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A Double-Pulse Approach For Electrotransfection

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Abstract Gene transfer and expression can be obtained by delivering calibrated electric pulses on cells in the presence of plasmids coding for the activity of interest. The electric treatment affects the plasma membrane and induces the formation of a transient complex between nucleic acids and the plasma membrane. It results in a delivery of the plasmid in the cytoplasm. Expression is only obtained if the plasmid is translocated inside the nucleus. This is a key limit in the process. We previously showed that delivery of a high-field short-duration electric pulse was inducing a structural alteration of the nuclear envelope. This study investigates if the double-pulse approach (first pulse to transfer the plasmid to the cytoplasm, and second pulse to induce the structural alteration of the envelope) was a way to enhance the protein expression using the green fluorescent protein as a reporter. We observed that not only the double-pulse approach induced the transfection of a lower number of cells but moreover, these transfected cells were less fluorescent than the cells treated only with the first pulse.

Keywords Electropulsation · Electroporation · Plasmid · Nuclear envelope · Green fluorescent protein

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Introduction

Exposition of mammalian cells to brief micro- to millisecond intense (hundreds of volts per centimeter) external electric field pulses can induce a transient permeability of their membrane (electropermeabilization) (Neumann and Rosenheck 1972). Under controlled electric field conditions, the permeabilization can be reversible, and cell viability can be preserved (Neumann et al. 1982). Electropulsation is now frequently used in cell biology to introduce exogenous active compounds such as drugs or nucleic acids (Electro-gene-transfer, EGT) (Golzio et al. 2004) into the cell cytoplasm. Targeted drug electrotransfer is now a routine clinical practice in oncology (electrochemotherapy, ECT) (Mir et al. 1996); (Miklavcic et al. 2012; Cadossi et al. 2014). Gene electrotransfer requires a so-called EGT protocol, i.e., 1–10 ms-pulse duration with an electric field intensity in the range of several hundred volts per cm, in order to (i) permeabilize the membrane and (ii) push DNA toward the permeabilized cell membrane by electric forces (electrophoresis) to form surface-associated pDNA clusters (Golzio et al. 2002b; Kanduser et al. 2009; Satkauskas et al. 2012). Gene expression would result from a two-barrier process at the cell level. First, plasmid DNA must cross from the cluster across the plasma membrane to the cytoplasm (Rosazza et al. 2012);(Pavlin et al. 2012), and second, after migration through the cytoplasm along the microtubule network associated with a protein complex (Badding et al. 2013), it must cross the nuclear envelop, presumably by the nuclear pore in order to be expressed (Young et al. 2003). Indeed, microinjection studies showed that only 1 out of 10^6 pDNA present in the cytoplasm would enter the nucleus (Utvik et al. 1999). Studies on the cell cycle have shown that gene expression efficiency resulting from the electrotransfer was increased when the

nuclear envelop was disrupted i.e., when cells were pulsed into G2 phase (Escoffre et al. 2010; Golzio et al. 2002a). Exposure of cell to EGT electric field led to plasma membrane permeabilization but did not affect nuclear permeabilization for nucleic acids meaning that transition across this second barrier remained a challenge.

From a biophysical point of view, (i) for organelles being smaller than the cell envelope, a higher field strength was needed to obtain their permeabilization, and (ii) as the nucleus was bearing large nuclear pores, the field effect was reduced (Kotnik et al. 2012). Therefore, to affect the nuclear envelope, the prediction was that high-field pulses must be delivered on the cell but to preserve the viability they must be short pulses (Weaver et al. 2012). Furthermore, a double-pulse procedure may enhance the effect of such a high-field pulse by inducing first the electropermeabilization of the plasma membrane (Esser et al. 2010). The associated high membrane conductance results in a higher magnitude in the cytoplasm of the second high pulse. An enhanced effect of the high-field pulse is expected.

Previous studies have shown that application of high electric field pulses (tens of KV/cm) with ultra-short duration (sub-microsecond range) might allow permeabilization not only of the plasma membrane but also of the membranes of intracellular organelles like the nuclear membrane (Batista Napotnik et al. 2012; Napotnik et al. 2010; Schoenbach et al. 2001). It was reported that association of "classical" plasma membrane electropermeabilization (EGT pulses) with these short high-voltage pulses (nano-pulses) could enhance the transfer and the resulting expression of a green fluorescent protein reporter gene in human cells (Beebe et al. 2003). A recent study did not report such an enhancement under their experimental conditions (Chopinet et al. 2013).

Thus, we hypothesized that combination of EGT and µs-pulses may potentiate the introduction of plasmid into the nucleus by electropermeabilization of both plasma and nuclear membrane. We previously reported that the nuclear membrane was affected by such a treatment by means of a digitized fluorescence microscopy approach (Bellard and Teissie 2009a, b). In this study, we compared the transfection rate and the efficiency of GFP plasmid expression in CHO cells treated by EGT alone or EGT plus µs-pulses.

Materials and Methods

Cell Culture

Chinese hamster ovary (CHO) cells (wild-type Toronto (WTT)) were grown as a monolayer culture on T75 flasks (Nunc, Denmark) in minimum essential Eagle medium with Earle's salts and nonessential amino acids (EMEM; Eurobio, Les Ulis, France), supplemented with 10 % fetal bovine serum (GIBCO/Life Technologies, Grand Island, NY), L-glutamine (0.58 g/l, GIBCO/Life Technologies), 2.95 g/l tryptose-phosphate (Sigma-Aldrich, St. Louis, MO), BME vitamins (Sigma-Aldrich), 3.5 g/l glucose (Sigma-Aldrich), and the antibiotics penicillin (100 U/ml) and streptomycin (100 µg/ml, both from GIBCO/Life Technologies), at 37 °C, 5 % CO_2 atmosphere in a humidified chamber until they reached 70 % confluence.

WTT CHO cells could grow in suspension. The plated cells were trypsinized, and the cells were cultured in suspension in a spinner. 0.5×10^6/mL cells were sown in the same culture medium in a hermetic closed spinner at 37 °C with a soft stirring. Each day, the cell culture was diluted twofold. Growing cells in suspension avoided the trypsinization step before the delivery of electric pulses.

Plasmid DNA

pCMV-EGFP-C1 (Clontech, Mountain View, CA), a 4.7-Kb plasmid DNA encoding GFP, was amplified in *Escherichia coli* DH5α and purified with the Maxiprep DNA Purification System (Qiagen, Germany) according to the manufacturer's protocol.

Gene Electrotransfection Protocols and Cell Viability Measurement

Cells in spinner were collected and suspended in phosphate buffer [PB; 10 mM KH_2PO_4/KH_2PO_4, 1 mM $MgCl_2$, 200 mM saccharose (pH 7.4)] at a concentration of 5×10^6 cells/ml, and 20 µg/ml pCMV-EGFP-C1 plasmid was added. 100 µl of cell solution was deposited between stainless steel, flat, parallel electrodes (0.4 cm gap) in contact with the bottom of a culture dish (Nunc, Denmark). 6 × 5 ms, 600 V/cm -1 Hz square-wave electric pulses were applied at room temperature using a pulse generator (electrocell S20; Betatech, St Orens, France). In the double-pulse condition, 30 s after the EGT protocol application, cells were pulsed with 1 × 5 µs-pulse of 5 kV/cm being kept between the electrodes. Pulse delivery was monitored online on the touch screen. Cells were transferred 5 min after pulses into 2-mL EMEM medium in 30-mm-diameter culture dishes (Nunc, Denmark) and incubated for 24 h at 37 °C with 5 % CO2.

Cell viability was analyzed 24 h after treatment by Crystal violet staining (Merck, Darmstadt, Germany) (Gabriel and Teissie 1995). Briefly, plated cells were washed with PBS 1× and incubated for 20 min with the 0.1 % of crystal violet solution. After three washes, a 10 % acetic acid solution was added to lyse stained cells.

Absorbance measurement was realized by spectrophotometric measurement at 595 nm (Novaspec II, Pharmacia biotech, Uppsala, Sweden).

Plasmid Expression Quantification by Flow Cytometry

The percentage of GFP-positive cells and the fluorescence intensity were determined by flow cytometry. Cells were trypsinized, centrifuged for 5 min at 500 × *g*, and transferred in 200 µL of FACS buffer (PBS 1× plus 2.5 % fetal bovine serum). Acquisition was performed on a FACScalibur cytometer (BD bioscience, San Jose, CA), and data were analyzed using FlowJo software (Tree Star, Ashland, OR). By gating on the fluorescence emission, it was possible to obtain the percentage of fluorescent cells and their mean fluorescence emission (see Fig. 2).

Plasmid Expression Observation by Microscopy

For microscopic observation of fluorescent protein expression, culture dishes were placed on the stage of an inverted digitized fluorescent microscope (Leica DMIRB, Wetzlar, Germany). Cells were observed with a Leica 20× objective and the L4 block filter (Exc: BP480/40; Em: BP527/40).

Statistical Analysis

Statistical analysis was carried out using Prism 5 statistical version (GraphPad Software Inc., San Diego, CA). We used one-way ANOVA with Bonferroni post tests. Each condition was tested four times (see the figures captions).

Results

Effect of µs-Pulses on CHO Cell Viability

The viability of CHO cells was determined 24 h after the treatment by Crystal Violet Coloration (Fig. 1). Long pulses in PB affected the viability as expected from the pulsing conditions (slightly hypo-osmotic solution) (Golzio et al. 1998). Long pulses in the presence of plasmid (EGT) drastically reduced cell viability as already reported (Li et al. 1999). The application of one short high electric field pulse after EGT did not change the viability of cells compared to EGT alone. Delaying the delivery of the microsecond pulse from a few seconds up to 20 min did not affect the level of the loss in viability. Accumulating the microsecond pulses (delivered 30 s after the long pulse treatment) up to five successive pulses did not significantly affect the loss in viability.

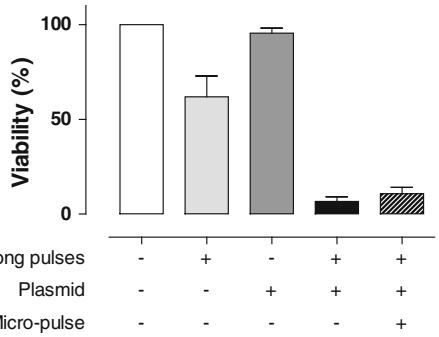

Fig. 1 CHO cell viability after short high electric field application. CHO cells cultured in spinner were transferred in phosphate buffer at a concentration of 5.10^6 cell/mL. When necessary, 20 µg/ml of EGFP plasmid was added to the solution. 100 µL of cell solution was put between stainless steel, flat, parallel electrodes (0.4 cm gap) on petri dish. 6 × 5 ms, 600 V/cm (240 V) −1 Hz square-wave electric pulses were applied at room temperature using a pulse generator (electrocell S20; Betatech, St Orens, France). In the indicated conditions, cells were pulsed with 1 × 5 µs-pulses of 5 kV/cm (2,000 V), 30 s after the long pulse protocol application, being kept between the electrodes. Cell viability was analyzed 24 h after treatment by Crystal Violet staining. ($n = 4$, 2 independent experiments)

The same observation was present in an iso-osmotic solution or in a slightly hypo-osmotic one (data not shown).

Effect of µs-Pulses on Transfection Rate and Expression of GFP Plasmid

To study if the application of a short high electric field pulse increased the expression of GFP plasmid, CHO cells were pulsed with EGT electrical parameters prior to the application of 1 × 5 µs-pulses of 5 kV/cm. A short delay of 30 s between the two treatments was observed to be sure that the cells were still permeabilized during the second treatment. This was the shortest delay available to adjust the electropulsator settings. The field effect of the microsecond would be more significant on the organelles as the plasma membrane was slightly conductive under this short delay conditions. 24 h after treatment, protein fluorescence was observed under the microscope and analyzed by flow cytometry (Fig. 2).

Under iso-osmotic conditions under the low concentration of pDNA that was used (Cepurniene et al. 2010), the percentage of fluorescent cells was low under the two procedures but the mean fluorescence intensity was strongly decreased by the microsecond pulse (from 447 down to 281).

As it was proposed that transfection was more effective under hypo-osmolar conditions (Golzio et al. 1998; Sukhorukov et al. 2005), experiments were further developed by reducing the amount of saccharose in the pulsing buffer (see the Materials and Methods section). Hypo-osmotic

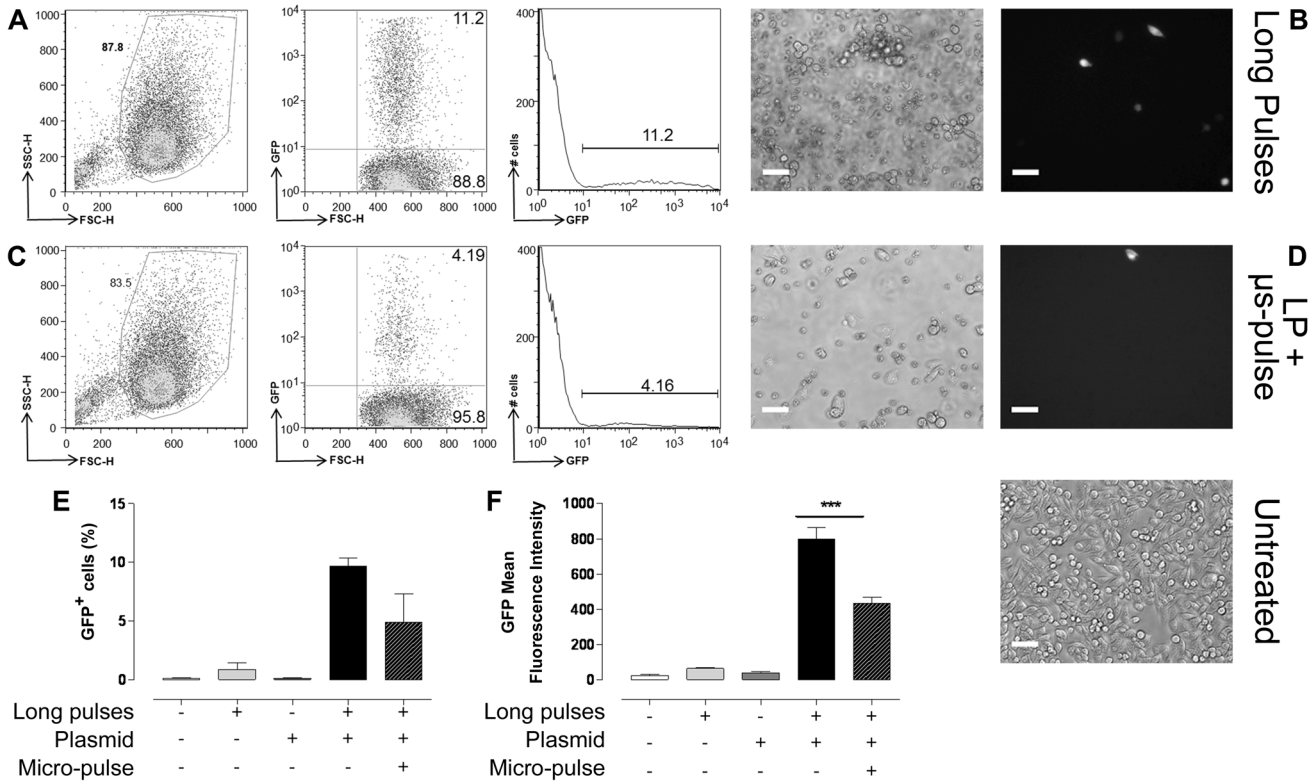

Fig. 2 Transfection rate is decreased with micro-pulses. CHO cells cultured in suspension (0.1 mL) were pulsed in the presence or absence of 2 µg of GFP plasmid DNA. 6 × 5 ms, 600 V/cm (240 V) −1 Hz square-wave electric pulses were applied at room temperature. To the indicated conditions, cells were pulsed with 1 × 5 µs-pulses of 5 kV/cm (2,000 V) 30 s after EGT. Percentage of GFP+ cells (**e**) and mean fluorescence intensity (**f**) of GFP CHO cells treated with EGT alone (**a**, **b**) or with EGT + µs-pulses (**c**, **d**) were determined 24 h after treatment by cytometry acquisition on a FACScalibur and analysis with FlowJo software. ($n = 4$, 2 independent experiments). Statistical analysis was conducted using 1-way ANOVA test with Bonnferroni correction. $***p < 0.05$

conditions were known to bring a higher electrotransfer. This was the case under our conditions. Under EGT conditions, 11.2 % of surviving cells expressed GFP plasmid while only 4.2 % with the EGT/µs-pulse association. Moreover, the fluorescence intensity of transfected cells is significantly reduced when the µs-pulse was added (434.66) compared to EGT alone (800.75) indicating a lower expression of GFP plasmid in cells after the double treatment.

Discussion

In this study, we hypothesized that the association of µs-electric pulses with "classical" EGT conditions could improve the accessibility of plasmid DNA to nucleus in order to increase its expression. We observed that not only EGT followed by a single µs-pulse induced the transfection of a lower number of cells but moreover, these transfected cells were less fluorescent than the cells treated with EGT alone.

We previously showed that while EGT alone induced a swelling of cells and nucleus, the combination with µs-pulses reduced this phenomenon or even induced shrinking of cells and nucleus. Moreover, we demonstrated that this shrinking was linked to the maintenance of the condensed form of DNA. This meant that if EGT help in the entry of plasmid DNA by creating pathways in the membrane, the shrinking induced by µs-pulses might affect those pathways and decreased those plasmid entries into the cell. Hyper-osmotic pulsing conditions were indeed shown to reduce the efficiency of electrotransfer (Golzio et al. 1998). Moreover, because the same phenomenon was observed on nucleus (Bellard and Teissie 2009a, b), the plasmid could be trapped in the cytoplasm after its transfer by the EGT treatment. Fluorescence intensity was directly correlated to the number of plasmids that reached the nucleus transcriptional machinery (Cohen et al. 2009). These two mechanisms could explain the reduced fluorescence intensity observed with the combination of EGT and µs-pulses.

Another explanation was linked to the complex network of reactions occurring in the cell after the plasmid transfer. The loss of cell viability due to the electric pulse-mediated pDNA transfer was previously mentioned (Hofmann et al. 1999; Li et al. 1999). pDNA in the cytoplasm either in an intact form or after its degradation by cytoplasmic nucleases affected the cell metabolism inducing apoptosis.

Our previous fluorescence investigations on the effects of the double-pulse treatment of mammalian cells pointed out that the nuclear envelope was seriously affected by the HV-microsecond treatment. There was a need for a repair of the damaged envelope to preserve the cell viability and to support expression. There were very few data on the involved processes. More information were present concerning the plasma membrane. It was shown that it was not a simple phenomenon as described in the case of pure lipid bilayer vesicles but an active process involving the cellular machinery (Huynh et al. 2004). This was dependent on the energy reserves of the cell (Rols et al. 1998). Furthermore, the expression of the transferred plasmids by the cellular machinery was also dependent on the remaining cytosolic ATP level (Rols et al. 1998). Our observation of a reduced level of expression (decrease in the fluorescence emission of the transfected proteins) could be a consequence of the enhanced decrease in cytoplasmic ATP induced by the HV-microsecond pulse.

Conclusion

As a final conclusion, while we recently showed that nsPEF delivered within at least 0.5 h after the EGT treatment did not induce any effect on the level of expression (Chopinet et al. 2013), our present approach with microsecond pulses, where we showed that the nuclear envelope was affected, was bringing a negative consequence. Electric pulse-mediated nuclear envelope permeabilization did not carry a positive effect on gene electrotransfection. This suggested that the induced structural defects were not improving the pDNA nuclear translocation or if the translocation was improved, the consequences at the cellular level brought negative conditions for the resulting gene expression.

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