Biochimica et Biophysica Acta 1840 (2014) 2139–2151

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Comparison of the effects of the repetition rate between microsecond and nanosecond pulses: Electropermeabilization-induced electro-desensitization?

A. Silve [a,b,c,d,*], A. Guimerà Brunet [e,f], B. Al-Sakere [a,b,c], A. Ivorra [g], L.M. Mir [a,b,c,**]

[a] Univ Paris-Sud, Laboratoire de Vectorologie et Thérapeutiques Anticancéreuses, UMR 8203, Villejuif F-94805, France
[b] CNRS, Villejuif, Laboratoire de Vectorologie et Thérapeutiques Anticancéreuses, UMR 8203, F-94805, France
[c] Gustave Roussy, Laboratoire de Vectorologie et Thérapeutiques Anticancéreuses, UMR 8203, Villejuif F-94805, France
[d] Karlsruhe Institute of Technology (KIT), Institute for Pulsed Power and Microwave Technology (IHM), 76344 Eggenstein-Leopoldshafen, Germany
[e] Institut de Microelectrònica de Barcelona IMB-CNM (CSIC), Bellaterra, 08193 Barcelona, Spain
[f] CIBER-BBN, Networking Center on Bioengineering, Biomaterials and Nanomedicine, Zaragoza, Spain
[g] Universitat Pompeu Fabra, Departament de Tecnologies de la Informació i les Comunicacions, Carrer Roc Boronat 138, E-08018 Barcelona, Spain

ARTICLE INFO

Article history:
Received 15 September 2013
Received in revised form 24 January 2014
Accepted 13 February 2014
Available online 28 February 2014

Keywords:
Bioimpedance
Repetition rate
Electropermeabilization
Potato
Liver
Electroporation

ABSTRACT

Background: Applications of cell electropermeabilization are rapidly growing but basic concepts are still unclear. In particular, the impact of electric pulse repetition rate in the efficiency of permeabilization has not yet been understood.
Methods: The impact of electric pulse repetition rate in the efficiency of permeabilization was analyzed in experiments performed on potato tissue and partially transposed on mice liver. On potato tissue, pulses with durations of 100 μs or 10 ns are applied. The intensity of permeabilization was quantified by means of bioimpedance changes and electric current measurements and a new index was defined.
Results: For the two pulse durations tested, very low repetition rates (below 0.1 Hz) are much more efficient to achieve cell permeabilization in potato tissue. In mice liver, using 100 μs pulses, the influence of the repetition rate is more complex. Indeed, repetition rates of 1 Hz and 10 Hz are more efficient than 100 Hz or 1 kHz, but not the repetition rate of 0.1 Hz for which there is an impact of the living mice organism response.
Conclusions: We propose that the effects reported here might be caused by an electroporation-induced cell membrane 'electro-desensitization' which requires seconds to dissipate due to membrane resealing.
General significance: This study not only reinforces previous observations, but moreover it sustains a new concept of 'electro-desensitization' which is the first unifying mechanism enabling to explain all the results obtained until now both *in vitro* and *in vivo*, with long and short pulses.

1. Introduction

Electropermeabilization, or electroporation, names the phenomenon by which cell membrane permeability increases when the cell is exposed to short and intense electric pulses [1]. Depending on the field intensity, the duration of the pulses or the number of pulses applied, the permeabilization can be either transient and reversible (which is useful for drug delivery or gene transfection [2–5]) or irreversible, leading to the death of the cell (which is used among other applications for tissue ablation [6,7] or bacterial inactivation [8–10]). Some standard values of the electric pulses (*e.g.*: 100 μs, 1 kV/cm for reversible electroporation of mammalian cells in suspension) are often used since they were proven to be very efficient. However, many combinations can result in the same final state of the cell even though it is difficult to give an exact rule regarding the relationship between the pulse parameters and the effects on the cells. It has appeared in many reports that a series of short pulses led to higher permeabilization than one single long pulse even if this pulse lasts as long as the cumulated duration of the series of shorter pulses [1]. For example, in almost all the electrochemotherapy treatments, as reported in the Standard Operating Protocols of Electrochemotherapy, eight pulses are applied instead of one [11]. Naturally, this observation has led to study the impact of the repetition rate used when a given number of pulses are delivered. Different studies *in vitro* [1,12–15], *in vivo* [7,16] and in plants [17,18] have indeed shown an impact of the repetition rate. Generally it appears that low rates are more efficient than high ones. However, there are up to now very few clues on whether an optimal rate does exist and on the mechanisms that could explain the impact of the repetition rate.

* Correspondence to: A. Silve, Hermann-von-Helmholtz-Platz 1, 76344 Eggenstein Leopoldshafen, Germany. Tel.: +49 721 608 23936.
** Correspondence to: L.M. Mir, UMR 8203 CNRS, Gustave Roussy, 114 rue Edouard Vaillant, Villejuif F-94805, France. Tel.: +33 142 11 47 92.
E-mail addresses: aude.silve@gmail.com (A. Silve), luismir@igr.fr (L.M. Mir).

http://dx.doi.org/10.1016/j.bbagen.2014.02.011
0304-4165/

More recently, different groups have shown that cell membrane permeabilization can be obtained by the application of pulses of only a few nanoseconds duration. The question of the impact of the repetition rate for such pulses has already been addressed in an *in vitro* study [13]. Authors have shown that for pulse duration ranging from 300 ns to 9 μs, the survival rate of cells exposed in suspension decreases when the repetition rate decreases. The authors proposed a concept of electrosensitation. Other *in vitro* studies have also pointed to an impact of the repetition rate during exposure to nanosecond pulses. They indicate either a higher efficiency of high repetition rate [19] or the existence of an optimal rate [20].

The objective of this study was to test the impact of the repetition rate but on a completely different model. We investigated the permeabilization of tissues by measuring the changes of electrical bioimpedance. We compared, on potato tuber, the permeabilization obtained by two types of pulses with duration of either 100 μs or 10 ns. They will be referred to as microsecond pulses (or micropulses) and nanosecond pulses (or nanopulses) respectively. In addition, the repetition rate impact on permeabilization in mice liver was investigated using micropulses.

2. Materials and methods

2.1. Microsecond pulse generator

Square-wave microsecond pulses were delivered by an electroporation power supply (Cliniporator™, Igea, Carpi, Italy) able to apply high-voltage pulses with repetition rates ranging from 1 Hz to 7 kHz. To obtain pulse repetition rates lower than 1 Hz, single pulses were manually triggered by the operator at the appropriate rate.

2.2. Nanosecond pulse generator

The generator used to expose the potato samples to nanosecond pulses was a commercial generator (FID Technology FPG 10-30MS, Russia) supplied with a DC source (Delta Electronika ES 0.300-0.45). It has four 100 Ω outputs that were connected by pairs in series and then globally in parallel.

In order to take into account the distortion of the electric field induced by wave reflection on the potato sample itself, the exact field applied was systematically measured using a D-dot probe directly mounted in the electrodes as described in [21].

2.3. Bioimpedance measurement system

In experiments designed to account for permeabilization, low signal impedance measurements from 100 Hz to 400 kHz were performed with the Bluetooth bioimpedance measurement system custom developed by the Centre Nacional de Microelectrònica (CNM, Barcelona, Spain) [22].

2.4. Potato sample preparation and treatment

Standard potatoes were bought from the local supermarket. Slices of 5 mm homogeneous thickness were first prepared and then small cylinders of 5 mm diameter were punched out from the peripheral part of the potato slice. Impedance measurements were performed with a four needle electrode set-up. The four needle electrodes (diameter 0.3 mm, length 4 mm) are arranged in a row with a 1 mm distance between each other. After the first impedance measurement the sample was placed between two stainless steel plate electrodes separated by a distance of 5 mm that were used to deliver the pulses. Immediately after the last pulse, the potato sample was removed from the plate electrodes and the impedance was measured again with the four needle electrodes at 7 s and 80 s after the last pulse.

2.5. Propidium iodide staining

Potato samples (previously cut at the right dimensions) were dipped for 24 h in a solution of PBS (Life Technologies, ref 14200-083) containing 0.1 mM propidium iodide. Samples were pulsed and, 1 min later, thin slices (thinner than 1 mm) were manually cut with a razor blade and placed on a microscope slide. Observations were made under an inverted microscope (Zeiss, Axiovert S100) at a magnification of ×10 and images were acquired with a CCD video camera (Zeiss, Axiocam HRc).

2.6. Surgical process for liver treatment

Very young nude Swiss mice were obtained from local production in the animal facility of Gustave Roussy. All the used animals were handled, and then sacrificed in the animal housing facility of Gustave Roussy according to the Experimental Animal Ethics Committees Guidelines and received the agreement of the CEEA26 committee. Mice of age between 8 and 10 days were used for these experiments as the size of their liver allowed to conveniently place it completely between the two plate electrodes separated by a distance of 2 mm. Mice were first anesthetized with a dose of 10 μl per g body weight of a mixture composed of xylazine 12.5 mg/kg (Bayer Pharma, Puteaux, France) and ketamine 125 mg/kg (Parke Davis, Courbevoie, France). The anesthetizing mixture was injected intraperitoneally. An incision was performed in the upper median abdominal (or epigastric) region of the anesthetized animal, and then the liver was pulled out gently and positioned in between the plate electrodes. The setup was performed in such a manner that movements were not possible during the procedure for the liver impedance measurement. A first electrical impedance measurement between the two stainless steel plate electrodes was performed and then, after disconnecting the impedance meter, the pulses were applied using the same electrodes. Approximately 5 s after the last pulse, electrodes were manually connected back to the impedance meter and the impedance was measured again. Voltage and current during pulses were systematically recorded.

3. Detection of permeabilization of tissue by electrical measurements: models and correlations established before data collection

3.1. Impedance of a biological tissue (bioimpedance)

Electrical passive properties of biological tissues have been described with very good precision up to several megahertz [23]. A simple electrical model of a biological tissue, adapted from [24,25], is presented in Fig. 1. It consists in a resistance (R_{ext} [ohm, Ω]) in parallel with a capacitance (C_w [farad, F]) and with a series association of a capacitance (C_m [farad, F]) and a resistance (R_{int} [ohm, Ω]). The resistances R_{ext} and R_{int} are directly linked to the conductive properties of the extracellular and intracellular media respectively which are both ionic solutions. The capacitance C_m represents the dielectric properties of the membranes of cells. In order to have a good representation of the impedance of biological sample, it

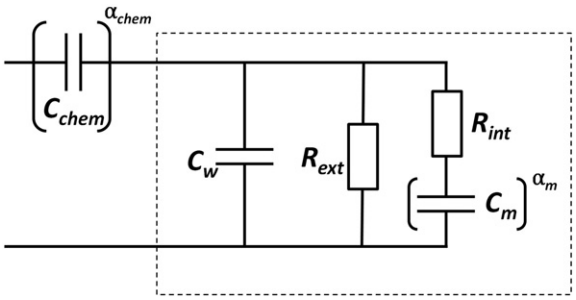

Fig. 1. An electrical model to interpret tissue bioimpedance measurements. The part of the model delimited by dotted lines describes the passive properties of the biological tissue. The additional capacitor C_{chem} is the electrochemical capacitance of the electrodes.