the value of the function f_S starts to decrease (from the asymptotic value of 1.5), and the value of the time constant τ increases (Fig. 2). As this figure shows, the dependence of f_S only becomes important with the decrease of the medium conductivity exceeding two orders of magnitude with respect to the physiological value, while τ is sensitive to smaller variations of the medium conductivity.

Besides the induced voltage, the resting transmembrane voltage (U_{TR}) is also present on cell membrane, but due to its relatively small value (~ -70 mV), it is often neglected in determination of the total transmembrane voltage. The resting transmembrane voltage is affected by extracellular conductivity, and since media of different ionic compositions have, in general, different conductivities, consequently, the resting transmembrane voltages of the cells suspended in different media are also different. In low conductivity media, the resting voltage can take values considerably higher than in physiological case and should not be neglected in determination of the total transmembrane voltage

$$U_T = U_{TI} + U_{TR}. \quad (6)$$

3.2. Calculations

3.2.1. Values for function f_S, time constant τ, and resting transmembrane voltage U_{TR}

Values for function f_S, and time constant τ were calculated using Eqs. (3) and (4), respectively, while the resting transmembrane voltage U_{TR} was calculated using Goldman's equation [23,24]. All values are shown in Table 2. Function f_S decreases considerably only in medium 3. Time constant increases with decrease of the medium conductivity and for media 2 and 3, it takes values consid-

Table 2
Calculated values for function f_S, time constant τ, and resting transmembrane voltage U_{TR}

	SMEM	m1	m2	m3
f_S	1.495	1.492	1.409	1.18
τ (μs)	0.31	0.51	6.1	21.4
U_{TR} (mV)	−78	−68	−159[a]	−209[a]

m1—medium 1, m2—medium 2, m3—medium 3.

[a] Because the resting transmembrane voltage was calculated under a questionable assumption of unchanged intracellular ionic concentration regardless of the extracellular ionic concentration, the calculated values might not reflect the actual situation (see Section 4 for details).

Table 3
Calculated values for resistivity of cell suspension R, temperature rise after one pulse ΔT_1 and after train of eight pulses ΔT_8, calculated at the highest voltage applied—400 V

	SMEM	m1	m2	m3
R (Ω)	51	587	17.1 k	72.9 k
ΔT_1 (°C)	1.5	0.13	0.0045	0.0011
ΔT_8 (°C)	12	1.04	0.036	0.0084

m1—medium 1, m2—medium 2, m3—medium 3.

erably higher than in the physiological case. Resting transmembrane voltage varies with decrease of the medium conductivity and may have an effect on the total transmembrane voltage.

3.2.2. Heating of cell suspension

Because during electropermeabilization, cell suspension is exposed to high-voltage pulses (up to 400 V), electric currents can take values up to several A (depending on medium conductivity) and the heating of cell suspension could influence cell survival. If we assume that electric energy from the pulse of amplitude U and duration t transmitted to cell suspension transforms into heat without loss and heat dissipation to the electrodes and the surrounding air, the change in temperature of the suspension ΔT can be estimated as

$$\Delta T = \frac{U^2 t}{R \rho V c_p} \quad (7)$$

with R, resistivity of cell suspension ($R = h^2/\lambda V$); h, distance between electrodes; ρ, specific density; V, volume; and c_p, the specific heat of droplet of cell suspension. Furthermore, the specific conductivity of cell suspension can be calculated as [24]

$$\lambda = \lambda_o \frac{1 - f}{1 + 0.5f} \quad (8)$$

with λ_o, the specific conductivity of the pure medium; f, the volume fraction occupied by the cells in suspension. Introducing Eq. (8) into Eq. (7), the maximum heating can be calculated (i.e., the heating for the highest voltage applied and after a train of eight pulses where no thermal dissipation between pulses is assumed). The results are shown in Table 3. The values of parameters used in calculations are shown in Appendix A.

Calculated values show a noticeable temperature rise of up to 12 °C in SMEM, while the temperature rise in other media is less than 1.04 °C. Because the cells were kept at 4 °C before the exposure to electric pulses, even the highest temperature rise due to heating (at highest voltage amplitudes) is too small to affect cell survival in any of the media used. This was verified using a water bath where cell suspension was heated from 4 to 30 °C and cooled back to 4 °C. The procedure was repeated twice. The cells were taken from the cell suspension before, during, and at the end of the experiment. The clonogenic test has shown that the cell survival remained unaffected.

3.3. Experimental results

3.3.1. Specific conductivity, osmolarity and pH of media used

Measured values for all parameters are shown in Table 4. Specific conductivities span three orders of magnitude which is sufficient for an observable change in induced transmembrane voltage, according to Eq. (2). Osmolarity results are within physiological values obtained from the literature (260–320 mOsm/kg H_2O) [25], which according to a study by Golzio et al. [26] should not affect cell electropermeabilization. Measured values for pH were within physiological values as well ($pH_{phys.} = 7.4$) [27]. Therefore, the effect of the variations of osmolarities and pH can be excluded from further examination.

3.3.2. Cell diameter

ANOVA test has shown no statistically significant difference between cell diameters in different media used. Therefore, all results were pooled and the median diameter was calculated to be 11.877 μm on measured population of 545 cells. The value of cell diameter did not change significantly during the experiment.

3.3.3. Survival and electropermeabilization of the cells

Experimental results for survival and electropermeabilization of the cells as a function of pulse amplitude are shown in Fig. 3A,B. Survival results show that cells in SMEM have the lowest 50% survival threshold (267 V), followed by other media in descending order of conductivities. According to the experimental results, electroperme-

Table 4
Measured values for specific conductivity, osmolarity and pH of media used

	SMEM	m1	m2	m3
Specific conductivity (S m^{-1})	1.61 ± 0.5%	0.14 ± 0.5%	0.0048 ± 0.5%	0.0011 ± 1%
Osmolarity (mOsm kg^{-1} H_2O)	299 ± 1%	286 ± 1%	269 ± 1%	259 ± 1%
pH	7.11 ± 0.01	7.40 ± 0.01	7.30 ± 0.01	7.12 ± 0.01

m1—medium 1, m2—medium 2, m3—medium 3.

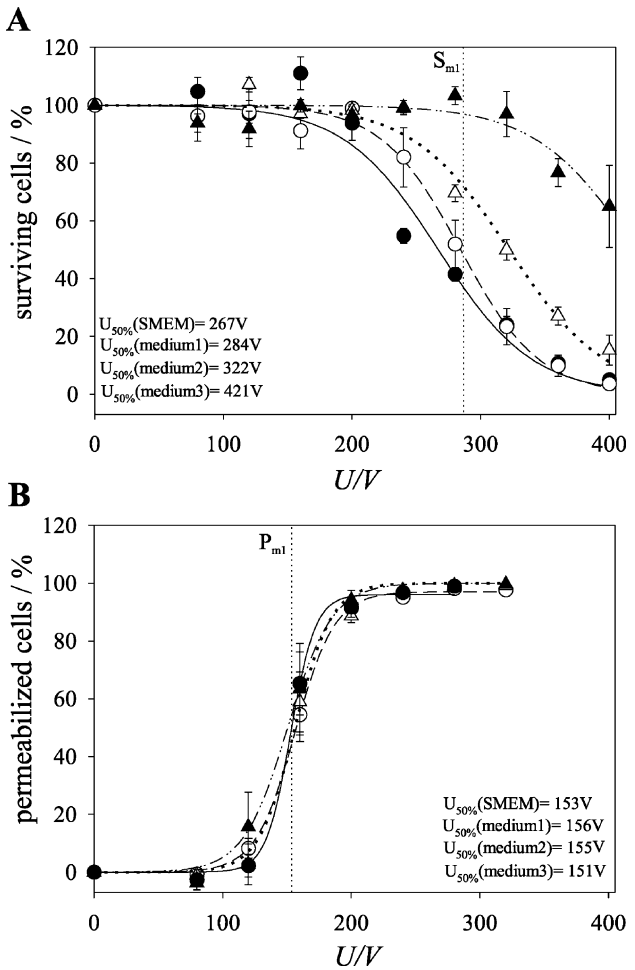

Fig. 3. Experimentally determined results for (A) survival and (B) permeabilization of the cells plotted as a function of pulse amplitude. Train of eight 100-μs rectangular pulses, repetition frequency 1 Hz was used for pulsation. Each point on the figure represents the mean of three values ± S.E. Conductivities of media used: SMEM (●): 1.61 S/m, medium 1 (○): 0.14 S/m, medium 2 (△): 0.0048 S/m, medium 3 (▲): 0.0011 S/m. S_{m1} denotes the pulse amplitude causing the death of 50% of the cells in medium 1. P_{m1} denotes the pulse amplitude needed for permeabilization of 50% of the cells in medium 1.

abilization of the cells is not affected by the conductivity of the medium.

4. Discussion

According to the experimental results (Fig. 3A,B), by decreasing the medium conductivity, we observed an influence on survival of the cells, but no effect was obtained on permeabilization of the cells. To explain why survival was affected by reduced medium conductivity while permeabilization remained unaffected, the influence of the reduction of the medium conductivity on the physiological parameters of the media and cells must be estimated.

The measurements have shown that the values of pH and osmolarity of the media are within physiological values, and cell diameter is unaffected by different media used. Moreover, estimated heating of cell suspension could not affect cell survival even at the highest voltages applied in the medium having the highest conductivity (this was verified experimentally by using water bath).

However, according to Eq. (2) and Goldman's equation [23,24], the induced and the resting transmembrane voltage both depend on the medium conductivity. Different values of these two voltages in different media could explain the experimentally obtained results for survival and permeabilization of the cells. To illustrate the influence of different medium conductivities on the induced and the resting transmembrane voltage at different pulse amplitudes in steady-state conditions, the total transmembrane voltage (Eq. (6)) is plotted as a function of pulse amplitude. It is therefore represented as a straight line with slope determined by the function f_S and the initial value determined by the resting transmembrane voltage (U_{TR}—absolute value). The total transmembrane voltages for different media have slopes and initial values corresponding to the conductivity of the specific medium (Table 2) (Fig. 4).

According to Fig. 4, at the pulse amplitudes between 100 and 200 V, the differences between the total transmembrane voltages of the cells in media with different conductivities are insignificant. If we consider that the value of the total transmembrane voltage determines the degree of permeabilization of the cell, cells exposed to pulses with amplitudes below 200 V should have the same degree of permeabilization independent of the media conductivity. This is in agreement with our experimentally obtained results for survival and permeabilization of the cells at mentioned pulse amplitudes (Fig. 3A,B).

At pulse amplitudes higher than 200 V, the difference between the calculated total transmembrane voltages for

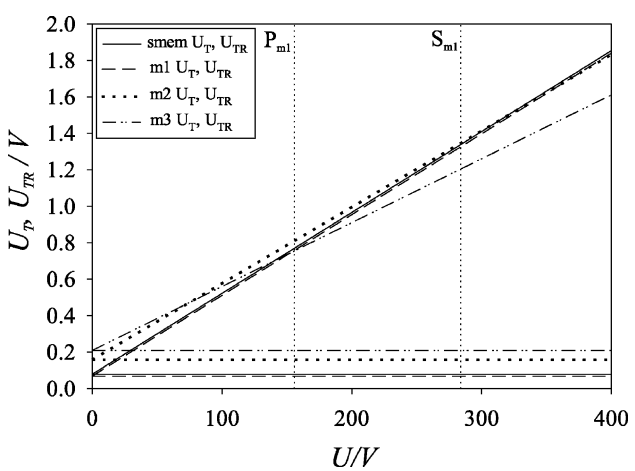

Fig. 4. The total transmembrane voltage (U_T, at $\varphi = 0°$, steady-state conditions) and the resting transmembrane voltage (U_{TR}) as a function of pulse amplitude (U) for each of the media used. The slope of U_T is determined by the value of the function f_S, while the initial value is determined by the value of the resting transmembrane voltage (U_{TR}—absolute value). S_{m1} denotes the pulse amplitude causing the death of 50% of the cells in medium 1. P_{m1} denotes the pulse amplitude needed for permeabilization of 50% of the cells in medium 1.

SMEM, medium 1, and medium 2 remains insignificant, while the total transmembrane voltage for medium 3 is significantly lower. Based on these theoretical considerations, for pulse amplitudes above 200 V, one would expect a similar degree of permeabilization and consequently, cell survival in SMEM and media 1 and 2, but a considerably lower degree of permeabilization and higher cell survival in medium 3. Experimental results confirm that at each given pulse amplitude, cell survival is much higher in medium 3 than in SMEM, medium 1, and medium 2, but they suggest that there are also differences between the latter three media (Fig. 3A).

Although our theoretical predictions for survival and permeabilization of the cells on the basis of the total transmembrane voltages are in a qualitative agreement with experimental results, the difference between the total transmembrane voltage for medium 3 and the other three media would suggest a smaller difference in cell survival of the cells than obtained by the experiments. Two plausible explanations for this can be stated.

In our calculations, the value of the resting transmembrane voltage was determined under the assumption of unchanged internal ionic concentration ($[K^+]_i = 140$ mM, $[Na^+]_i = 5$ mM) regardless of external ionic concentration (e.g.: $[K^+]_o = 0.044$ mM in medium 3). Because of the high ionic concentration difference, especially when low conductivity media are used, it is likely that ionic flux occurs (mostly K^+efflux) which decreases the resting transmembrane voltage [15,28,29]. Moreover, a living cell will pump ions as long as ATP is present and it is improbable that the resting potential of the living cell could change as dramatically as the calculations suggest (from -78 to -209 mV). Taking this into account, reducing the resting transmembrane voltage would shift the straight lines of the total transmembrane voltages towards lower values (Fig. 4), thus increasing the survival of the cells in low conductivity media. Reduction of the resting transmembrane voltage due to ionic flux is more emphasized with higher pulse amplitudes and during the pulses subsequent to electropermeabilization.

Perhaps the most plausible explanation for the disagreement between theoretically predicted and experimentally obtained survival of the cells in medium 3 is the dynamics of permeabilization, which was not accounted for in Fig. 4. Because of the large value of time constant τ in low conductivity media (Table 2), the membrane becomes permeabilized before the total transmembrane voltage reaches the maximum value predicted by Eq. (2) (Fig. 5A, phase 2). A substantial increase of membrane permeability results in an increase of its conductivity λ_m, and consequently, in the decrease of the function f_S (Fig. 5B). This hinders further increase of the total transmembrane voltage, which then even slightly decreases, as confirmed by experimental observations made by Hibino et al. [30] on sea urchin eggs, and by the modeling study made by DeBruin and Krassowska [31] (Fig. 5A, phase 3).

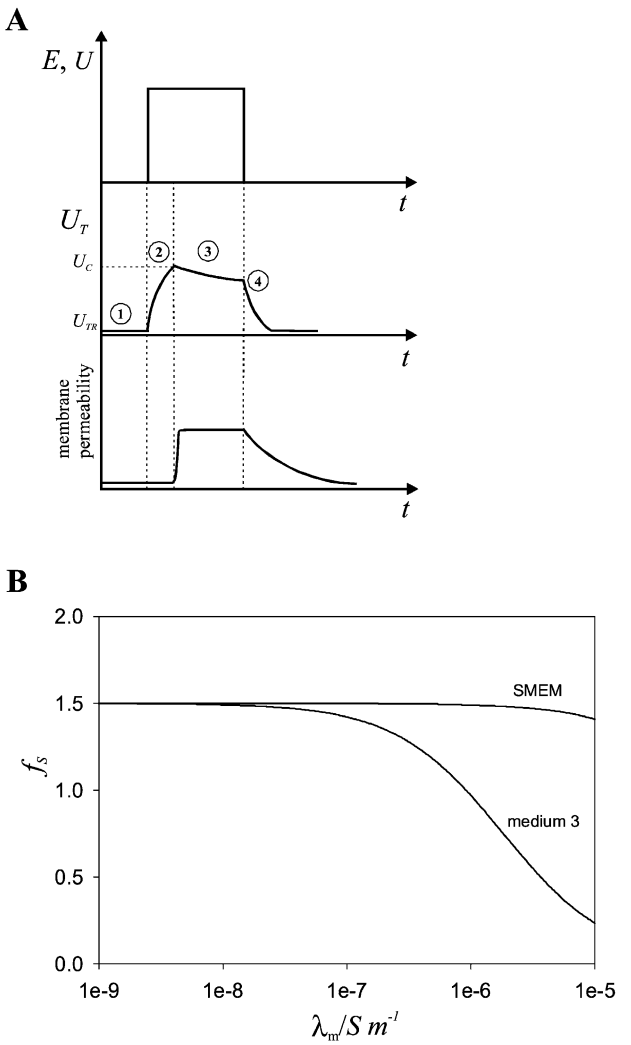

Fig. 5. (A) shematic presentation of electropermeabilization dynamics. (1) $U_T = U_{TR}$, membrane permeability is low; (2) $U_T = U_{TR} + U_{TI}$, and U_{TI} increases with time constant τ; (3) when U_T exceeds a critical value U_C, the permeability of the membrane rapidly increases and prevents further increase of U_T, which then even slightly decreases; (4) after the end of the pulse, U_T returns to U_{TR} with time constant τ, while membrane permeability decreases with a time constant in the range of seconds or even minutes. (U_{TR}—resting transmembrane voltage, U_{TI}—induced transmembrane voltage, U_T—total transmembrane voltage) (B) The dependence of function f_S on membrane conductivity λ_m for two media (SMEM: 1.61 S/m, medium 3: 0.0011 S/m).

In contrast to cell survival, the fraction of permeabilized cells seems to be unaffected by the time constant of voltage inducement, as long as the pulse duration exceeds this time constant considerably. We should note that the method used for detection of permeabilization, although very precise in determining the threshold of permeabilization, does not evaluate the quantity of molecules taken up per cell. Therefore, our results do not exclude the possibility that the amount of the uptake depends on medium conductivity.

A detailed study on the effect of ionic composition and medium conductivity on viability and PI-uptake of the

cells was made by Djuzenova et al. [15]. This study implies that reducing the medium conductivity results in lower viability of the cells in this medium, which is in disagreement with our experimental results, where reducing the medium conductivity resulted in higher cell survival (see Fig. 3A). However, the range of media conductivities investigated by Djuzenova et al. [15] was between 0.08 and 0.37 S/m, whereas we investigated a much broader range of conductivities between 0.0011 and 1.61 S/m.

In summary, our study has shown that medium conductivity influences the survival of the cells while no detectable effect was obtained on electropermeabilization of the cells. To interpret the experimental results, we have analyzed the influence of the medium conductivity on the induced and the resting transmembrane voltage. This analysis suggests that different values of these two voltages in media with different conductivities are the main reason for the experimentally obtained results, taking into account the time constant of the voltage inducement. Some practical guidelines for experiments involving electropermeabilization can also be made from our study. Use of extracellular media with lower conductivities reduces the electric current and consequently, the heating of cell suspension is reduced. Moreover, because of the lower electric current, less power is required of the electropulsator. Nevertheless, the reduction of the medium conductivity should be moderate, because in the opposite case, the time constant of voltage inducement τ increases and the function f_S decreases, and this increases the pulse duration and the amplitude required for successful electropermeabilization.

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Appendix A

Symbol	Description	Value
C_m^*	Membrane capacitance	1×10^{-2} F/m^2
d^*	Membrane thickness	5 nm
λ_i^*	Cytoplasmic conductivity	0.2 S/m
λ_m^*	Membrane conductivity	5×10^{-7} S/m
r	Cell radius	5.94 μm
t	Pulse duration	100 μs
h	Distance between the electrodes	2 mm
U	Applied voltage	400 V
R	Gas constant	8.3 J/mol K
T	Temperature	300 K
F	Faraday constant	9.6×10^4 As
$[Na^+]_i^\#$	Concentration of Na^+ ions inside the cell	5 mM
$[K^+]_i^\#$	Concentration of K^+ ions inside the cell	140 mM
c_p	Specific heating	4.18 J/kg K
V	Volume of droplet	50 μl
ρ	Specific density	1 g/cm^3
N	Number of cells	10^6 cells
V_C	Volume of cell	8.77×10^{-16} m^3
f	The volume fraction occupied by the cells in suspension	0.0018
q^{**}	The permeability ratio (P_{Na}/P_K)	0.01

* Values taken from Ref. [32].
** Values taken from Ref. [24].
# Values taken from Ref. [27].

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