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Electric Field Orientation for Gene Delivery Using High-Voltage and Low-Voltage Pulses

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Abstract Electropermeabilization is a biological physical process in response to the presence of an applied electric field that is used for the transfer of hydrophilic molecules such as anticancer drugs or DNA across the plasma membranes of living cells. The molecular processes that support the transfer are poorly known. The aim of our study was to investigate the effect of high-voltage and low-voltage (HVLV) pulses in vitro with different orientations on cell permeabilization, viability and gene transfection. We monitored the permeabilization with unipolar and bipolar HVLV pulses with different train repetition pulses, showing that HVLV pulses increase cell permeabilization and cell viability. Gene transfer was also observed by measuring green fluorescent protein (GFP) expression. The expression was the same for HVLV pulses and electrogenotherapy pulses for in vitro experimentation. As the viability was better preserved for HVLV-pulsed cells, we managed to increase the number of GFP-expressing cells by up to 65 % under this condition. The use of bipolar HVLV train pulses increased gene expression to a higher extent, probably by affecting a larger part of the cell surface.

Keywords High-voltage and low-voltage pulses · Electropermeabilization · CHO cell · Electrotransfection · Field orientation · Gene transfer

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Introduction

The cell membrane represents a physical barrier which isolates the cell content from the external medium. A calibrated electric field pulse (electropulsation) can be applied to cells in suspension to induce a permeabilization of the cell membrane (electropermeabilization). This permeabilized state is obtained when the field strength is higher than a threshold function to the cell size, shape and orientation (Bellard and Teissie 2009; Valic et al. 2003).

Plasmid electrotransfer and resulting expression (electrotransfection) was first described 30 years ago (Neumann et al. 1982). Because electropermeabilization represents an efficient and safe method for transmembrane transfer, it is now a routine technique for the delivery of various types of molecules (RNA, DNA, drugs, etc.). Gene delivery is a complex phenomenon which is still poorly understood, with pertinent studies performed in the 1990s (Neumann et al. 1999; Smith et al. 2004; Sukharev et al. 1992). Intense activity is still present, combining biophysical and cellular approaches (Pavlin et al. 2010, 2012; Wu and Yuan 2011; Yu et al. 2012). Gene delivery is not the result of direct plasmid diffusion into the cytoplasm. DNA molecules are accumulated by electrophoretic forces at the membrane level, where they remain trapped after pulse application and then are slowly translocated into the cytoplasm. Due to the electrophoretic drift of DNA, this accumulation is present only on the cell side facing the cathode (Escoffre et al. 2011; Golzio et al. 2002). Changing the orientation of the electric field was indeed described to increase the interaction surface between DNA and the membrane (Faurie et al. 2004). As a consequence, a higher level of expression was observed.

Several studies have shown the crucial role of electrophoretic forces in gene transfer, but alone those forces do

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not provide any transfection (Cepurniene et al. 2010): electropermeabilization must be present. For a given electrode width, using high-voltage, short pulses followed by a low-voltage, long pulse (HVLV), gene expression is observed in vivo (Andre et al. 2008). After an HV pulse, all cells are permeabilized and the LV pulse brings plasmids into contact with cells due to electrophoretic forces. This double-pulse method was shown to be valid in vitro when using suboptimal plasmid concentration (Kandušer et al. 2009). In the present study we observed the effect of electric field orientation using HVLV pulse trains with different modulations of the pulse polarities.

Materials and Methods

Cell Culture

Chinese hamster ovary (CHO) cells in early passage were used. The WTT clone was selected for its ability to grow in suspension or plated. Cells were grown in minimum Eagle's medium (MEM) supplemented with 8 % fetal calf serum. Cells were mycoplasma-free.

Plasmid

A 4.7-kbp plasmid (pEGFP-C1; Clontech, Mountain View, CA) carrying the gene of the green fluorescent protein (GFP) controlled by the CMV promoter was used. It was prepared from transfected *Escherichia coli* cells using the Maxiprep DNA purification system according to the manufacturer's instructions (Qiagen, Valencia City, CA).

Electropulsation Device

A S20b pulse generator was used (Betatech, L'Union, France). The output was connected to a pulse polarity inverter, which was controlled by the pulse generator through a TTL signal. The double pulse from the S20b kept the same polarity as for the classical HVLV protocol. But in a train of pulses, an inversion can be obtained between each (HVLV) pulse couple (Fig. 1).This was called "bipolar" HVLV pulses. The output was controlled at two levels: either directly on the internal monitor of the S20b or by a current follower (Chauvin Arnoux, Paris, France) on a laptop using a Picoscope (Pico Technology, St. Neots, UK) to follow the polarity.

Electropermeabilization

Cells were cultured by plating in a flask (Easy Flask; Nunc, Rochester, NY), and just before the experiment cells were trypsinized and counted with a Neubauer chamber. Cells were centrifugated at $800 \times g$ for 5 min at room temperature. Culture medium was removed. Cells were resuspended in an electropulsation buffer (10 mM phosphate, 1 mM $MgCl_2$, 250 mM sucrose, pH 7.4) complemented with propidium iodide (100 μM) (Sigma-Aldrich, St. Louis, MO) at 5×10^6 cells/ml. Penetration of the propidium iodide was used to monitor cell permeability (Kennedy et al. 2008). This suspension (100 μl) was poured between two plated parallel stainless-steel electrodes (distance between electrodes 4 mm). After electropulsation, this suspension was transferred in a culture chamber (Lab-Tek I system, Nunc) and observed under an inverted digitized video fluorescence microscope (DMIRB; Leica, Wetzlar, Germany). Several images of each condition were taken and treated with ImageJ (ImageJ 1.4n; Wayne Rasband, NIH, Bethesda, MD).

Electrotransfection

The same protocol was applied for the electrotransfection test. Cells were resuspended in a pulsation buffer complemented with 5 μg/ml of plasmid pEGFP-C1. After pulse delivery, 1 ml of complete culture medium was added, and the cells were incubated for 24 h in an incubator at 37 °C, 5 % CO_2. Cells were trypsinized and analyzed by flow cytometry (BD Biosciences, San Jose, CA; FACScalibur) to determine the percentage of transfected cells and the fluorescence intensity (average value) of the expression of GFP.

Viability

For viability experiments, cells were imaged 24 h after treatment by bright field illumination under an inverted digitized video microscope (DMIRB). We acquired six random images. Adherent and morphologically nonaffected cells were counted for each condition and expressed as the relative percentage to the counting of nonpulsated cells.

Statistical Analysis

Statistical analysis was carried out using statistical software (Prism 4.01; GraphPad Software, San Diego, CA). Each experiment under the different pulsing conditions was performed three times. Errors bars represent the standard error of the mean. The statistical significance of differences between the means was evaluated by two-sided, unpaired Student's *t* test (NS = nonspecific, $*P < 0.05$, $**P < 0.01$).

Results and Discussion

Pulse parameters were selected by taking into account previous data (Faurie et al. 2010). This reference was our

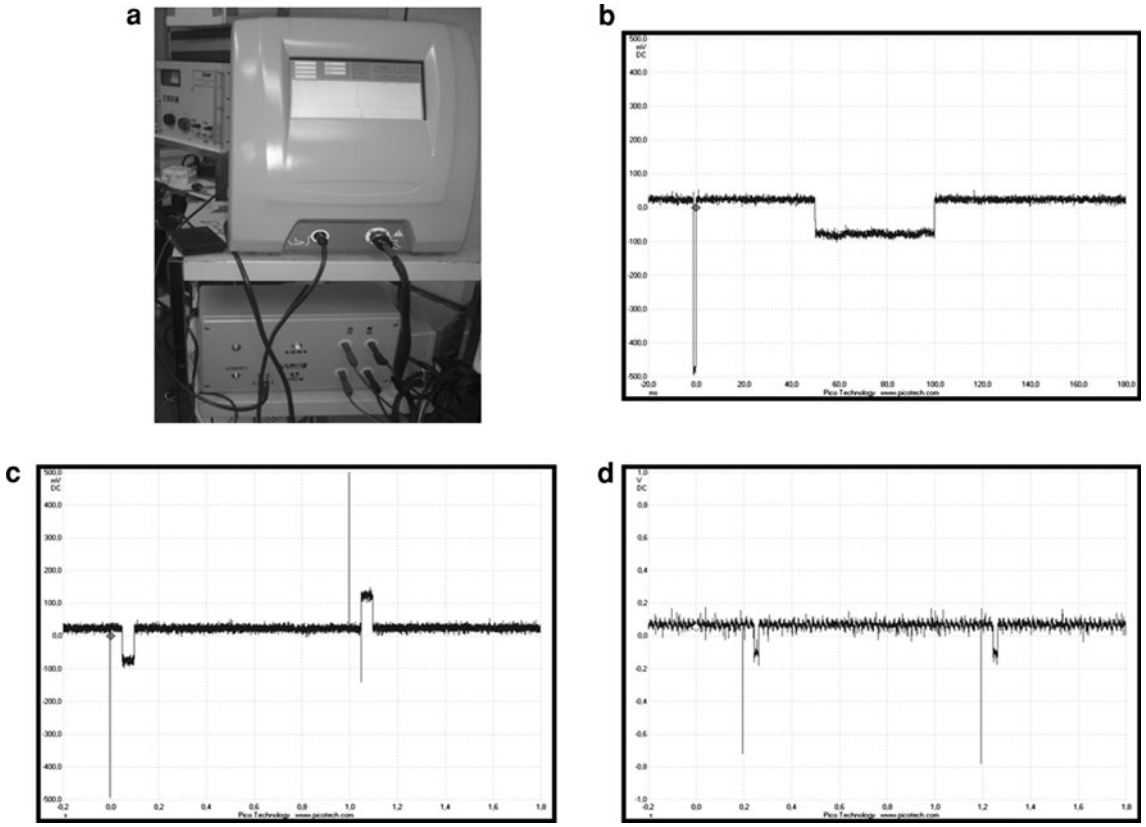

Fig. 1 Multipolarity pulse generator. A bipolar Betatech S20b was coupled to a homemade automatic pulse inverter (*gray box* on the stage below the pulse generator) (**a**). The output (*black* and *grey* banana plugs) was connected to plate parallel electrodes (not shown). View on the picoscope of one single HVLV pulse current (one HV, $E = 1{,}300$ V/cm, $t = 0.1$ ms, followed by one LV of $E = 150$ V/cm, $t = 50$ ms after a delay of 50 ms) (**b**). A train of two pulses (each was as displayed in **b**) in a bipolar sequence. A polarity inversion is present. The delay was 1 s (**c**). A train of two pulses (each was as displayed in **b**) in a unipolar sequence. The pulse polarity is conserved. The delay was 0.5 s (**d**)

guideline for the choice in the delay between successive pulses in a train for the pulse polarity inversion. We therefore selected 1-s delay. For technical reasons (the present specifications of the S20b pulsator), the LV (150 V/cm) pulse duration remained limited to 50 ms, but this second pulse was nevertheless delivered only 50 ms after the HV (1,300 V/cm, duration 0.1 ms) one. Pulse parameters were therefore slightly different from in previous reports (Cepurniene et al. 2010; Kandušer et al. 2009). We compared the effect of the polarity inversion in trains of HVLV pulses, bipolar (Fig. 1c) and unipolar trains (Fig. 1d), to a train of electrogenotherapy (EGT) "classical" pulses (long-lasting pulse with a medium voltage, 700 V/cm) on the permeabilization, viability and transfection.

Permeabilization was quantified by monitoring propidium iodide uptake in CHO cells (Kennedy et al. 2008). We observed a higher rate of permeabilized cells with HVLV pulses when compared to classical pulses (EGT) (Fig. 2a). Fluorescence intensity was also measured (Fig. 2b). Whatever the number of train repetitions, HVLV pulses provided a higher fluorescence level ("higher" permeabilization). Using HVLV pulses increased the percentage of propidium iodide-positive cells and the entry of small molecules. The EGT conditions were selected to preserve the viability, so the field strength was reduced. The consequence was permeabilization of only a subpopulation, due to the size selectivity by the electric field strength, which affected only the largest cells. The HV pulse affected all cells (whatever their size) and the LV electrophoretically accumulated the dye (Pucihar et al. 2008).

When the viability 24 h after electric pulses (Fig. 3) was observed, as already known, classical electric pulses (EGT) reduced the number of viable cells. Furthermore, HVLV pulses (with and without polarity inversion) do not affect the viability of CHO cells even for an eight-repetition train. As after the electric pulse, cells have to keep their integrity to express the transfected gene. This is a clear advantage of using HVLV pulses.

Taking into account previous results, a suboptimal concentration of plasmid was used (Kandušer et al. 2009). Cells electropulsated with eight HVLV pulses with no polarity inversion provide a more efficient transfer of

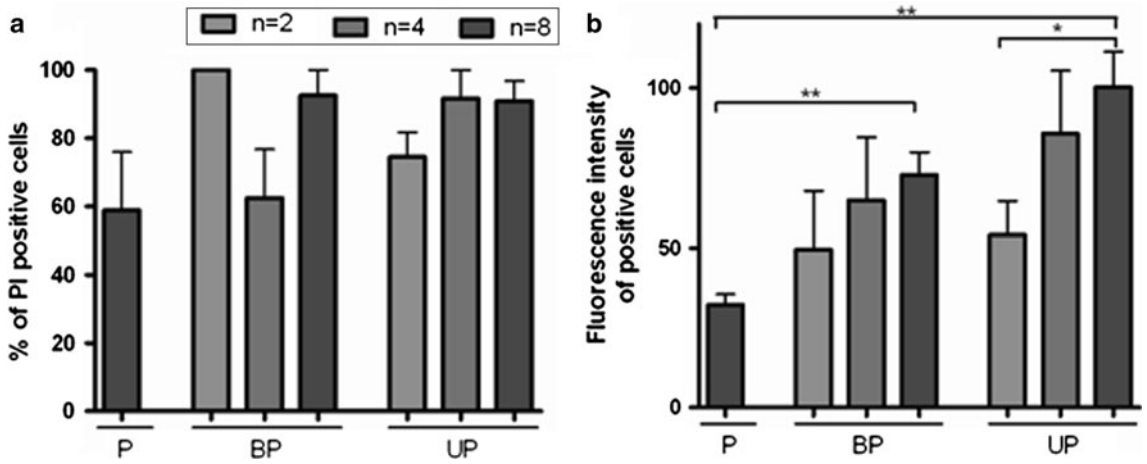

Fig. 2 Effect of high- and low-voltage pulses on cell permeabilization. CHO cells were pulsed with electric pulses of $E = 700$ V/cm, $t = 5$ ms, $n = 8$, $F = 1$ Hz (*P*); with bipolar trains of two, four or eight HVLV pulses (*BP*); or with unipolar trains of two, four or eight HVLV pulses (*UP*) in the presence of propidium iodide to monitor the permeabilization. We observed the percentage of permeabilized cells (**a**) and the associated mean fluorescence level of propidium iodide-positive cells (**b**) by microscopy

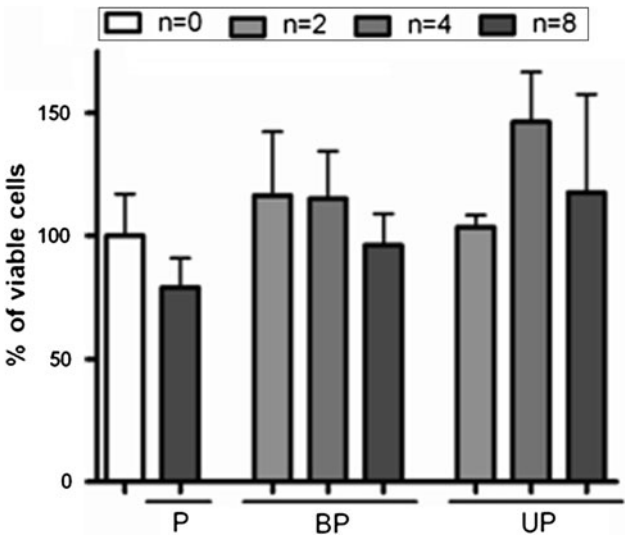

Fig. 3 Viability of CHO cells after electropulsation. CHO cells were pulsed with electric pulses of $E = 700$ V/cm, $t = 5$ ms, $n = 8$, $F = 1$ Hz (*P*); with bipolar trains of two, four or eight HVLV pulses (*BP*); or with unipolar trains of two, four or eight HVLV pulses (*UP*). Cells were counted 24 h after electropulsation under the microscope

plasmid than classical (EGT) pulses (Fig. 4a) as reported (Cepurniene et al. 2010; Kandušer et al. 2009; Pavlin et al. 2010). But the pulse duration, sequence and shape (a sharper voltage rise time [200 ns], a delay of 50 ms is delivered by the S20b) were different from these published data (several microseconds with a Cliniporator used by Kandušer et al., the delay between HV and LV pulses was 1 s in Cepurniene et al.'s experiments). This is strongly illustrative of the flexibility of the approach. Taking into account the key role of the electric field pulse-induced electrophoretic drift of DNA in electrotransfection, this was tentatively explained in the 2009 study by the hypothesis that LV pulses could bring the plasmid in the neighborhood of the membrane due to electric forces (Pavlin et al. 2010). This effect was masked by an excess of plasmid in the cell suspension (Kandušer et al. 2009) but detected with a suboptimal concentration.

Our technology (giving a polarity inversion in the HVLV train delivery) (Fig. 1) allows us to show that bipolar HVLV pulses bring a higher level of plasmid expression than unipolar HVLV pulses (Fig. 4b). Under EGT conditions, polarity inversion was previously shown to provide DNA–membrane interaction on a larger part of the cell surface (Faurie et al. 2004) and more plasmid transfer to the cytoplasm. This was proposed to explain the higher expression of GFP. Again, under the bipolar train condition, DNA would interact on both sides of the cell membrane, facing the electrodes. A larger part of the cell surface acts in the DNA transfer across the plasma membrane to the cytoplasm. As expected, the number of GFP-positive cells increased with the number of pulse couples and was larger under the bipolar condition.

The mean GFP fluorescence level was only slightly lower than under the EGT conditions (in a nonstatistically significant way). Nevertheless, the computed electrophoretic DNA accumulation at the cell surface was always larger under the HVLV conditions. It is proportional to ETN (E = field strength, t = pulse duration, n = cumulated number of pulses). In all HVLV conditions, mean fluorescence was observed to fairly increase linearly with the number of pulses, in agreement with a key role of the electrophoretic DNA drift in the control of expression. Another key feature of the HVLV train is that cell viability appears not to be affected by the electrical treatment. From the initial population, EGT conditions bring a 17 % value

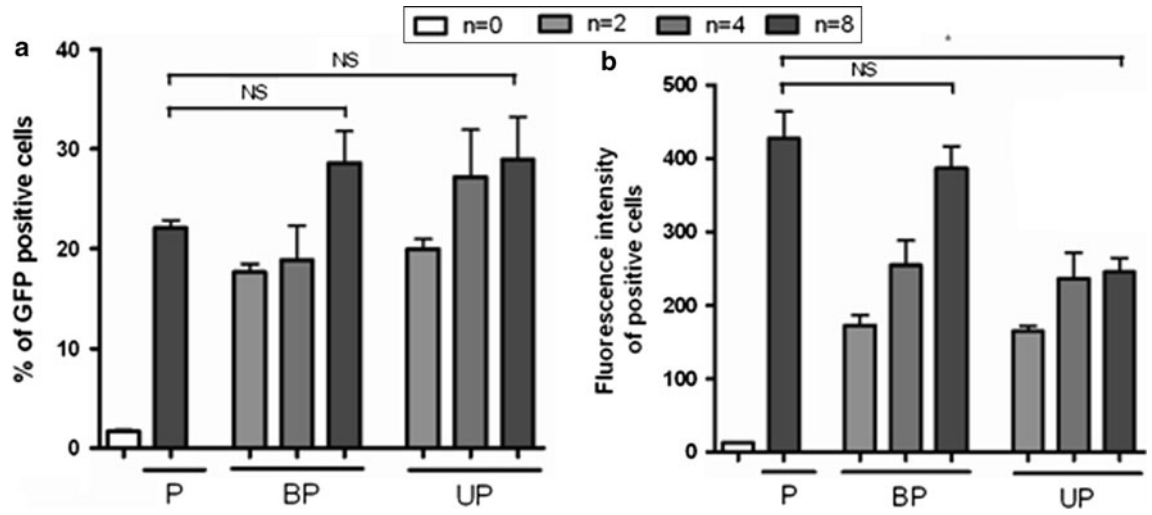

Fig. 4 Effect of unipolar and bipolar pulse trains on CHO cell gene transfection. Percentage of transfected cell with plasmid pEGFP-C1 (**a**) and the associated median fluorescence level of GFP-expressing cells (**b**). CHO cells were pulsed with electric pulses of $E = 700$ V/cm, $t = 5$ ms, $n = 8$, $F = 1$ Hz (*P*); with bipolar trains of two, four or eight HVLV pulses (*BP*); or with unipolar trains of two, four or eight HVLV pulses (*UP*). GFP expression was assayed 24 h after electropulsation and analyzed by flow cytometry

of GFP-positive cells (from Figs. 3, 4), while eight-train bipolar HVLV conditions give 28 % (i.e., a 65 % increase).

As a final conclusion, the LV pulse in the HVLV couple acts on the DNA–membrane interaction under nonpermeabilizing conditions (Rols and Teissie 1990). Under our conditions, the interaction can occur up to 100 ms after the permeabilizing HV pulse (50-ms delay and 50-ms duration). The use of bipolar conditions brings this interaction to a larger part of the cell surface and results in higher expression. This improvement is associated with preserving the viability of the pulsed population.

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