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RESEARCH ARTICLE

Electro-mediated gene transfer and expression are controlled by the life-time of DNA/membrane complex formation

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Abstract

Background Electroporation is a physical method used to transfer molecules into cells and tissues. Clinical applications have been developed for antitumor drug delivery. Clinical trials of gene electrotransfer are under investigation. However, knowledge about how DNA enters cells is not complete. By contrast to small molecules that have direct access to the cytoplasm, DNA forms a long lived complex with the plasma membrane and is transferred into the cytoplasm with a considerable delay.

Methods To increase our understanding of the key step of DNA/membrane complex formation, we investigated the dependence of DNA/membrane interaction and gene expression on electric pulse polarity and repetition frequency.

Results We observed that both are affected by reversing the polarity and by increasing the repetition frequency of pulses. The results obtained in the present study reveal the existence of two classes of DNA/membrane interaction: (i) a metastable DNA/membrane complex from which DNA can leave and return to external medium and (ii) a stable DNA/membrane complex, where DNA cannot be removed, even by applying electric pulses of reversed polarity. Only DNA belonging to the second class leads to effective gene expression.

Conclusions The life-time of DNA/membrane complex formation is of the order of 1 s and has to be taken into account to improve protocols of electro-mediated gene delivery. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords electroporation; gene delivery; electric field; pulse repetition frequency; electropermeabilization; membrane

Introduction

The cell plasma membrane acts as a selective barrier that regulates the exchange of molecules between the cell and the external medium. Therefore, the delivery of therapeutic molecules such as DNA into living cells and tissues represents an outstanding challenge in molecular biology and medicine. Delivery can be achieved using biological vectors such as viruses or using chemical approaches. However, the use of these methods in the field of gene therapy is limited because of their lack of efficiency and/or safety [1]. A physical approach, electropermeabilization also named electroporation,

was introduced in the 1970s and was subsequently developed in the 1980s for gene delivery [2]. This method has been used with increasing popularity [3–6]. Applications have been successfully developed for antitumor drug delivery [7–9] and the transfer of genes [4,10–14]. Electrochemotherapy is accepted in a number of countries as a palliative treatment and clinical trials of gene electrotransfer are under investigation [15–18]. A phase I dose escalation trial of plasmid interleukin electroporation was carried out in patients with metastatic melanoma. Biopsies showed plasmid dose proportional increases in interleukin-12 protein levels as well as marked tumor necrosis and lymphocytic infiltrate, indicating that this modality is safe, effective and titrable [19].

Despite numerous studies, much remains unknown about the basic mechanisms of how DNA enters cells [20]. Several theoretical models postulate that DNA crosses the plasma membrane during electric pulse application through 'electropores' [2,21–23]. However, such models are not supported by experimental data [24,25]. They necessitate the creation of pores that have never experimentally been observed. Performed at the single cell level, studies have yielded a phenomenological description of gene electrotransfer. Small and large molecules enter the cell through well-defined permeabilized caps of the plasma membrane. Small molecules enter the cell across part of the membrane facing the two electrodes via the concentration gradient difference between the exterior and cell interior by a diffusion process [26–28]. By contrast, the introduction of DNA only occurs in the part of the membrane facing the cathode and requires a number of consecutive steps: electrophoretic migration of DNA towards the cell, DNA insertion into the membrane, translocation across the membrane, migration of DNA towards the nucleus and, finally, transfer of DNA across the nuclear envelope [29–32]. Therefore, DNA does not enter into the cell during electric pulses but it is 'trapped into' the plasma membrane. Its presence inside the cytoplasm is only detected several minutes after pulse application [33]. The electric field induces a key reaction between the membrane under the electrical stress and the DNA, which is electrophoretically accumulated at the interface, leading to its insertion [29,34–36]. The present study aimed to characterize the interaction of the DNA with the membrane. Indeed, the plasmid DNA has two possibilities: either to penetrate into the cell or to return into the bulk [29]. However, in our previous model [33], the plasmid was assumed to move only towards the cytoplasm, and we therefore changed the electric field orientation to evaluate the role of the electropermeabilized caps and the DNA electrophoretic migration [37]. This was achieved by studying, at the single cell membrane level, the role of the electromigration of plasmid DNA towards (or from) the membrane, as well as the kinetics of DNA interaction with the membrane during the process of its entrance into the cells. We have designed experiments where the direction of electric field could be alternated in a way that each consecutive pulse was delivered with reversed polarity. If the interaction of the DNA with the cell membrane is transient, it must be affected by changing the pulse polarity at various pulse repetition frequencies. Quantitative observations of DNA/membrane interactions were made as a function of pulse polarity and repetition frequency. The results obtained support the existence of two classes of DNA interaction with the plasma membrane: one leading to a metastable DNA/membrane complex from which the DNA can escape and go back to external medium and the other leading to a stable DNA/membrane complex, where the plasmid cannot be removed, even by applying electric pulses of reversed polarity. These results have to be taken into account for protocols of electro-mediated gene delivery.

Materials and methods

Cells

Chinese hamster ovary (CHO) cells were used. The WTT clone was selected for its ability to grow in suspension or plated on Petri dishes or on microscope glass cover slip. They were grown in minimal essential medium as previously described [37]. Their ability to grow on a support after being maintained in suspension comprises direct evidence of their viability.

Plasmid

A 4.7-kb plasmid (pEGFP-C1) carrying the green fluorescent protein (GFP) gene controlled by the cytomegalovirus promoter was obtained from Clonetech (Palo Alto, CA, USA). It was purified from *Escherichia coli* transfected cells using the Maxiprep DNA purification system according to the manufacturer's instructions (Qiagen, Courtaboeuf, France). This plasmid was used both for the direct visualization at the single cell level of its electrotransfer into the cells (by labeling as shown below) and for gene expression.

Electropulsation

Electropulsation was performed using a new system of electroporator developed at the University of Ljubljana. This electroporator delivers between one and 32 square-wave electric pulses of 80–400 V amplitude, with a duration of 10 μs to 1 ms, and a pulse repetition frequency in the range 0.1–5000 Hz. A particularity of this electroporator is an embedded electrode commutator. This commutator applies one of three possible states to each of the electrodes: positive, negative or high impedance state; thus, the electric field direction between the electrodes can be changed. Electrode state change is accomplished within 12 ms. Therefore, for bipolar pulses lasting 1 ms,

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the maximum repetition frequency, corresponding to the minimum time required for switching the electrodes, is 76.9 Hz. The electrodes were designed as cylindrical rods made of stainless steel allowing delivery of electric field in different directions and, at the same time, providing a relatively homogeneous electric field distribution [38]. In the present study, two opposite electrodes, 5 mm apart (diameter 3.5 mm), were used for the delivery of either unipolar or bipolar pulses. Six electric pulses lasting 1 ms were applied at 0.8 kV/cm; these parameters allowed high permeabilization and transfection efficiencies at the same time as preserving cell viability.

Electropermeabilization

Cells in suspension were centrifuged for 5 min at 120 *g* and resuspended in 100 µl of pulsing buffer (10 mM K_2HPO_4/KH_2PO_4 buffer, 1 mM $MgCl_2$, 250 mM sucrose, pH 7.4) at a 0.5×10^6 cells/ml cell density.

Penetration of propidium iodide (100 µM, in pulsing buffer) was used to monitor permeabilization. Six unipolar or bipolar rectangular pulses lasting 1 ms at various repetition frequencies were applied at 400 V amplitude (e.g. at a 800 V/cm voltage to distance ratio) at room temperature. Accordingly, cells that had already been pulsed were diluted in 1 ml of phosphate-buffered saline (PBS) buffer and analysed 10 min after the application of pulses by flow cytometry (Becton Dickinson FACScan; Becton-Dickinson Biosciences, Franklin Lakes, NJ, USA) to determine both the percentage of fluorescent cells (i.e. the percentage of permeabilized cells) and the level of fluorescence associated with this permeabilization (i.e. the efficiency of permeabilization). In all experiments, cells treated exactly the same way apart from being exposed to electric pulses were used as control cells. Cells were resuspended in 1 ml of buffer and analysed by flow cytometry, gating with the scatters (forward scatter and side scatter) to exclude debris. The excitation wavelength was 488 nm (argon laser) and the fluorescence of intracellular propidium iodide was collected in FL-2 channel (bandpass 585 ± 42 nm). A minimum of 5×10^3 events were acquired in list mode and analysed with Cellquest software (Becton Dickinson).

Cell viability

Viability was measured by quantifying the growth of cells over more than one generation after application of electric pulses (approximately 24 h) as previously described [37]. Briefly, cells were pulsed as above in absence of propidium iodide and kept for 10 min at room temperature, with the delay allowing the membrane to become impermeable again. They were then cultured on Petri dish with 2 ml of culture medium. Viability was measured by quantifying the number of cells over 24 h after electropulsation (more than one generation) by crystal violet staining.

Electrotransfection

Cells in suspension were centrifuged for 5 min at 120 *g* and resuspended in 100 µl of plasmid DNA containing pulsing buffer at a 0.5×10^6 cells/ml density. For each condition, 4 µg of pEGFP-C1 plasmid was used. As in the electropermeabilization protocol, six unipolar or bipolar rectangular pulses lasting 1 ms at various repetition frequencies were applied at a 400 V amplitude (800 V/cm voltage to electrode distance ratio) at room temperature. Cells were incubated for 10 min at room temperature and cultured in Petri dish with 2 ml of culture medium at 37 °C in a 5% CO_2 incubator. Twenty-four hours later, the cell monolayer was washed with PBS to remove all non-adherent cells. Cells were harvested by trypsinization, resuspended in 1 ml of PBS and analysed by flow cytometry to evaluate both the percentage of fluorescent cells (i.e. the percentage of GFP expressing cells) and the mean level of fluorescence associated with GFP expressing cells (i.e. the efficiency of gene expression). The excitation wavelength was 488 nm (argon laser) and the fluorescence of GFP was collected in FL-1 channel (bandpass 520 ± 42 nm). A minimum of 5×10^3 events were acquired in list mode and analysed with Cellquest software (Becton Dickinson).

DNA staining

Plasmid pEGFP-C1 was stained stoichiometrically with the DNA intercalating thiazole orange homodimer dye TOTO-1. The pEGFP-C1 plasmid was stained with TOTO-1 dye at a DNA concentration of 1 µg/µl for 60 min on ice. The TOTO-1 concentration was 2.3×10^{-4} M, yielding an average base pair to dye ratio of 5 [33]. TOTO-1 was obtained from Molecular Probes (Eugene, OR, USA).

Microscopy

For on line microscopic observations, an electropulsation chamber was designed using the electrodes in contact with a microscope glass coverslip chamber (Lab-Tek I system; Nunc I1, Dutsher SA, Brumath, France). The electrodes were connected to the voltage generator. The chamber was placed on the stage of an inverted digitized video microscope (DMIRB; Leica, Wetzlar, Germany). Cells were observed with a Leica ×100, 1.3 NA oil immersion objective. The wavelengths were selected using the Leica L4 filter block ($450 \text{ nm} \leq \lambda_{ex} \leq 490$ nm; dichromatic mirror pass band: $515 \text{ nm} \leq \lambda_{em} \leq 560$ nm) for the TOTO-1-labeled DNA. Images were recorded with the CELLscan System from Scanalytics (Billerica, MA, USA) equipped with a Photometrics cooled charge-coupled device camera (Princeton Research Instruments, Inc., Princeton, NJ, USA) [33]. This digitizing set up allowed a quantitative localized analysis of the fluorescence emission along the cell membrane. Plot histograms

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detected local increase above the background level outside of the cells. Two characteristic parameters were used: the peak intensity and the integral under the peak. Both were directly related to the number of fluorescent molecules locally present. The light haze contributed by fluorescent-labeled structures located above and below the plane of optimal focus was mathematically reassigned to its proper places origin (Exhaustive Photon Reassignment EPR software; Scanalytics) after accurate characterization of the blurring function of the optical system [33].

Statistical analysis

Errors bars represent the standard error of the mean. The statistical significance of differences between the means of unipolar and bipolar was evaluated by an unpaired Student's *t*-test. All statistics tests were two sided (NS, not significant; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$).

Results

We analysed the effect of electric pulses polarity and repetition frequency on membrane permeability, gene transfer and expression by affecting the DNA plasma membrane interaction. Cells were pulsed under electric field conditions of strength, number and duration that were known from previous studies to lead to efficient gene expression [37,39]. The use of the new square-wave pulse generator [38] made it possible to deliver repeated pulses at various frequencies with normal or reversed polarities (i.e. unipolar or bipolar pulses, respectively).

In the present study, we compared the effect of a train of six unipolar or bipolar rectangular electrical pulses lasting 1 ms delivered with a delay between pulses varying from 12 ms to 10 s (corresponding to a pulse repetition frequency of 76.9 Hz to 0.1 Hz, respectively).

Effect of pulse repetition frequency on membrane permeabilization

The results obtained regarding the uptake of propidium iodide into cells submitted to unipolar or bipolar electric pulses as a function of repetition frequency are shown in Figure 1. The percentage of permeabilized cells was not affected when frequency of pulse repetition was increased, regardless of the polarity used (unipolar or bipolar) (Figure 1A). Almost 80% of cells were propidium iodide positive (i.e. permeabilized).

The associated fluorescence intensity, related to the number of molecules incorporated into the electropermeabilized cells, was not dramatically affected. Indeed, it only slightly decreased above 1 Hz under unipolar conditions, whereas it did not significantly change under bipolar conditions (Figure 1B).

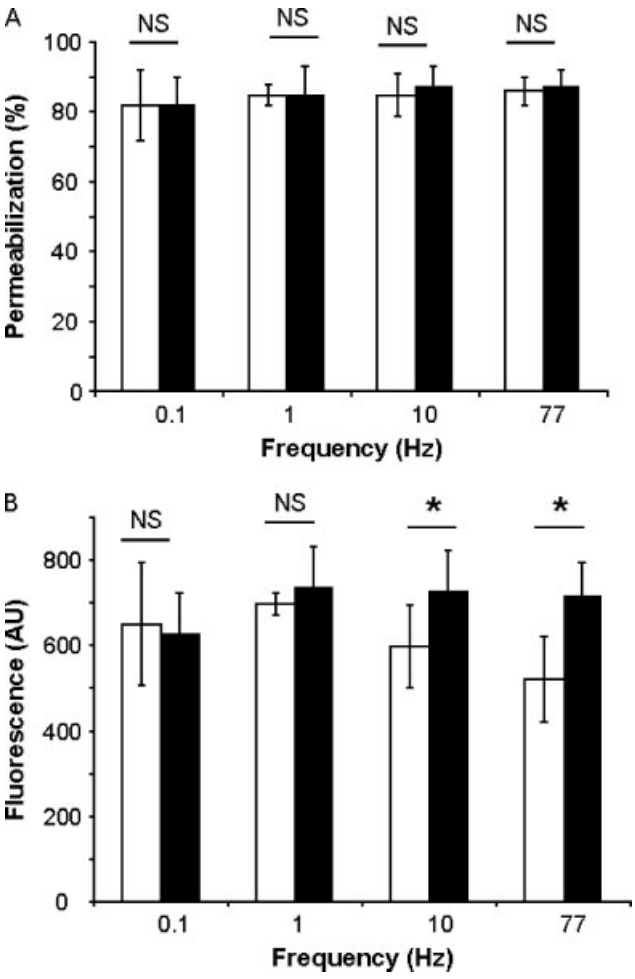

Figure 1. Effect of unipolar and bipolar pulse frequency on CHO cell permeabilization. (A) Percentage of permeabilized cells. (B) Mean fluorescence intensity associated with pulsed cells. Train of six pulses of 1 ms in duration at 400 V were applied as a unipolar (white symbol) and bipolar train (black symbol) to cells in pulsing buffer containing propidium iodide. Permeabilization was quantified by flow cytometry

Effect of pulse repetition frequency on cell viability

Cell viability was decreased when increasing the pulse repetition frequency, regardless of whether the pulses in the train were unipolar or bipolar (Figure 2). Under unipolar conditions, 60% of cells were viable at 0.1 Hz, although this percentage decreased to 25% of survival at 10 Hz to reach a plateau. The same profile was observed under bipolar conditions. No significant difference was observed between unipolar and bipolar conditions.

Effect of pulse repetition frequency on DNA/membrane interaction

Videomicroscopy at the single cell level offers direct access to the early events of DNA transfer across the electropermeabilized membrane. Images of DNA/membrane

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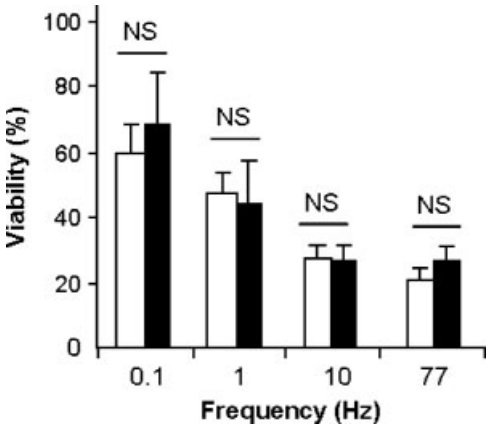

Figure 2. Effect of unipolar and bipolar pulse frequency on CHO cell viability. Train of six pulses of 1 ms in duration was applied at 400 V to cells. Cell survival was assessed by cristal violet staining 24 h after electropulsation. White symbols, unipolar conditions; black symbols, bipolar conditions

interactions were obtained by fluorescence microscopy with the TOTO-1 fluorescent-labeled plasmid DNA in the minutes after pulse application. As previously described [33], and as shown in Figure 3, DNA interacted as 'aggregates' only with the electropermeabilized part of the membrane facing the cathode under unipolar conditions. In bipolar conditions, DNA interacted on both sides of the cell surface facing the electrodes [37]. DNA does not enter the cell during electric pulses but it is 'trapped into' the membrane. Its presence inside the cytoplasm is only detected minutes after pulses delivery. Therefore, the interaction of plasmid DNA at the membrane level can be quantified. Moreover, as shown in Figure 3, the amount of DNA interacting with the membrane varied according to the pulse repetition frequency. The quantitative analysis of micrographs obtained under unipolar and bipolar conditions was performed as reported in Figure 4. Two distinct pieces of information were obtained: the percentage of positive cells (corresponding to the cells that DNA interacted with) and the fluorescence intensity (corresponding to the mean amount of DNA interacting with cells). Forty-seven percent of cells were positive under 0.1 Hz unipolar conditions. When increasing the frequency, this percentage increased by a factor of two to reach 92% at 10 Hz and decreased to 80% for higher frequencies. At the same time, a quantitative fluorescence analysis was performed to determine the quantity of DNA interacting with the permeabilized membrane. The mean fluorescence increased with increase in frequency up to a factor of four. However, under bipolar conditions, the percentage of cells interacting with DNA decreased from 78% to 40% (a factor of two) when the pulse repetition frequency was larger than 1 Hz. Under those bipolar conditions, the fluorescence slightly increased from 0.1 Hz to 10 Hz and then decreased with further increases in pulse repetition frequency.

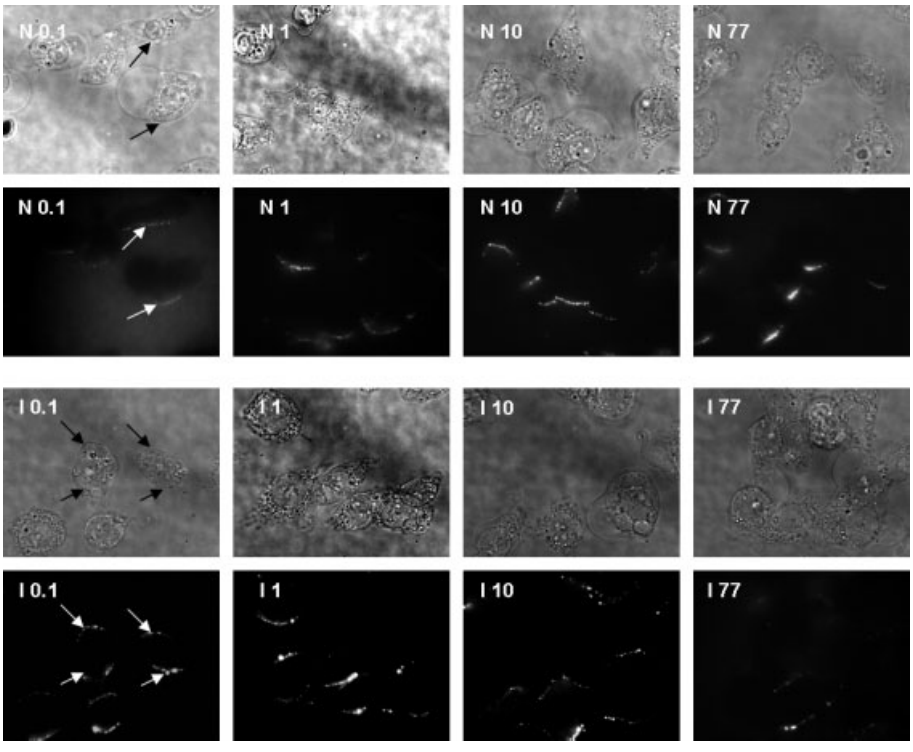

Figure 3. Effect of pulse polarities on DNA/membrane interaction. CHO cells were incubated in the presence of TOTO-1-labeled DNA (pEGFP-C1) in the pulsing buffer. Six pulses of 1 ms in duration at 400 V were applied to cells for unipolar and bipolar pulses sequences at a 0.5 Hz pulse repetition frequency. The time exposure of the camera was set at 1 s. Images of cells where DNA interacts with are given under phase contrast and fluorescence for different pulse frequencies (i.e. 0.1, 1, 10 and 77 Hz). N, normal conditions; I, inverted conditions. White arrows show DNA/membrane complexes, black arrows show the corresponding areas under phase contrast

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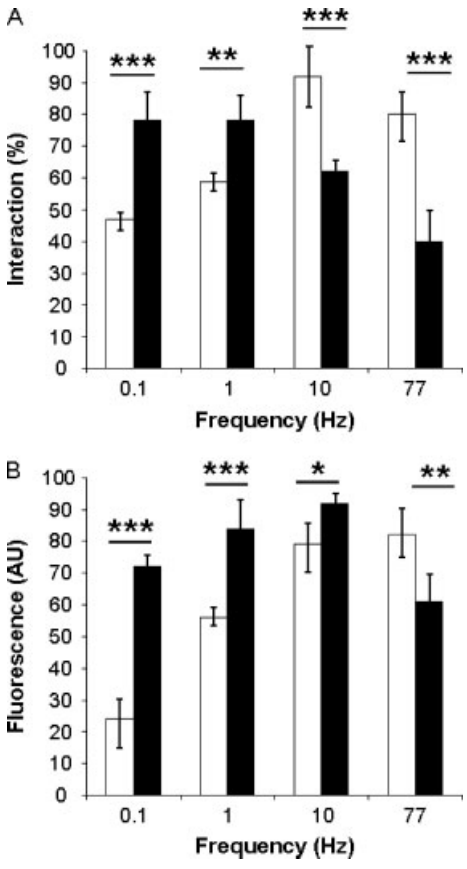

Figure 4. Effect of pulse polarities on DNA/membrane interaction. CHO cells were incubated in the presence of TOTO-1-labeled DNA (pEGFP-C1) in the pulsing buffer. Six pulses of 1 ms in duration at 400 V were applied to cells for unipolar and bipolar pulses sequences at a 0.5 Hz pulse repetition frequency. The time exposure of the camera was set at 1 s. Percentage of cells where DNA interacts with (A) as well as the mean fluorescence level of labeled DNA associated with their membrane (B) were quantified. White symbols, unipolar conditions; black symbols, bipolar conditions

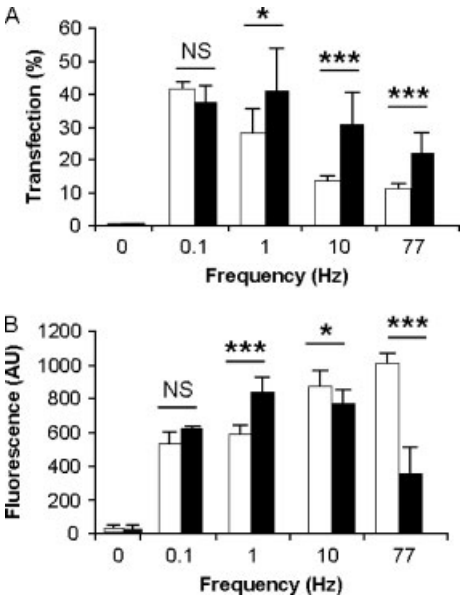

Figure 5. Effect of a train of unipolar and bipolar pulses with a different repetition frequency on CHO cell gene expression. (A) Percentage of electrotransfected cells. (B) Associated mean fluorescence level of GFP expressing cells. Six pulses of 1 ms in duration were applied at 400 V to cells in pulsing buffer containing a plasmid coding for the GFP reporter gene. GFP expression was detected 24 h after electric treatment by flow cytometry. White symbols, unipolar conditions; black symbols, bipolar conditions

Effect of pulse repetition frequency on gene expression

Gene expression was accessed by using a plasmid carrying the GFP gene. The results of GFP expression, measured 24 h after pulse application, in cells submitted to electric pulses of the same or reversed polarity as a function of repetition frequency are shown in Figure 5. The percentage of transfected cells was 40% at 0.1 Hz under unipolar conditions (Figure 5A). The increase of pulse repetition frequency significantly decreased the percentage of transfected cells to 10% at 77 Hz. The same profile was observed under bipolar conditions, with approximately 40% of transfected cells at 0.1 Hz and a slight but significant decrease to 25% at 77 Hz (Figure 5A).

The associated fluorescence intensity, related to the level of GFP expression in the electrotransfected cells, has a more complex behaviour (Figure 5B). With respect to a pulse repetition frequency larger then 1 Hz, the GFP expression was observed to increase up to two times under unipolar conditions (Figure 5B). Under bipolar conditions, however, pulse repetition frequency values of approximately 1 Hz appear to be optimal for the gene expression. Outside this apparent optimum (i.e. pulse repetition frequencies higher or lower than 1 Hz), GFP expression was decreased (Figure 5B).

Discussion

To be of use clinically, gene delivery methods have to be safe and efficient. For that, it is a necessity to be able to control each step of the DNA delivery process. Electroporation shows promise in that field because proofs of concept of its *in vivo* use and, more recently, of its first clinical trial have been published [12,19,40]. The method is safe because DNA is specifically delivered to the cells and organs in between the electrodes. However, compared to viral methods, it is less efficient because higher quantities of plasmid DNA are required. To better understand the mechanism involved in electro-mediated DNA transport across the cell membrane, and therefore to improve efficiency of the method, we designed experiments where the direction of the electric field was reversed between each consecutive electric pulse that was applied. The electric field was thus either delivered in a single direction (unipolar conditions), or its direction was reversed between consecutive pulses (bipolar conditions). The pulses were delivered (and the polarity changed in bipolar conditions) with different delays between pulses

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by varying pulse repetition frequencies, so that we were able to experimentally determine the characteristic time of DNA plasma membrane association and the consequences in gene expression. Electric pulse parameters (i.e. pulse intensity, number and duration) were chosen in a regime where both membrane permeabilization (Figure 1) and cell viability (Figure 2) were not significantly affected by the pulse polarities. Indeed, under these experimental conditions, 80% of cells were permeabilized irrespective of the pulse repetition frequency. The cell viability decreased with an increasing pulse repetition frequency, in agreement with previous studies [41].

Under unipolar conditions, increasing the pulse repetition frequency (i.e. decreasing the delay between electric pulses) directly enhances the amount of DNA interacting with electropermeabilized membrane (Figure 4B). This increase was directly correlated with an increase in gene expression (Figure 5B), showing a direct relationship between the two phenomena. However, as far as the percentage of cells is concerned, the data appear to be contradictory. It would be expected that an increase in the number of cells with DNA interacting with the membrane would have a higher number of transfected cells. However, as shown Figure 4A, even if the number of cells increased with pulse frequency up to 10 Hz, the number of cells that expressed the reporter gene decreased with pulse frequency (Figure 5A). These results are a direct illustration of the complexity of gene delivery phenomena. If DNA interaction with the membrane is a necessary condition to obtain gene expression, it is not a sufficient condition. One possible explanation to explain these data is the loss in cell viability. Cell viability was indeed shown to decrease by increasing the pulse repetition frequency (Figure 2) as a direct effect of electric field application. Moreover, under given pulses parameters, this can also be the result of a high number of DNA molecules interacting with the membrane and/or entering cells; the toxicity of DNA in electroporated cells has been previously shown to be concentration-dependent [42].

Under bipolar conditions, the effects of pulse repetition frequency are biphasic: (i) for frequencies below 1 Hz, the same effects as those observed under unipolar conditions were observed, but (ii) for frequencies above 1 Hz, the opposite effects were observed. Namely, under bipolar conditions, increasing the pulse repetition frequency (and decreasing the delay between pulses) and pulse inversion resulted in a reduction of the quantity of DNA interacting with the membrane (Figure 4B), which was also accompanied by a decrease in gene expression (Figure 5B), but still showing a direct correlation between the two phenomena. Moreover, in that case, there was a fair agreement between the percentage of cells with DNA interacting with the membrane (Figure 4A) and expressing GFP (Figure 5A) and the efficiencies (Figures 4B and 5B). All these data can be interpreted by considering that, under bipolar conditions, a short delay between pulses (i.e. high pulse repetition frequency) prevents the formation of a stable DNA/membrane complex by uncoupling DNA with the membrane, leading to a decrease in gene expression. The results obtained show that a delay of approximately 1 s, corresponding to the breaking point of the curve, is necessary for DNA to form this stable complex with the membrane. This delay indeed varies from 0.1 s to 1 s (i.e. 10 Hz and 1 Hz as shown in Figure 4B and Figures 4A, 5A and 5B, respectively).

From a mechanism point of view, the present stusy provides new insight into the mechanisms of DNA electrotransfer by addressing one of the key steps of the process, namely DNA/membrane complex formation. It demonstrates the existence of two classes of DNA/membrane interactions: (i) a metastable DNA/membrane complex from which the DNA can leave and return to external medium as a result of electrophoretic stress (caused by the reversal of the electric field direction within less than 1 s after the previous pulse) and (ii) a stable DNA/membrane complex, where the plasmid cannot be removed even by applying electric pulses of reversed polarity. Only DNA belonging to the second class may be effective for gene expression. This suggest the formation of anchoring points/structures within the cell surface, allowing DNA not to be desorbed to form the stable complex with the membrane. DNA takes an order of seconds to become trapped in them. Electro-mediated gene transfer and expression can therefore be described as a complex process with different successive steps, with time scales that can be considered to range from μs to days (Table 1).

Table 1. Kinetics of the different steps involved in DNA electro-mediated delivery into cells

Time scale	Steps involved in DNA electro-mediated delivery	Reference
Microsecond*	Plasma membrane permeabilization	Kinosita and Tsong (1979) Neumann *et al*. [2]
Millisecond*	Electrophoretic migration of DNA towards the membrane DNA/membrane complex formation	Wolf *et al*. [29] Hristova *et al*. [32] Golzio *et al*. [33]
Second	Conversion of the metastable form of the DNA/membrane complex to a stable one	Present study
Minute	DNA translocation/diffusion across the membrane	Eynard *et al*. [35]
Hour	DNA migration towards the nucleus	Bertling *et al*. (J Biochem Biophys Methods, 1987)
Day	Gene expression	Rols and Teissié (Biophys J, 1998) Kubiniec *et al*. [39]

*Electric field applied.

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These data highlight the role of plasmid DNA migration towards the membrane and the importance of designing pulse parameters for electric field-mediated gene delivery applications. It is possible that, when keeping all the pulse parameters constant, pulse duration and number, as well as pulse intensity, alter gene expression. Gene expression will be improved by increasing the number of interaction sites between plasmid DNA and the cell membrane. This can be carried out either by applying electric pulses of reversed polarity or by changing electric field direction [38]. However, the delay between pulses (i.e. pulse repetition frequency) will have to be carefully chosen to insure DNA/membrane complex formation. Optimization of the electric pulses parameters (direction, frequency) has already demonstrated an improvement of the electroporation method in the field of its clinical applications. For example, in electrochemotherapy experiments, reversing the pulse polarity and changing the electric field orientation was associated with an improvement of tumor eradication [43,44]. Because a train of electric pulses with repetition frequency of 1 Hz is usually applied to the tumors, each pulse in the train excites the underlying nerves and provokes muscle contractions. Therefore, for patients involved in electrochemotherapy, the use of pulses with a repetition frequency higher than the frequency of tetanic contraction would represent a reduced number of muscle contractions and the associated unpleasant sensations [45]. Indeed, this is what is presently carried out, as reported in a recent clinical study [46] that supports the results of the ESOPE study [47] where 5 kHz repetition frequency of electric pulses were used. Electrochemotherapy also gives excellent results in veterinary medicine [48]. A very recent study performed on dogs confirms that electroconvulsive threapy comprises an easy, effective and safe local treatment for tumors. It represents an alternative treatment to surgery, specifically for smaller nodules in which a complete response with long duration can be obtained after only one treatment session, or when the nodule is unresectable because of the location [49]. The possibilities for the clinical use of pulses with a high repetition frequency in electro-mediated gene transfer and expression are now considered. However, as far as the DNA electrotransfer process is concerned, the data obtained in the present study demonstrate that the frequency of the pulses when applied under inverted polarities should be carefully adjusted to lead to efficient gene expression. Moreover, as shown in the present study, the correlation between the percentage of transfected cells and the efficiency of expression (i.e. level of expression) can be highly affected by electric field parameters, depending on the way that the electric pulses (polarity, frequency) are applied and most likely also on the amount of electrotransfered plasmid DNA. The DNA concentration has to be adjusted to lead to a high expression level without any severe cell damage. Once again, the DNA concentration will have to be adjusted to each cell type and tissue. For gene therapy applications, one therefore has to optimize the electrotransfer method to favor either the percentage of positive cells and/or the amount of gene expression. Accordingly, a reporter gene such as GFP is a convenient method that can be used *in vivo* in combination with more global detection such as Luciferase activity [50]. This will be particularly important in tissues where new constraints are present, such as the high cell density and the environment of cells in specific organs. One can expect that, in the near future, by improving our knowledge of all the steps involved in DNA electrotransfer, which comprises a process that is more complex than simply pushing DNA through electropores, we will be able to define new protocols (based on a refinement of electric pulses parameters) that will lead not only to a safer method, but also one of the more efficient gene therapy protocols.

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