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Mechanisms involved in gene electrotransfer using high- and low-voltage pulses — An in vitro study

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ABSTRACT

Gene electrotransfer is an established method for gene delivery which uses high-voltage pulses to increase permeability of cell membrane and thus enables transfer of genes. Currently, majority of research is focused on improving in vivo transfection efficiency, while mechanisms involved in gene electrotransfer are not completely understood.

In this paper we analyze the mechanisms of gene electrotransfer by using combinations of high-voltage (HV) and low-voltage pulses (LV) in vitro. We applied different combinations of HV and LV pulses to CHO cells and determined the transfection efficiency. We obtained that short HV pulses alone were sufficient to deliver DNA into cells for optimal plasmid concentrations and that LV pulse did not increase transfection efficiency, in contrast to reported studies in vivo. However, for sub-optimal plasmid concentrations combining HV and LV pulses increased transfection rate. Our results suggest that low-voltage pulses increase transfection in conditions where plasmid concentration is low, typically in vivo where mobility of DNA is limited by the extracellular matrix. LV pulses provide additional electrophoretic force which drags DNA toward the cell membrane and consequently increase transfection efficiency, while for sufficiently high concentrations of the plasmid (usually used in vitro) electrophoretic LV pulses do not have an important role.

1. Introduction

Gene therapy is a relatively new method for treatment of cancer and other diseases [1–4] and for DNA vaccination [5–7]. Viruses are the most efficient vectors for gene transfer to target cell however, due to the risk of insertional mutagenesis, immune response and potential replication such vectors still need improvement before they can be safely used in clinics [8,9]. Viral vectors are increasingly being replaced by non-viral plasmid DNA vectors. Since the efficiency of direct injection of DNA is relatively low, methods for improved DNA administrations using cationic lipids and polymers are being developed [3,4,10]. Nevertheless, the toxicity of these methods is still an obstacle to their application in gene therapy [2]. Another promising non-viral gene delivery method, which is gaining importance for therapeutic use is gene electrotransfer. It involves the injection of plasmid DNA followed by the electric pulse application that enhance gene transfer into the cells and tissues [11–13].

Gene electrotransfer is caused by temporary increase in membrane permeability, however the exact molecular mechanisms of DNA transport across the membrane are still not known [13–15]. Different mechanisms that explain DNA entry to the cytosol have been proposed. Among them, free diffusion of DNA through long-lived electropores [11,16] and diffusion coupled to the water flow caused by exposure of the cell to electric field and related cell swelling [17,18]. Several following studies showed that the process is more complex than free diffusion and that several steps are crucial for efficient DNA electrotransfer, where one of the key steps is interaction of a DNA molecule with the cell membrane [19,20]. The only direct visualization of the process was done by Golzio et al. where they observed DNA accumulation and formation of DNA–membrane complexes on the cathodic side of the cell membrane [20–23].

It was also shown that properties of the buffer (osmolarity and ionic composition) used during and after electropermeabilization affect gene electrotransfer efficiency [19,24–27]. Also electric field stimulated endocytosis was suggested as a mechanism for gene electrotransfer [28–30].

As most experiments of gene electrotransfer were performed using long millisecond pulses or exponentially decaying pulses, electrophoretic movement of DNA during the pulse was proposed as an important mechanism for more efficient gene electrotransfer. In a single in vitro study of Sukharev et al., DNA transfection was enhanced when a combination of short high-voltage (HV) and long low-voltage (LV) pulses was applied [31,32]. It was suggested that HV pulses are crucial for permeabilization, while LV pulses electrophoretically drag DNA to or/and into the cell. In vivo similar results were obtained with combinations of HV and LV pulses in muscle [33–35] and in skin [36].

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However, the role of electrophoresis in in vivo gene electrotransfer was questioned in the recent study by Liu et al. since reversing the polarity of electric pulses did not affect gene expression [37].

In our present study, we investigated the effect of HV and LV pulses on the efficiency of in vitro gene electrotransfer in order to determine their role in the processes involved in electric field mediated transfer of genes. In order to compare gene electrotransfer in in vivo and in vitro conditions we used different plasmid concentrations from optimal (typically used in vitro) to sub-optimal, which are usually present in in vivo conditions. We analyze the role of electrophoresis for efficient gene electrotransfer and compare our results with other reported studies.

2. Theory

2.1. The induced transmembrane potential

Even though the exact physicochemical mechanisms of cell electroporation and related gene electrotransfer are not fully understood it is generally accepted that one of the key parameters for successful electroporation and increased membrane permeability is the induced transmembrane voltage. This voltage is generated by an external electric field due to the difference in the electric properties of the membrane, cytoplasm and the external medium. If the induced transmembrane voltage is above a certain critical value increased permeability of the membrane is observed [15,38]. The transmembrane voltage induces strong electric field inside the cell membrane which is crucial for destabilization of the membrane and formation of structural changes (pores) inside the lipid bilayer, which consequently increases membrane permeability for ions and molecules. It was shown by several studies [38–40] that the process of gene electrotransfer is similarly as "classical" electroporation a threshold process which occurs only above a certain critical electric field $E_0 > E_c$, where E_c is in range of few hundreds V/cm depending on the length and number of pulses. Wolf et al. reported that the observed threshold value of the field strength for gene electrotransfer is the same as the one needed to induce permeabilization. The extent of transfection increases sharply when higher field intensities are used [14,39,41]. Therefore electric field induced destabilization of the cell membrane is a critical step of gene electrotransfer.

However, it was further shown that the process of gene electrotransfer is more complex than simple diffusion of DNA molecules through membrane pores [20,21,38]. Several authors showed that crucial step in DNA delivery is the interaction (adsorption) of a DNA molecule with the cell membrane which is followed by DNA translocation. How a DNA molecule crosses the membrane has not been directly visualized and there is yet no description on the molecular level.

2.2. Electrophoresis

Electrophoresis is another mechanism which was shown to be important for the delivery of DNA molecules into cells by electric pulses [32]. The electrophoretic driving force acts on the negatively charged DNA molecules and drags it toward the cathodic side of the membrane, and is unlike diffusion present only during the pulse. For this reason protocols for gene transfection most often use much longer pulses (a few to tens of milliseconds) or additional long low-voltage pulses compared to relatively short electric pulses used for uptake of smaller molecules. Electrophoretic force depends on the local electric field (E_{loc}) and on the effective charge of a given molecule (e_{eff}): $F = e_{eff} E_{loc}$, where the effective charge depends on the ionic strength of the solution and length of the plasmid. The velocity of the molecular movement and the final distance traveled due to electrophoresis depend also on the mobility of the molecule, friction forces and duration of the electric pulses as well as on other forces involved during the DNA interaction with the cell membrane, therefore theoretical quantification of electrophoresis during gene electrotransfer is very complex.

Currently both theoretical models [42] and experimental quantification of DNA electrophoresis were studied on model gel systems [43] and ex-vivo on tumors [44,45], however full quantitative description of this process in different tissues is very complex and is still lacking [46].

3. Materials and methods

3.1. Cells

Chinese hamster ovary CHO cells (European Collection of Cell Cultures) were grown as a monolayer culture in a nutrient mixture F12 HAM (Gibco) supplemented with 2 mM glutamine, 10% fetal bovine serum (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) and antibiotics crystacylin and gentamycin, at 37 °C in a humidified 5% CO_2 atmosphere in the incubator.

3.2. Electrotransfection protocol

To generate electric pulses high-voltage generator Cliniporator™ was used which enabled use of different combinations of high- (HV) and low-voltage (LV) pulse. Two types of pulsing protocols were used as shown in Fig. 1, and for both protocols either only HV pulses, only LV pulses or combination of both pulses (HV+LV) was used.

A pair of parallel wire electrodes was used with the distance d between the electrodes being 2 mm (see Fig. 2). In pulsing protocols we used: a) one HV pulse of E = 1.5 kV/cm (applied voltage U = 300 V) with duration of 100 μs and in protocol b) four HV pulses of 200 μs duration with two amplitudes: E = 0.8 kV/cm (U = 160 V) and E = 1.0 kV/cm (U = 200 V). For both pulsing protocols (a and b) the amplitude of LV pulse was set to E = 0.075 kV/cm (U = 15 V) the value that is below the observed threshold for membrane permeabilization.

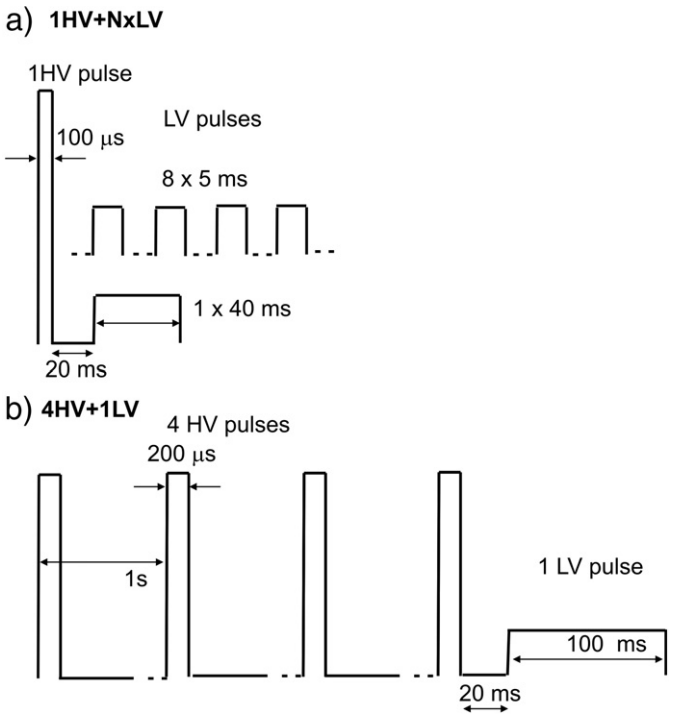

Fig. 1. Schematic representation of different pulsing protocols. In protocols a) either 1HV pulse, single or several LV pulses of total duration of 40 ms or combination 1HV+8×LV was used. In protocols b) we used either 4HV pulses, only 1LV pulse or combination 4HV+1LV, the duration of LV pulse was 100 ms.

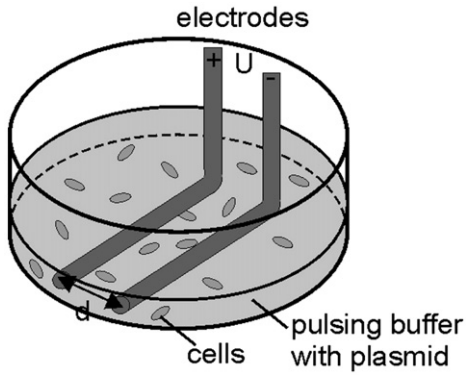

Fig. 2. Schematic representation of electrode configuration. Cells between the electrodes were exposed to approximately homogeneous applied electric field $E=U/d$, where U is applied voltage and d distance between the electrodes.

3.3. Experiments

Plasmid DNA pEGFP-N_1 purified with Endofree Plasmid mega kit (Qiagen) coding for GFP (green fluorescent protein) was used to analyze efficiency of gene electrotransfer. The experiments were performed on plated cells seeded in 24 multiwell plates. Initial concentration of plated cells was 5×10^4 cells per well. On the day of experiment the growth media was removed and replaced with the mixture of plasmid DNA and isoosmolar pulsing buffer (pH 7.4, 10 mM Na_2HPO_4/NaH_2PO_4, 1 mM $MgCl_2$ and 250 mM sucrose). Final concentration of plasmid was 10 μg/ml, except in the experiments where three different concentrations of plasmid were used: sub-optimal concentrations 2 μg/ml and 5 μg/ml, and optimal concentration 10 μg/ml. The optimal concentration was determined experimentally as the concentration above which the increase in concentration of the plasmid did not further increase the transfection efficiency, i.e. no increase in transfection was observed for 40 μg/ml.

We incubated cells in the pulsing buffer for 2–3 min at room temperature (22 °C). Then different combinations of high-voltage and low-voltage pulses were delivered (Fig. 1). Treated cells were incubated for 5 min at 37 °C to allow cell membrane resealing and then grown for 24 h in cell culture medium at 37 °C in a humidified 5% CO_2 atmosphere in the incubator to allow GFP expression.

Efficiency of transfection was determined by fluorescent microscopy (Zeiss 200, Axiovert, ZR Germany) with excitation light at 445 nm generated with a monochromator system (PolyChrome IV, Visitron, Germany) and emission was detected at 488 nm. The images (see Fig. 3) were recorded using imaging system (MetaMorph imaging system, Visitron, ZR Germany) and at least five phase contrast and five fluorescence images were acquired in the area between the electrodes (see Fig. 2) at 20× objective magnification per each parameter. The cells were counted manually and the relative transfection efficiency was determined by the ratio between the number of green fluorescent cells (successfully transfected) and the total number of cells counted under the phase contrast. The cell survival was obtained from phase contrast images as the ratio between the number of viable cells in the treated sample and the number of viable cells in the control sample. At least three independent experiments were performed for each parameter and results are presented as a mean values ± standard deviation.

4. Results

In this study we analyzed the effect high-voltage (HV) and low-voltage pulses (LV) on gene electrotransfer in vitro. We determined the percentage of transfected cells and cell viability using plasmid coding for GFP protein. In the first part of our study we analyzed different combinations of HV and LV pulses for pulsing protocols a) and b) as presented in Fig. 1 for optimal plasmid concentration 10 μg/ml (no increase in transfection was observed for higher concentrations). In the second part of our study we investigated the role of HV and LV pulses at sub-optimal (2 μg/ml and 5 μg/ml) plasmid concentrations.

4.1. The effect of HV and LV pulses on the transfection efficiency at optimal plasmid concentrations

In Fig. 4 the transfection efficiency is presented for pulsing protocols a) (see Fig. 1a), where single HV pulse in combination with 1LV pulse 40 ms long or 8LV pulses 5 ms long were applied. In general LV pulses did not contribute to gene transfection, either when using single LV pulse or several LV pulses for these pulse parameters. The cell survival was around 80% for all the parameters tested (results not shown). With only LV pulse no transfection was obtained. It can be seen that for this pulsing protocol even a single 100 μs HV pulse was sufficient to obtain transfection efficiency of around 17%.

We further investigated the role of HV and LV pulses in gene electrotransfer by applying pulsing protocol b) (see Fig. 1b). Instead of using a single HV pulse of U = 300 V (E_{HV} = 1.5 kV/cm) as used in protocol a), we applied several HV pulses of either E_{HV} = 0.8 kV/cm (U = 160 V) or E_{HV} = 1 kV/cm (U = 200 V) followed by a single 100 ms long LV pulse E_{LV} = 0.075 kV/cm (U = 15 V). In Fig. 5 transfection efficiency for different combinations of 4HV and 1LV pulses is compared. Similarly as in Fig. 4 with only LV pulse no transfection was obtained. The high-voltage pulses alone lead to substantial transfection efficiency of 15% at E_{HV} = 0.8 kV/cm and 21% at E_{HV} = 1 kV/cm. Interestingly, also for this pulsing protocol the transfection efficiency was not affected significantly by addition of LV pulse compared to using only HV pulses.

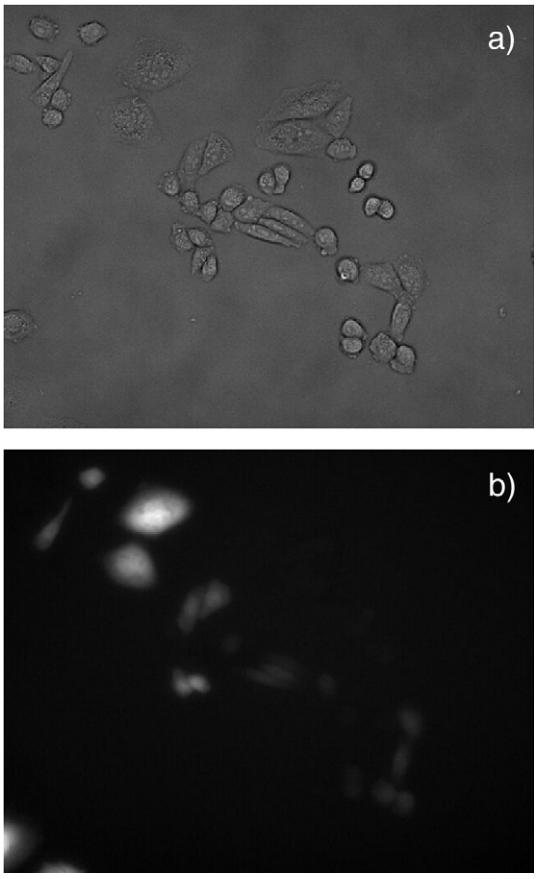

Fig. 3. Gene electrotransfer of plasmid DNA in vitro to CHO cells 24 h after pulse application. a) phase contrast image of treated cells b) fluorescent image of the same cells expressing GFP protein (white). Intensity of fluorescence correlates with expression of GFP.

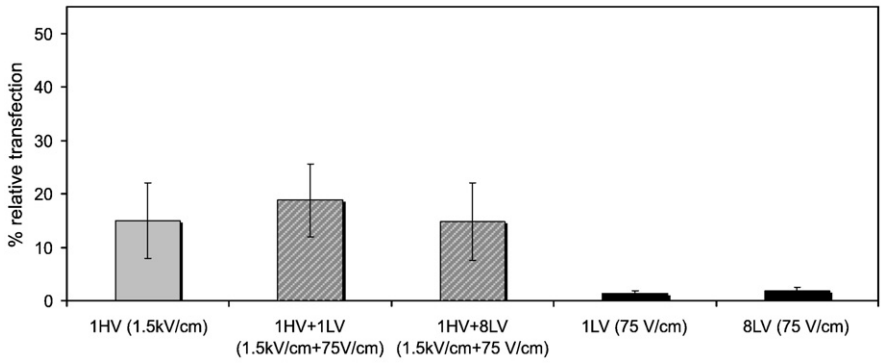

Fig. 4. Relative transfection efficiency for protocol a) (see Fig. 1a) with combinations of 1HV pulse of E = 1.5 kV/cm (300 V) and duration of 100 μs and either 1LV pulse with duration 40 ms or 8LV pulses 5 ms long were applied, E_{LV} = 0.075 kV/cm (15 V). Results are presented as a mean and vertical bars represent standard deviation.

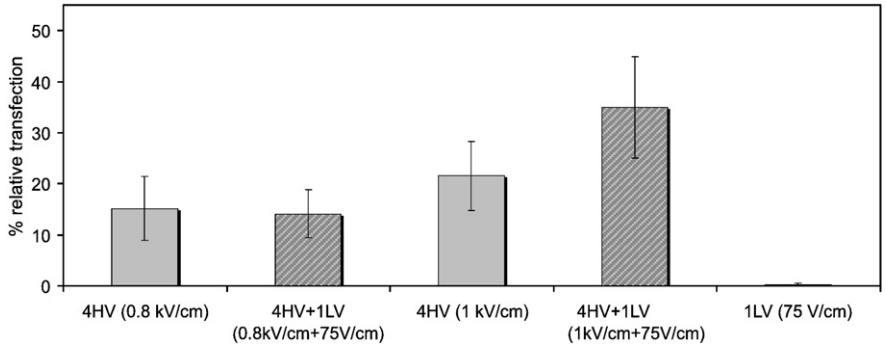

Fig. 5. Relative transfection efficiency for protocol b) (see Fig. 1b) with combinations of 4HV pulses, 200 μs duration of two amplitudes: 160 V and 200 V (E = 0.8 kV/cm and E = 1.0 kV/cm), repetition frequency 1 Hz, and 1LV pulse 15 V (E = 0.075 kV/cm), 100 ms duration. Results are presented as a mean and vertical bars represent standard deviation.

At E_{HV} = 0.8 kV/cm no effect of LV pulse was observed, while at higher amplitude of HV pulses (E_{HV} = 1 kV/cm) there was an increase in transfection efficiency for HV+LV pulses compared to only HV pulses, however it was statistically not significant (paired t-test, p<0.05). To demonstrate this more clearly we additionally calculated the difference in the transfection efficiency (TE) between HV and LV and only HV pulses for each experiment i: $\Delta_i\% = TE_{HV+LV} - TE_{HV}$ as shown in Table 1.

The differences shown in Table 1 further demonstrate that results obtained with 4HV+1LV pulses are not statistically different from the results obtained with 4HV pulses alone.

Since we obtained for both pulsing protocols (a and b) that HV pulses alone are crucial for efficient transfection for optimal plasmid concentration we investigated the field dependence of transfection efficiency for only 4HV pulses as defined in protocol b). In Fig. 6 we present the results of the effect of different pulse amplitudes on cell survival and gene transfection for 4HV pulses (4×200 μs, 1 Hz repetition frequency). It can be seen that gene electrotransfer is a threshold phenomena with E_c = 0.3 kV/cm. This critical field can be understood as a phenomenological threshold at which permeabilization of the membrane is sufficient to allow uptake of DNA molecules into the cell. We obtained gradual increase in the transfection efficiency with increasing field strength with 30% of transfected cells at 1 kV/cm and around 40% at 1.4 kV/cm. Cell viability decreased from 95% at 0.3 kV/cm to around 60% at the highest field strength.

Table 1
The difference in the transfection efficiency (TE) between 4HV+1LV and only 4HV pulses for each experiment i: $\Delta_i\% = TE_{HV+LV} - TE_{HV}$ at optimal plasmid concentration

$\Delta\% = TE_{HV+LV} - TE_{HV}$	EHV = 0.8 kV/cm	EHV = 1 kV/cm
Average Δ ± std	0.6 ± 6.9	13.4 ± 13

From this the average difference Δ and standard deviation of the differences were obtained.

4.2. The analysis of the role of HV and LV pulses at sub-optimal plasmid concentrations

In order to determine the effect of HV and LV pulsing protocols on the transfection efficiency at sub-optimal plasmid concentrations we reduced plasmid concentration from 10 μg/ml (optimal concentration), used for experiments presented in previous section (Figs. 4 and 5), to sub-optimal concentrations 5 μg/ml and 2 μg/ml. In the experiments with sub-optimal plasmid concentrations combinations of 4HV pulses with E_{HV} = 1.0 kV/cm (U = 200 V) and 1LV pulse E_{LV} = 0.075 kV/cm (U = 15 V), 100 ms duration were used (the same pulsing protocol as for the results presented in Fig. 5).

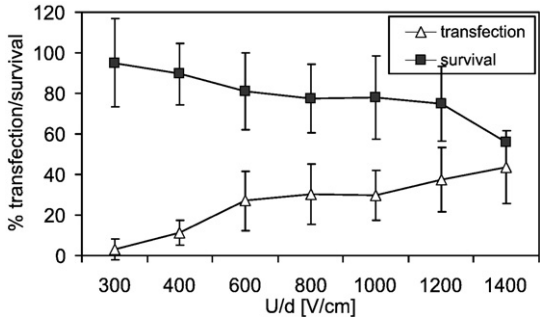

Fig. 6. Relative transfection efficiency (triangles) and cell survival (squares) for 4HV electric pulses 4×200 μs with repetition frequency 1 Hz.

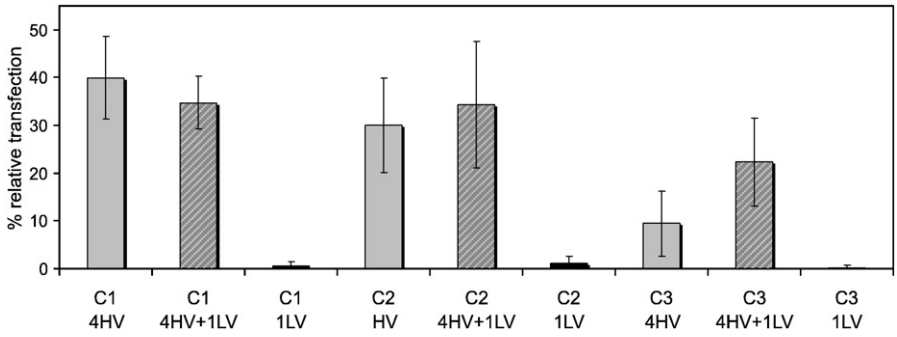

Fig. 7. Relative transfection efficiency for protocol b) (Fig. 1b) with different plasmid concentrations (C1 = 10 μg/ml, C2 = 5 and C3 = 2 μg/ml. Combinations of 4HV pulses, 200 μs duration pulse amplitudes E_{HV} = 1.0 kV/cm (200 V), repetition frequency 1 Hz and 1LV pulse E_{LV} = 0.075 kV/cm (15 V), 100 ms duration were used. Results are presented as a mean and vertical bars represent standard deviation.

As seen in Fig. 7 for sub-optimal plasmid concentration we obtained a significant increase in transfection efficiency if combination of HV and LV pulses was used compared to the application of only HV pulses. For optimal plasmid concentration (10 μg/ml) the results were similar as in our previous experiments shown in Fig. 5, where LV pulse did not contribute significantly to gene expression. Cell survival was around 80% for HV or HV + LV pulses for all plasmid concentrations (results not shown).

Only at the lowest plasmid concentration C3 = 2 μg/ml we obtained statistically significant effect when using combination of 4HV + 1LV pulses (paired *t*-test, p = 0.01). In order to present this effect more clearly we additionally calculated the difference in the transfection efficiency (TE) between 4HV + 1LV and only 4HV pulses for each experiment i: $\Delta_i\% = TE_{HV+LV} - TE_{HV}$ and calculated the average difference Δ and standard deviation of the differences as shown in Table 2 for different plasmid concentrations.

5. Discussion and conclusions

Gene electrotransfer is an efficient method for introducing genes into cells in vitro and in vivo even though the mechanisms of gene electrotransfer are not completely understood. Several studies showed that the process is more complex than pure diffusion through membrane pores and that several steps are involved in DNA electrotransfer: i) interaction of a DNA molecule with the cell membrane, ii) translocation across the cell membrane, iii) transport from the cytoplasm into the nucleus and iv) expression of the gene [14,20,21]. Furthermore, different studies which used high-voltage (HV) pulses followed by milliseconds long low-voltage pulses (LV) showed increased transfection efficiency for this specific combination. They suggested that HV pulses destabilize the cell membrane and induce pore formation in the cell membrane whereas LV pulses produce electrophoretic force which drives negatively charged DNA molecule into the cell/across cell membrane [32–35] and actively adds to pore formation which is the result of mechanical interaction between the pores and DNA [32].

The aim of our study was to analyze the effect of HV and LV pulses on efficiency of in vitro gene electrotransfer in order to determine their specific role in process of electric field mediated gene transfer. We used different plasmid concentrations: optimal – typically used in in vitro studies and sub-optimal – typically present in in vivo conditions.

We analyzed effect of different pulsing combinations with only HV pulses, only LV pulses (LV) or HV pulses (HV) followed by LV pulses (HV + LV) on the transfection efficiency in vitro on CHO cells. For our experimental conditions we obtained that transfection is obtained only at electric fields above 0.3 kV/cm (Fig. 6). Therefore HV pulses are crucial for increasing permeability of the cell membrane and consequentially critical also for gene electrotransfer as already observed in several other studies [34,35,39,41,47].

Our results also show that HV pulses alone are sufficient to allow transport of DNA into the cell (see Figs. 4 and 5). Even if a relatively short single pulse (1.5 kV/cm, 100 μs duration) was used as a HV pulse a single or several LV pulses did not increase transfection efficiency as it can be seen from Fig. 4. Our observations are in agreement with in vivo study by Lucas and Heller where they obtained that also short pulses alone are capable of delivering plasmid DNA into the cell [48]. Slight increase in transfection with additional LV pulses was observed only for protocol b) with four HV pulses 1 kV/cm followed by one LV long pulse where increase from 20% to approximately 33% relative transfection was obtained but this difference was not statistically significant (see Table 1).

Altogether, the results presented in Figs. 4 and 5 show that at optimal plasmid concentration, even short HV pulses alone are sufficient to obtain gene electrotransfer in vitro and that LV pulses do not contribute significantly to the transfection efficiency. Since our results contradicted the reported results in vitro and in vivo where using combinations of HV and LV pulses increased transfection efficiency [32–35], we hypothesized that this discrepancy is due to the low local concentration of plasmid DNA in vivo which limits the access of DNA to the cell membrane. To check this hypothesis we further performed experiments with lower, sub-optimal, plasmid concentration in order to simulate more realistically the in vivo conditions.

For sub-optimal concentration (2 μg/ml) we obtained that there is indeed a significant increase in transfection efficiency for combination of HV and LV pulses compared to application of only HV pulses (Fig. 7). Our results therefore suggest that LV pulses do not increase transfection efficiency for our optimal plasmid concentrations in vitro, however in conditions where plasmid concentration is a limiting factor (typically in vivo) electrophoretic force of LV pulses significantly increase transfection efficiency. This is in agreement with studies by Bureau et al. and Satkauskas et al. [33–35] where LV pulses markedly increased transfection in vivo conditions. In vivo we can expect that local plasmid concentrations are sub-optimal due to limited plasmid mobility in the tissue and availability of plasmid DNA at the cell membrane level [43–46,49].

Our results obtained for optimal plasmid concentrations are in apparent contradiction to the only similar in vitro studies by Sukharev et al. and Klenchin et al. [31,32] where they obtained increase in transfection rate for combinations of HV and LV pulses. However, the

Table 2
The difference in the transfection efficiency (TE) between 4HV + 1LV and only 4HV pulses for each experiment i: $\Delta_i\% = TE_{HV+LV} - TE_{HV}$ at sub-optimal plasmid concentration

$\Delta\% = TE_{HV+LV} - TE_{HV}$	C1 = 10 μg/ml	C2 = 5 μg/ml	C3 = 2 μg/ml
Average Δ ± std	−5.2 ± 10.2	4.4 ± 7.4	12.9 ± 4.8

From this the average difference Δ and standard deviation of the differences were obtained.

experimental conditions in their study cannot be directly compared to our results since they used cells in a suspension while we used plated cells. We can assume that in their experimental conditions the effective number of DNA molecules per cell was reduced compared to our conditions (plated cells) even though similar concentrations of plasmid (10–30 μg/ml) were used. Namely, in a cell suspension the concentrations of cells are much higher compared to plated cells and therefore the number of DNA molecules per cell is reduced for similar plasmid concentration. Since the minimal number of DNA molecules per cell is crucial for successful gene transfer we can therefore presume that in the study of Sukharev et al. they in fact used sub-optimal plasmid concentration, which consequently might explain the additive effect of LV pulse observed in their study.

Based on our results in vitro and different reported results in vivo [33–35] we can therefore explain the observed effect of HV and LV pulses in the following way. We obtained for different pulsing protocols that LV pulses do not significantly increase the transfection (see Figs. 5 and 6) for our optimal plasmid concentration of 10 μg/ml, which demonstrates that HV pulses alone are sufficient for efficient transfection. This suggest that HV pulses could provide electrophoretic force large enough to obtain contact with the cell membrane and/or insertion of the DNA into the phospholipid bilayer, since at optimal plasmid concentrations there are enough DNA molecules in close proximity to the cell membrane. The fact that relatively short HV pulses alone enable gene electrotransfer also suggest that DNA translocation is a slow process [20,50] which does not require electrophoretic force and that after the contact or/and insertion in the cell membrane DNA presumably enters by other mechanisms such as entropic and diffusion forces [20,51], or by a process similar to electrostimulated endocytosis [22,25,28–30,50]. This however has to be further examined in future studies.

Our hypothesis is that at sub-optimal plasmid concentrations the number of DNA molecules in close proximity of the membrane is small, while low mobility in tissues limits the access of DNA molecules to the cell membrane. Therefore in in vivo conditions LV pulses are crucial for efficient transfection as they provide electrophoretic force needed to drag negatively charged DNA molecules toward the cell membrane. Our explanation is also in agreement with conclusions of Golzio et al. [20,50] that crucial step in gene electrotransfer is the interaction of the DNA molecule with the cell membrane, as well as with the experimental and theoretical studies of Zaharoff et al. that long pulses are important for electrophoretic movement of DNA in gels [43] and for improving interstitial transport of DNA during gene delivery in tissues [43,44,46].

To conclude, in our study we demonstrated that short HV pulses are not only crucial for efficient permeabilization of the cell membrane which enables transfection, but are by themselves sufficient to successfully deliver DNA into cells at optimal plasmid concentrations. Therefore it is in general difficult to separate the role of HV pulses as being only permeabilizing and LV pulses as being electrophoretic since both effects can be present during HV pulses. We further suggest that electrophoretic force of LV pulses is crucial in in vivo condition where sub-optimal plasmid concentration is the limiting factor for efficient transfection. At low local DNA concentrations electrophoretic force contributes to better interaction of the DNA with the cell membrane and is therefore crucial for efficient gene electrotransfer in vivo.

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