ELSEVIER

Bioelectrochemistry 54 (2001) 91–95

Bioelectrochemistry

www.elsevier.com/locate/bioelechem

Cell membrane electropermeabilization by symmetrical bipolar rectangular pulses ☆ Part II. Reduced electrolytic contamination

Tadej Kotnik [a,b,*], Damijan Miklavčič [a], Lluis M. Mir [b]

[a] *Faculty of Electrical Engineering, University of Ljubljana, Trzaska 25, SI-1000 Ljubljana, Slovenia*
[b] *UMR 8532 CNRS, Institute Gustave-Roussy, F-94805 Villejuif, France*

Received 16 February 2001; accepted 18 April 2001

Abstract

The paper presents a comparative study of the contamination of a cell suspension by ions released from aluminum cuvettes (Al^{3+}) and stainless steel electrodes (Fe^{2+}/Fe^{3+}) during cell membrane electropermeabilization by unipolar and by symmetrical bipolar rectangular electric pulses. A single pulse and a train of eight pulses were delivered to electrodes at a 2-mm distance, with 100-μs and 1-ms pulse durations, and amplitudes ranging from 0 to 400 V for unipolar, and from 0 to 280 V for bipolar pulses. We found that the released concentrations of Al^{3+} and Fe^{2+}/Fe^{3+} were always more than one order of magnitude lower with bipolar pulses than with unipolar pulses of the same amplitude and duration. We then investigated the viability of DC-3F cells after 1 h of incubation in the medium containing different concentrations of Al^{3+} or Fe^{2+}/Fe^{3+} within the range of measured released concentrations (up to 2.5 mM for both ions), thus separating the effects of electrolytic contamination from the effects of electropermeabilization itself. For Fe^{2+}/Fe^{3+}, loss of cell viability became significant at concentrations above 1.5 mM, while for Al^{3+}, no effect on cell survival was detected within the investigated range. Still, reports on the biochemical effects of released Al^{3+} also suggest that with aluminum cuvettes, electrolytic contamination can be detrimental. Our study shows that electrolytic contamination and its detrimental effects can be largely reduced with no loss in efficiency of electropermeabilization, if bipolar rectangular pulses of the same amplitude and duration are used instead of the commonly applied unipolar pulses. © 2001 Published by Elsevier Science B.V.

Keywords: Electropermeabilization; Electroporation; Bipolar pulses; Electrolytic contamination; Metal ions

1. Introduction

Electropermeabilization, also referred to as electroporation, is an effective method for the internalization of various molecules into biological cells, with an increasing number of applications in oncology, genetics, immunology, and cell biology. A short list of bibliographical sources, in which this phenomenon and its diverse applications are described in detail, can be found in the Introduction of Part I of this study [1].

Besides the parameters of electric pulses and various physical and chemical parameters, the efficiency of electropermeabilization in vitro also depends on the composition of the extracellular medium in which the cells are suspended [2]. Electrolytic action of the pulses alters this composition, both by release of metal ions from the electrodes and by additional ionization and dissociation of the medium constituents.

To date, several reports have been published on the detrimental effects of metal ions released from the electrodes [3–5]. Loomis-Husselbee et al. [3] have reported that Al^{3+} released from aluminum electroporation cuvettes can significantly affect biochemical processes involving inositol phosphates. Friedrich et al. [4] have measured the release of Al^{3+} in concentrations up to 1 mM, and have observed that this release also affects the pH of the suspension. Similarly, Stapulionis [5] has measured the release of Fe^{2+}/Fe^{3+} from stainless steel electrodes in concentrations up to 1.2 mM, as well as the release of Al^{3+} from aluminum electrodes and of Cu^{2+} from copper electrodes, and has reported that the release of each of these ions can

☆ Part of this paper was presented by Tadej Kotnik as the Luigi Galvani prize lecture 2001 at the XVIth International Symposium on Bioelectrochemistry and Bioenergetics in Bratislava, Slovakia.

* Corresponding author. Faculty of Electrical Engineering, University of Ljubljana, Trzaska 25, SI-1000 Ljubljana, Slovenia.
Tel.: +36-614-768-768. *Fax:* +38-614-264-658.
E-mail address: tadej@svarun.fe.uni-lj.si (T. Kotnik).

1567-5394/01/$ - see front matter
PII: S1567-5394(01)00115-3

cause substantial precipitation of nucleic acids and proteins from the solution. The release of Fe^{2+}/Fe^{3+} from stainless steel electrodes has also been confirmed by Tomov and Tsoneva [6].

In our study, we have measured concentrations of metal ions released into the cell suspension from aluminum electroporation cuvettes (release of Al^{3+}) and stainless steel electrodes (release of Fe^{2+}/Fe^{3+}) during cell membrane electropermeabilization by unipolar and symmetrical bipolar rectangular electric pulses. We show that in both cases, 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, the detrimental effects of electrolytic contamination on cells can be largely reduced by the use of bipolar instead of unipolar pulses.

2. Materials and methods

2.1. Electric pulses and the medium

Unipolar electric pulses were generated by a PS 15/GHT 1287 B electropulsator (Jouan, St. Herblain, France), while bipolar pulses were generated by an AFG 310 programmable function generator (Tektronix, Wilsonville, OR, USA) and were amplified to the required voltages in the range from 0 to 280 V using a bipolar amplifier built in the Laboratory of Biocybernetics at the Faculty of Electrical Engineering of the University of Ljubljana. A 50-μl droplet of Spinner minimum essential medium SMEM 21385 (Life Technologies, Rockville, MD, USA), which contains salts of neither aluminum nor iron, was placed either into an aluminum electroporation cuvette (Eppendorf, Hamburg, Germany) or between a pair of flat stainless steel electrodes (in both cases, the distance between the electrodes was 2 mm) to which the pulses were then delivered. We have used a single pulse and a train of eight pulses, with 100-μs and 1-ms pulse durations, and amplitudes ranging from 0 to 400 V for unipolar, and from 0 to 280 V for bipolar pulses. Within seconds after the delivery of pulses, the droplet was removed from the electrodes. To allow for comparability of our results with experiments performed with electrodes at various distances, the pulse amplitude was given as the ratio between the voltage and the distance between the electrodes.

2.2. Measurements of ionic concentrations

The concentrations of Al^{3+} were determined using an ICP-MS 4500 Series inductively coupled plasma-mass spectrometer (Hewlett-Packard, Wilmington, DE, USA), while the concentrations of Fe^{2+}/Fe^{3+} were measured on a CX7 clinical system for chemical analysis (Beckman Coulter, Fullerton, CA, USA).

2.3. Cell culture

DC-3F cells, a line of spontaneously transformed Chinese hamster fibroblasts [7], 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, and 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), 100 units/ml of penicillin and 125 μg/ml of streptomycin (both from Sarbach/Solvay Pharma, Brussels, Belgium).

2.4. Determination of cell viability

After trypsination with trypsin-EDTA (Life Technologies), cells were centrifuged for 5 min at 1000 rpm in a C312 centrifuge (Jouan), and suspended at 2×10^7 cells/ml in SMEM to which $AlCl_3$ (resp. $FeCl_3$) was previously added in quantities giving concentrations of Al^{3+} (resp. Fe^{2+}/Fe^{3+}) in the range from 0 to 2.5 mM. One milliliter of cell suspension was incubated for 1 h at room temperature, then diluted in the culture medium (see

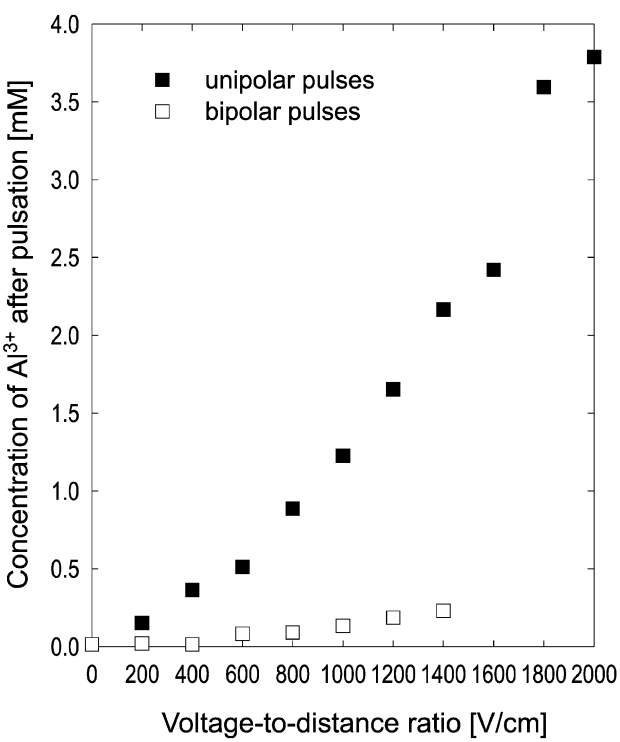

Fig. 1. The concentration of Al^{3+} ions in the suspension as the function of pulse amplitude (the voltage-to-distance ratio) for eight unipolar (■) and symmetrical bipolar (□) rectangular electric pulses. Each pulse was of 1 ms total duration (for bipolar pulses, 500 μs of one polarity followed by 500 μs of the opposite polarity), and the pulses were delivered in 1 s intervals.

above) to 100 cells/ml, and 4 ml of suspension was transferred into each 60-mm petri dish. The cells were grown for 5 days, 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). Colonies were counted under a light microscope (Leica, Wetzlar, Germany) and normalized to the control (cells incubated in pure SMEM, in which 1 h incubation does not cause any detectable reduction in the cloning efficiency of these cells) to obtain the percentage of cells surviving a 1-h exposure to different concentrations of Al^{3+} or Fe^{2+}/Fe^{3+}. Each experiment was repeated three times at intervals of at least several days.

3. Results and discussion

Fig. 1 shows the concentration of Al^{3+} ions released from aluminum electroporation cuvettes by an application of eight unipolar, and of eight bipolar rectangular pulses. Pulses, each of 1 ms duration, were delivered at intervals of 1 s. In bipolar pulses, the polarity was reversed after 500 μs. Fig. 2 shows the results of an analogous study performed for Fe^{2+}/Fe^{3+} ions released from the stainless steel electrodes.

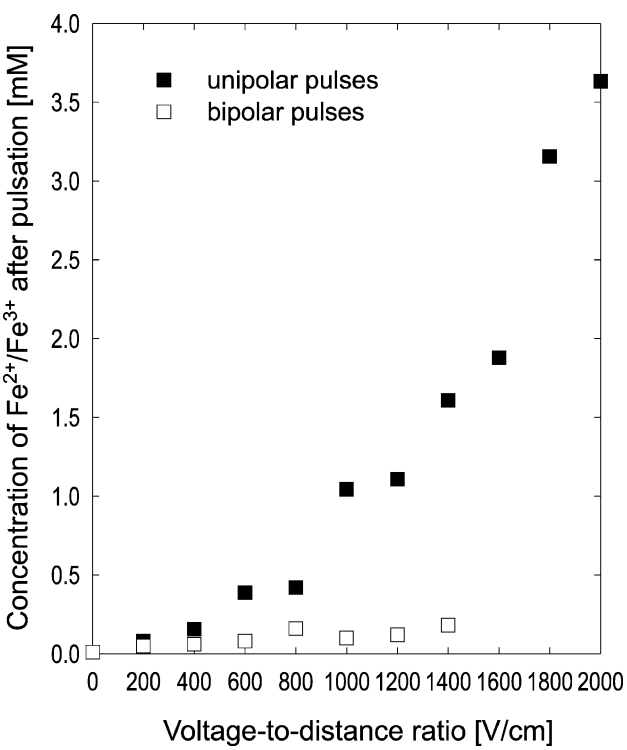

Fig. 2. The concentration of Fe^{2+} and Fe^{3+} ions in the suspension as the function of pulse amplitude (the voltage-to-distance ratio) for eight unipolar (■) and symmetrical bipolar (□) rectangular electric pulses. Each pulse was of 1 ms total duration (for bipolar pulses, 500 μs of one polarity followed by 500 μs of the opposite polarity), and the pulses were delivered in 1 s intervals.

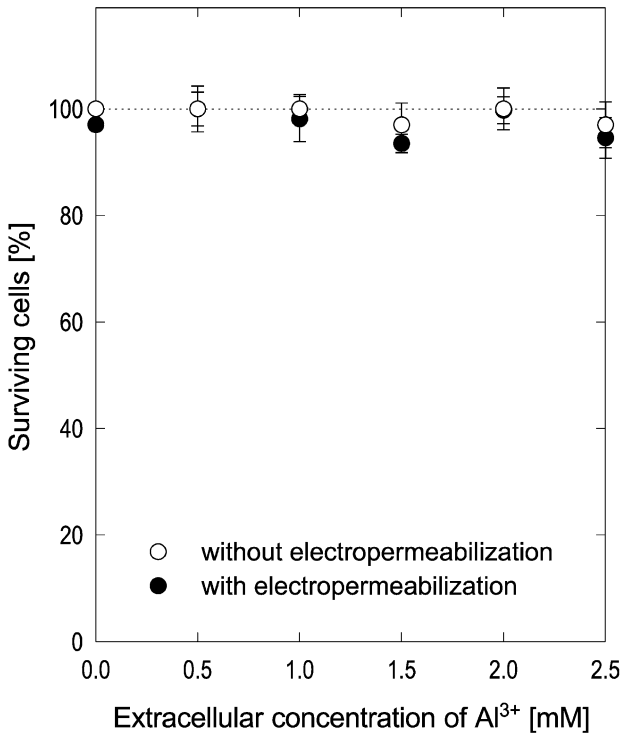

Fig. 3. Cell survival (mean ± S.D.) without (○) and with electropermeabilization (●) as the function of Al^{3+} concentration in the suspension. The cells were incubated for 1 h at room temperature. Electropermeabilization was performed at the beginning of the incubation using a train of eight unipolar rectangular pulses, each of 100 μs duration and 240 V amplitude (1200 V/cm voltage-to-distance ratio), delivered in 1 s intervals.

These results show that with bipolar pulses, the released concentrations of both Al^{3+} and Fe^{2+}/Fe^{3+} are more than one order of magnitude lower than with unipolar pulses of the same amplitude and duration. For unipolar pulses, the released concentrations are also roughly proportional to the number, duration, and amplitude of pulses.

To date, there are no commercially available generators of bipolar rectangular pulses with adequate amplitude and duration for electropermeabilization. While Figs. 1 and 2 testify that electrolytic erosion of the electrodes is decreased if bipolar pulses are applied instead of unipolar ones, it is unlikely that this improvement alone would provide sufficient motivation for the development of bipolar pulse generators for electropermeabilization. On the other hand, if electrolytic contamination of the suspension by unipolar pulses leads to detrimental effects on the suspended cells, this can provide a considerable impetus for such a development.

The most obvious detrimental effect of the released metal ions would be the loss of cell viability, and to investigate whether such an effect actually exists, we have determined the viability of DC-3F cells after 1 h of incubation in the medium containing concentrations of Al^{3+} and Fe^{2+}/Fe^{3+}, respectively, ranging from 0 to 2.5 mM (Figs. 3 and 4, hollow circles). As can be seen from

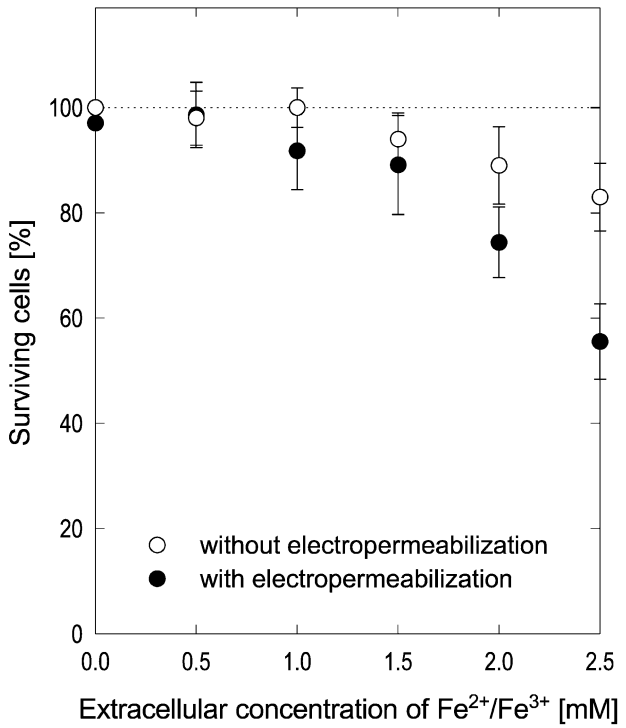

Fig. 4. Cell survival (mean ± S.D.) without (○) and with electropermeabilization (●) as the function of Fe^{2+}/Fe^{3+} concentration in the suspension. The cells were incubated for 1 h at room temperature. Electropermeabilization was performed at the beginning of the incubation using a train of eight unipolar rectangular pulses, each of 100 μs duration and 240 V amplitude (1200 V/cm voltage-to-distance ratio), delivered in 1 s intervals.

these figures, no effect on cell survival was detected within the investigated range for Al^{3+} ions, while for Fe^{2+}/Fe^{3+} ions, loss of cell viability became significant at concentrations above 1.5 mM. This study reveals that with stainless steel electrodes, even nonpermeabilized cells can be affected by the released concentrations of Fe^{2+}/Fe^{3+} ions. To investigate if electropermeabilization increases the vulnerability of the cells to Al^{3+} and Fe^{2+}/Fe^{3+} ions in the medium, an additional experiment was performed, in which at the beginning of incubation, the cells were permeabilized by eight unipolar pulses of 100-μs duration and 240-V amplitude (1200 V/cm voltage-to-distance ratio). Under our experimental conditions, this established protocol caused permeabilization of ≈94% of the cells accompanied by only ≈4% cell death (data not shown), but if the cells were electropermeabilized in a medium containing Fe^{2+}/Fe^{3+} ions, the cytotoxic effect was more pronounced (Fig. 4, filled circles) than for nonpermeabilized cells (Fig. 4, hollow circles). For Al^{3+} ions, no cytotoxic effect was observed even when the cells were electropermeabilized (Fig. 3, filled circles).

These results, which focus on the most obvious detrimental effect of the released ions—loss of cell viability—show that in the case of stainless steel electrodes, decrease of electrolytic contamination of the medium can lead to an improvement of efficiency of electropermeabilization, since in most of the applications, only the cells that remain viable after the treatment are of interest. In this respect, the study of cell survival alone offers no evidence for a need to decrease the electrolytic contamination of the medium in the case of aluminum cuvettes. However, the reports on the effects of released Al^{3+} on biochemistry of inositol phosphates [3], pH of the suspension [4], and precipitation of macromolecules [5] also suggest that with aluminum electrodes, electrolytic contamination can be detrimental, as well as biasing for the outcome of the experiments.

As our study shows, electrolytic contamination from both stainless steel electrodes and aluminum cuvettes is largely reduced if unipolar rectangular pulses are replaced by symmetrical bipolar pulses of the same amplitude and duration. It is important to stress that this substitution does not lead to any loss in the efficiency of electropermeabilization. On the contrary, as we have demonstrated in Part I of this study [1], replacing unipolar pulses by bipolar pulses of the same amplitude and duration can even lead to a noticeable increase of both the percentage of electropermeabilized cells and the uptake of molecules into these cells. Together with the results presented in this paper, this offers a strong motivation for the use of bipolar instead of unipolar rectangular pulses, and consequently for the development of commercial bipolar pulse generators which could, in due course, gain a wide use in cell membrane electropermeabilization.

Acknowledgements

This work was supported in part by CNRS, Institute Gustave-Roussy, University of Paris XI, and Ministry of Science and Technology of the Republic of Slovenia. The high-voltage amplifier has been developed within the frame of the Cliniporator project (Grant QLK3-1999-00484) under the framework of the 5th PCRD of the European Commission. The authors would like to thank Dr. M. Bonnay from the Department of Clinical Biology of Institute Gustave-Roussy, who kindly provided the equipment for the measurements of Fe^{2+}/Fe^{3+} concentrations. T.K. was also a recipient of a part-time PhD Scholarship of the French Government (CNOUS).

References

[1] T. Kotnik, L.M. Mir, K. Flisar, M. Puc, D. Miklavčič, Cell membrane electropermeabilization by symmetrical bipolar rectangular pulses: Part I. Increased efficiency of permabilization, Bioelectrochemistry 54 (2001) 83–90.

[2] S. Orlowski, L.M. Mir, Cell electropermeabilization: a new tool for biochemical and pharmacological studies, Biochim. Biophys. Acta 1154 (1993) 51–63.

[3] J.W. Loomis-Husselbee, P.J. Cullen, R.F. Irvine, A.P. Dawson, Electroporation can cause artifacts due to solubilization of cations from the electrode plates, Biochem. J. 277 (1991) 883–885.
[4] U. Friedrich, N. Stachowicz, A. Simm, G. Fuhr, K. Lucas, U. Zimmermann, High efficiency electrotransfection with aluminum electrodes using microsecond-controlled pulses, Bioelectrochem. Bioenerg. 47 (1998) 103–111.
[5] R. Stapulionis, Electric pulse-induced precipitation of biological macromolecules in electroporation. Bioelectrochem. Bioenerg. 48 (1999) 249–254.
[6] T. Tomov, I. Tsoneva, Are the stainless steel electrodes inert? Bioelectrochemistry 51 (2000) 207–209.
[7] J.L. Biedler, H. Riehm, Cellular resistance to actinomycin D in Chinese hamster cells in vitro, Cancer Res. 30 (1970) 1174–1184.