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Cell membrane permeabilization by 12-ns electric pulses: Not a purely dielectric, but a charge-dependent phenomenon

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ABSTRACT

Electric pulses of a few nanoseconds in duration can induce reversible permeabilization of cell membrane and cell death. Whether these effects are caused by ionic or purely dielectric phenomena is still discussed. We address this question by studying the impact of conductivity of the pulsing buffer on the effect of pulses of 12 ns and 3.2 MV/m on the DC-3F mammalian cell line. When pulses were applied in a high-conductivity medium (1.5 S/m), cells experienced both reversible electropermeabilization and cell death. On the contrary, no effect was observed in the low-conductivity medium (0.1 S/m). Possible artifacts due to differences in viscosity, temperature increase or electrochemical reactions were excluded. The influence of conductivity reported here suggests that charges still play a role, even for 12-ns pulses. All theoretical models agree with this experimental observation, since all suggest that only high-conductivity medium can induce a transmembrane voltage high enough to induce pore creation, in turn. However, most models fail to describe why pulse accumulation is experimentally required to observe biological effects. They mostly show no increase of permeabilization with accumulation of pulses. Currently, only one model properly describes pulse accumulation by modeling diffusion of the altered membrane regions.

1. Introduction

Electric pulses can induce reversible or irreversible defects in a cell membrane, thus allowing direct access of external molecules to the cell cytosol [1–3]. This effect is referred to as electropermeabilization or electroporation. This technique is nowadays routinely used in research laboratories and clinics e.g. to treat cancerous tissues [4–8]. In traditional electropermeabilization, pulse parameters can be chosen over a wide range and still lead to a successful permeabilization of cells. Values usually range between a few tens or hundreds of μs (pulses which are referred to as micropulses) and a few ms (pulses referred to as millipulses). Field magnitude is chosen depending on the cell type and desired effect, but usually lies between a few tens of kV/m and a few hundreds of kV/m. More recently, different groups found that much shorter and more intense pulses can also lead to a membrane permeabilization [9–13]. Typical electrical parameters mentioned in literature are a few tens of nanoseconds for the duration and at least 2 MV/m for the electric field magnitude. Nomenclature to refer to various types of pulses in literature, however, is not yet homogeneous. Frequently, a distinction is made between 'short pulses' and 'long pulses'. These names usually refer to the charging time of the cell plasma (external) membrane. Indeed, when a cell is subjected to an external field, its plasma membrane charges like a capacitor, with an exponential time course that is characterized by the charging time constant τ. 'Short pulses' and 'long pulses' usually mean pulses shorter and longer than the cell plasma membrane charging time ($\sim 5 * \tau$), respectively. Several electromagnetic models have been developed to precisely describe the behavior of a spherical cell in a homogeneous unidirectional electric field [14–17]. Those models can predict the charging time constant τ of the cell plasma membrane in the linear regime (Eq. (1) [14]). Most of the parameters influencing this charging time constant are intrinsic to the cell and are (almost) inaccessible by the experimental design. This is the case for the cell radius r_c (m), the intracellular conductivity σ_i (S/m), the membrane surface capacitance C_m (F/m^2), and the

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membrane surface conductance S_0 (S/m^2). However, the charging time also depends on extracellular conductivity σ_e (S/m), which is a parameter that can be set to different values during an experiment (within the limit of physiologically acceptable values).

$$\tau = r_c C_m \frac{\sigma_i + 2\sigma_e}{2\sigma_e\sigma_i + r(\sigma_i + 2\sigma_e)S_0} \quad (1)$$

The time constant is plotted as a function of the extracellular conductivity in Fig. 1. Other parameters were kept constant and their values are given in the legend of the figure. Values for the time constant range from approximately 93 ns to 420 ns at external conductivities ranging from 1.5 S/m to 0.1 S/m.

Whether the values of Fig. 1 are accurate or not, it can still be assumed that when a cell with a radius of 7 μm is subjected to a 12-ns pulse, its membrane will not reach the stationary value imposed by the pulse, since the charging time is much longer than 12 ns. Moreover, Eq. (1) and Fig. 1 suggest that the charging time of the membrane is highly dependent on the conductivity of the extracellular medium. As a consequence of those two observations, an important influence of extracellular conductivity can be expected. In this study we focused on pulses of 12 ns duration and 3.2 MV/m. In a previous study, it was shown that these types of pulses can induce membrane permeabilization as well as cell death [13]. Here, these effects are shown to correlate with the conductivity of the extracellular medium. We additionally demonstrate that the higher efficiency of the high-conductivity medium is not due to an elevation of temperature.

Experiments were performed on DC-3F cells which grow attached, but can be kept in suspension for the time of the experiments. The cell death caused by the exposure of the cells to the pulses was assessed by cell survival quantified by a cloning efficiency test. Moreover, pulse treatment combined with a nonpermeant cytotoxic agent in the extracellular medium (namely bleomycin [18–22]) provided a robust and quantitative method for determining reversible permeabilization (such a method was already described in [13,23] and the concept of the approach is detailed in [5]).

2. Material and methods

2.1. Cell culture

Chinese hamster lung cell line DC-3F [24] was grown in complete medium, consisting of MEM — Minimum Essential Medium (31095-052, Life Technologies, Saint Aubin, France) with the addition of 10% fetal bovine serum (10270-106, Life Technologies) and supplemented by antibiotics (500 U/ml penicillin and 500 μg/ml streptomycin). Cells were kept in a humidified atmosphere at 37 °C and 5% CO_2 and routinely passed every two days. When they are placed in a suspension, those cells have an average diameter of 13.2 ± 1.3 μm (mean ± standard deviation evaluated from bright light microscopy images; 380 cells were analyzed in five independent experiments).

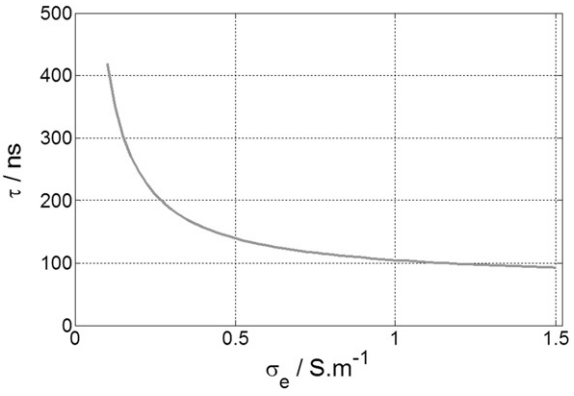

Fig. 1. Charging time as a function of extra-cellular conductivity. The parameters used for the computation are: $r_c = 7$ μm, $\sigma_i = 1$ S/m, $C_m = 0.01$ F/m^2, $S_0 = 1.9$ S/m^2.

2.2. Pulsing media

Two different media were mainly used: Either S-MEM (a Minimum Essential Medium modified for the cultivation of cells in suspension, 11380-037 Life Technologies) or STM (250 mM sucrose, 10 mM Tris HCl pH 7.0, 1 mM $MgCl_2$). Conductivity of the solutions was measured with a *Conductivity Meter CLM 381* (Endress and Hauser, Weil am Rhein, Germany). To measure the dependence of conductivity on temperature, a beaker containing the solution to be tested was heated with a standard heating plate (VHP-C7, VWR International). The two media will hereinafter be referred to as high-conductivity (1.5 S/m) and low-conductivity (0.1 S/m) medium, respectively.

Lyophilized bleomycin was dissolved in S-MEM (or STM) and stored at −20 °C at 300 μM. Aliquots were taken just before the experiments and dissolved in S-MEM (or STM) to obtain media with a concentration of 30 nM of bleomycin. As STM is more viscous than SMEM due to the high concentration of sucrose, "viscous S-MEM" was prepared by adding 0.1% or 0.15% of agar (30391-023, Life Technologies) to the standard S-MEM. These solutions were sterilized by autoclaving prior to the experiments. Viscosity measurements were performed with a viscometer of the type Brookfield DV2T at 24 °C. Results are the mean ± standard deviation of three independent measurements.

2.3. Assessment of cell viability

After trypsinization of exponentially growing cells and inactivation of trypsin (25300-054, Life Technologies) by the serum factors of the complete medium, cells were centrifuged for 10 min at 150 *g* and resuspended at a density of 5×10^6 cells/ml in the appropriate medium containing 30 nM bleomycin or not. Cells were immediately deposited between the two electrodes and exposed to the electric pulses. Cells were kept for 10 min at room temperature after the application of the electric pulses. The cells were then diluted in complete medium. After dilution, cells were seeded in triplicate in complete culture medium (250 cells per cell culture dish, 35 mm in diameter) to measure their viability through a quantitative cloning efficacy test. After 5 days in a humidified, 5% CO_2 atmosphere, colonies were fixed and stained (with a solution of 5% formaldehyde containing crystal violet) and the number of clones N was counted for each condition. Viability was then normalized to the number of clones in the control $N_{control}$ and reported as a percentage of survival: $N/N_{control} \cdot 10^2$. In experiments without bleomycin, the control condition refers to cells subjected to no treatment. In experiments with bleomycin, the control condition refers to cells in contact with the bleomycin, which did not receive any pulses. Viability of unpulsed controls was typically between 90% and 95% after exposure to 30 nM bleomycin. Final results are represented as mean values + SD (standard deviation) of three to five independent experiments.

2.4. Nanosecond-pulse delivery

Unless stated otherwise, a pulse-forming line generator based on spark gap technology and designed by Europulse (Cressensac, France) was used. Cells were exposed in suspension in conventional electroporation cuvettes from Cell project (Harrietsham, United Kingdom). The 4 mm cuvettes were used and precise measurements of the distance between the electrodes indicated $d_{4mm} = 4.19 \pm 0.02$ mm. The measured distance was used to evaluate the electric field inside the cuvette.

During the experiments designed to test the influence of conductivity, special care was taken to impose exactly the same electric field in both the high- and low-conductivity media. To this end, the two cuvettes containing the two different media were exposed in a parallel arrangement as depicted in Fig. 2. This ensures that an identical voltage is