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The role of electrically stimulated endocytosis in gene electrotransfer

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ABSTRACT

Gene electrotransfer is an established method for transfer of genes into cells, however, the mechanism of transfer of DNA across the cell membrane is still not known. Some studies suggest that DNA is translocated through membrane pores while others propose that DNA enters the cell via electro-endocytosis, but no direct observation was performed. In this paper we investigated the second hypothesis. Cells were stained with membrane dye FM 1-43FX, which is used for observation of endocytosis, and then exposed to electric pulses. We analyzed if endocytosis was stimulated by applying electric pulses with intensities below and above the threshold value for gene electrotransfer. No increase in endocytosis from 20 min or even up to 2 h after the pulse delivery was observed, regardless of the electric field strength. These observations do not correlate with electrotransfer efficiency, which increases with field strength and is observed only above the threshold value. Our results suggest that electro-endocytosis is not a crucial mechanism for gene electrotransfer and that the hypothesis of DNA entry by translocation through permeabilized membrane is more plausible. The presented results are important for better understanding of the mechanisms of gene electrotransfer and for its optimization for clinical applications.

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1. Introduction

Gene electrotransfer of cells was first achieved 25 years ago [1,2] when it was shown that high-voltage pulses applied to cells in the presence of DNA, enable delivery of DNA into the cells resulting in gene expression. Gene electrotransfer is already an established method for transfer of foreign genes *in vitro* and *in vivo* [3–9] and first clinical trials are in progress [10,11]. It presents a safer alternative method to viral transfection [12] and is also the most versatile and efficient method compared to other nonviral methods. For example, gene gun delivery is limited to exposed tissues, while complexes of DNA and cationic lipids or polymers can be unstable, inflammatory and toxic. Recent studies show that gene electrotransfer is a promising method for cancer gene therapy [6,13], DNA vaccination [14–17], autoimmune and inflammatory diseases [18], and several other illnesses [19].

Several studies have shown that gene electrotransfer is a threshold process where electric field has to be higher than some critical value [20–22] for given pulse parameters. Such pulses create pores in the cell membrane, a process known as electroporation, and these pores enable the transfer of DNA into the cell [1]. Further studies demonstrated that transfer of DNA molecules across the cell membrane is a more complex process than diffusion through pores created during electroporation [23,24]. It was shown that several steps are involved in gene electrotransfer: (i) the interaction of DNA molecules with the cell membrane (formation of a DNA-membrane complex) [24–28], (ii) transfer of DNA into the cytoplasm by some yet unknown process [3,13,25], (iii) trafficking/transport of DNA to nucleus [29] and (iv) gene expression. Regarding the steps (i) and (ii), electrophoresis was demonstrated *in vivo* and *in vitro* to be an important mechanism, since it provides electrophoretic force, which drags DNA towards the membrane and enhances insertion of DNA into the electroporated region of the cell membrane [30–34]. However, direct visualization of DNA transfer into the cell has not been done yet and up to now there is still no clear explanation of the mechanisms involved in gene electrotransfer.

One of biological mechanisms for the uptake of foreign material into the cell is endocytosis. Several different pathways of endocytosis were identified depending on the scale of initial membrane invagination: ingestion of particles larger than 500 nm typically occurs via triggered processes called ‘phagocytosis or macropinocytosis’; while molecules and particles below this size are internalized by other possible endocytotic processes, which include specific receptor-mediated clathrin-dependent as well as non-specific clathrin-independent ‘pinocytotic’ pathways [35–37]. Some authors proposed that electric fields could stimulate molecular uptake by endocytosis [38–40]. Furthermore, endocytosis [41,42] and electrically stimulated endocytosis [43–45] were suggested as possible mechanisms for DNA entry into the cell. Already in 1990, Zimmerman and co-workers showed that electric pulses stimulate endocytotic uptake of labeled bovine serum (FITC-BSA) in mouse L cells and DNA uptake in yeast cells [38]. Furthermore, Glogauer with co-authors demonstrated that electroporation pulses cause an actin-dependent increase in internalization of FITC-BSA in human fibroblasts [39], while Rols and colleagues [40] obtained induced

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non-specific macropinocytosis of enzyme beta-galactosidase during cell electroporation. It was also shown later [43] that a train of low electric pulses could induce endocytosis processes and by this the uptake of macromolecules, which was confirmed in further studies [45]. Also, for gene electrotransfer *in vivo*, receptor-mediated endocytosis was suggested as a possible mechanism of DNA transfer into muscle cells [44].

While electrically stimulated endocytosis, or electro-endocytosis, was suggested several times as a possible mechanism for the uptake of DNA during gene electrotransfer [37,43–45], none of these studies clearly demonstrated that endocytosis was indeed the dominant process for DNA entry into the cytoplasm. For this reason we set to systematically observe the possible changes in the formation of endocytotic vesicles after the exposure of cells to electric pulses used for gene electrotransfer [33]. In this manner we wanted to analyze the role of endocytosis in gene electrotransfer and to determine whether endocytosis is the dominant process for DNA transfer into the cell. With a set of experiments we first demonstrated that endocytosis can be observed with a membrane dye FM 1-43FX, which enables real-time monitoring of the processes occurring on the cell membrane [46]. We then exposed the cells to electric pulses with amplitudes below and above the threshold value of electric field for gene electrotransfer and analyzed if these pulses affect the process of endocytosis.

2. Materials & methods

2.1. Cells

Chinese hamster ovary cells (CHO-K1, European Collection of Cell Cultures) were grown in monolayers in culture medium HAM-F12 supplemented with 2 mM glutamine, 10% fetal calf serum (all three from Sigma-Aldrich, Deisenhofen, Germany), 200 units/ml benzylpenicillin (Pliva, Zagreb, Croatia) and 16 mg/ml gentamicin (Sigma-Aldrich, Steinheim, Germany). Cells were kept in a humid atmosphere at 5% CO_2 and 37 °C.

2.2. Gene electrotransfer

A detailed description of the experiment can be found in [33,34]. Briefly, plasmid DNA pEGFP-N1 purified with Endofree Plasmid mega kit (Qiagen, Hilden, Germany) coding for GFP (green fluorescent protein) was used to analyze the efficiency of gene electrotransfer. The experiments were performed on plated cells seeded in 24 multiwell plates in concentration of 5×10^4 cells per well. Before the experiments the culture medium was replaced with isoosmolar pulsing buffer (10 mM Na_2HPO_4/NaH_2PO_4, 1 mM $MgCl_2$, 250 mM sucrose, pH 7.4) supplemented with plasmid DNA in a quantity leading to a final concentration of 10 μg/ml. In a separate experiment, the plasmid was added at different time intervals (1, 5, and 10 min) after pulse delivery.

After incubation for 2 to 3 min at 22 °C cells were exposed to a train of four rectangular electric pulses with 200 μs duration and 1 Hz pulse repetition frequency, generated with a Cliniporator™ device (IGEA s.r.l., Carpi, Modena, Italy). The pulses were delivered to a pair of parallel Pt/Ir wire electrodes with 4 mm distance between them (d) and positioned at the bottom of the chamber. Such electrode geometry results in approximately homogeneous electric field between the electrodes $E=U/d$, where U is the applied voltage. The amplitude of the pulses and consequently the applied electric field was varied from 0 to 1400 V/cm in 200 V/cm steps. After pulse delivery, cells were left for 5 min at 37 °C to allow for cell membrane resealing. Then, the pulsing buffer was replaced with a culture medium and the cells were placed in the incubator (37 °C, 5% CO_2) for 24 h to allow for GFP expression.

Cells were observed with the imaging system consisting of a fluorescence microscope (Axiovert 200, Zeiss, Germany) a CCD camera (VisiCam 1280) and a monochromator (Polychrome IV) (both from Visitron, Germany). For analysis of GFP expression the excitation wavelength was set to 445 nm and emission was detected at 488 nm. For each pulse parameter at least five sets of images, each set consisting of a phase contrast and a fluorescence image, were acquired using MetaMorph 7.1.1 imaging software (Molecular Devices, Downingtown, PA, USA).

The relative transfection efficiency was determined by manually counting the cells in fluorescence and phase contrast images acquired 24 h after pulse delivery. The percentage of transfected cells (% Transfection) in a given sample was determined as the ratio between the number of fluorescent cells expressing GFP – $N_{GFP}(E)$ counted in the fluorescence image and the number of viable cells – $N(E)$ counted in the corresponding phase contrast image:

$$\%Transfection = 100\times N_{GFP}(E)/N(E). \qquad (1)$$

Survival of cells after electrotransfection (% Survival) was determined as the ratio between the number of viable cells, which were exposed to electric field – $N(E)$ and the number of cells in the control sample – N_0 that were not exposed to electric field:

$$\%\,Survival = 100\times N(E)/N_0. \qquad (2)$$

Results obtained from five independent experiments performed on different days were averaged and are presented in Fig. 3 as means ± standard error (SE). The described method of determining the percentage of transfected cells and the percentage of cell survival is consistent with our previously published results [22,33,34].

2.3. Monitoring of endocytosis

Endocytosis was observed using a fluorescent lipophilic styryl dye FM 1-43FX (Invitrogen, Eugene, Oregon, USA), which stains membranes and is used as a standard endocytotic probe [46]. One day prior to experiments, cells were plated in Lab-Tek II chambers (Nalge Nunc International, USA) at 5×10^4 cells per chamber in the culture medium. On the day of the experiments the culture medium was replaced with 500 μl of isoosmolar pulsing buffer. Cell staining with FM 1-43FX was performed either before (Protocol A) or after (Protocol B) the pulse delivery as described in detail below. Both protocols follow the protocol used for gene electrotransfer (described in Section 2.2 Gene electrotransfer) as close as possible.

2.3.1. Protocol A

After 5 minutes of incubation in the pulsing buffer 6 μl stock solution of FM 1-43FX (100 μg/ml) was added to the cells. Cells were stained for several minutes and then washed. Plasmid DNA was added to the pulsing buffer (final concentration 10 μg/ml) and the cells were incubated for additional 2 to 3 min at 22 °C. Cells were then exposed to the same train of pulses as described in Section 2.2 Gene electrotransfer, except for the pulse amplitude, which was set to either 0.2 kV/cm or 1 kV/cm, a value below or above the critical amplitude for gene electrotransfer, respectively (see Fig. 3) [22,33,34]. Five minutes after pulse delivery the pulsing buffer was replaced with 1 ml of culture medium.

2.3.2. Protocol B

After 2 to 3 min incubation of cells in pulsing buffer mixed with plasmid DNA (concentration 10 μg/ml), cells were exposed to the same train of pulses as described in Section 2.2 Gene electrotransfer, except for the pulse amplitude, which was set to either 0.2 kV/cm or 1 kV/cm. After incubation of cells for 5 min at 37 °C, 1 ml of culture medium was added to the cells. The cells were transferred back to the incubator (5% CO_2, 37 °C) for additional 15 min and then stained with FM 1-43FX and subsequently washed with fresh buffer.

Endocytosis was monitored with the same imaging system as described in Section 2.2 Gene electrotransfer. The excitation wavelength of FM 1-43FX was set to 510 nm and the emission was measured at 605 nm. In Protocol A, the images of cells were acquired before and after the pulses in 1 minute time intervals for a duration of 30 min. In Protocol B, the first images were acquired 30 min after pulse delivery and the cells were observed for additional 30 min in 1 minute time intervals. Such experimental design allowed us to observe the possible short-term (Protocol A) and long-term (Protocol B) endocytotic response.

2.4. Temperature dependent visualization of endocytosis

To determine the influence of the temperature on the endocytosis the procedures (incubation, staining, washing) described in Section 2.3 Monitoring of endocytosis, Protocol A, were performed at either 4 °C, 24 °C or 37 °C, and the cells were kept at these temperatures throughout the experiments. For this purpose, the experiments were performed inside the plexiglass chamber mounted on the heated stage of the inverted microscope. Temperature was monitored with temperature sensor. Since endocytosis is a temperature dependent process, cells maintained at 4 °C provided the reference for the absence of endocytosis (negative control), while cells at 37 °C provided the reference for endocytosis occurring at physiological temperatures (positive control).

3. Results

3.1. Effect of temperature on endocytosis

We first analyzed the influence of temperature on formation of endocytotic vesicles. Cells in these experiments were not exposed to electric pulses. For cells maintained at 4 °C practically no endocytosis was detected (Fig. 1A). With a gradual increase in the temperature the endocytotic vesicles started to appear, and at 37 °C a number of vesicles could be observed (Fig. 1B). Cells kept at 4 °C provided a reference for the absence of endocytosis, while cells at 37 °C provided a reference for endocytosis occurring at physiological temperatures.

To evaluate the time-dependent formation of endocytotic vesicles, another experiment was performed at 22 °C, using protocol A (see Section 2.3 Monitoring of endocytosis). The purpose of this experiment was to determine the baseline of endocytosis for our experimental conditions. Fig. 2 shows a pair of stained cells with clearly visible endocytotic vesicles. During the 30 min observation the number of vesicles did not change significantly. Therefore, in further experiments this experimental protocol allowed us to detect any changes in endocytosis stimulated by electric pulses.

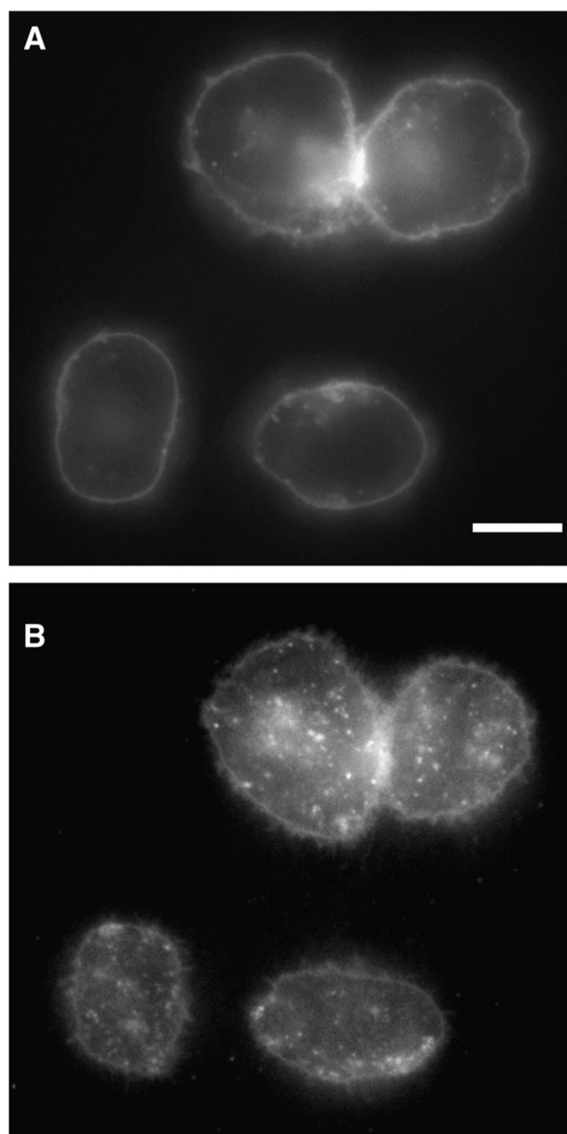

Fig. 1. The influence of temperature on endocytosis of CHO cells stained with membrane dye FM 1-43FX. Cells were first kept at (A) 4° and (B) gradually heated to 37 °C. Image in panel A was acquired 15 min after staining, while image in panel B was recorded app. 30 min after staining. The bar in panel A represents 10 μm.

3.2. Relation between gene electrotransfer and endocytosis

In the second part of our study we investigated the hypothesis that endocytosis is the dominant mechanism for the uptake of plasmid DNA during gene electrotransfer. We first present the results of transfection efficiency and then proceed to observations of endocytosis.

Fig. 3 shows how the amplitude of the electric field affects cell survival and gene transfection, after exposure of cells to 4×200 μs pulses with repetition frequency of 1 Hz [33]. It can be seen that transfection efficiency gradually increases with electric fields above 0.3 kV/cm, reaching 40% at 1.4 kV/cm, while cell viability at this field drops to 60%. Since no transfection was observed below $E=0.3$ kV/cm, this value presents a threshold for gene electrotransfer — E_c for pulse parameters used in our study (4×200 μs, 1 Hz). This threshold electric field can be understood as a phenomenological threshold at which electroporation of the membrane is sufficient to allow the uptake of DNA molecules into the cell [22].

In our standard protocol for gene electrotransfer plasmid DNA is always added a few minutes before pulse application. In order to test if adding the plasmid at different times after pulse delivery could lead to some observable GFP expression due to electro-endocytotic uptake, additional experiments were performed where plasmid was added 1, 5 and 10 min after pulse delivery. The number of transfected cells in all these cases was insignificant. In Fig. 3 the data are presented for DNA added 5 min after pulse application.

In order to study the effect of electric pulses on the process of endocytosis we exposed cells to: (i) electric field below the threshold value for gene electrotransfer $E<E_c$ ($E=0.2$ kV/cm), where no gene expression was observed and (ii) electric field above the threshold value $E>E_c$ ($E=1$ kV/cm), where approximately 30% of gene expression can be observed (Fig. 3).

The experimental observations of endocytosis were divided into two sets of experiments, depending on the experimental protocol used (see Section 2.3 Monitoring of endocytosis). Protocol A and Protocol B allowed us to observe the possible short-term and long-term endocytotic response, respectively.

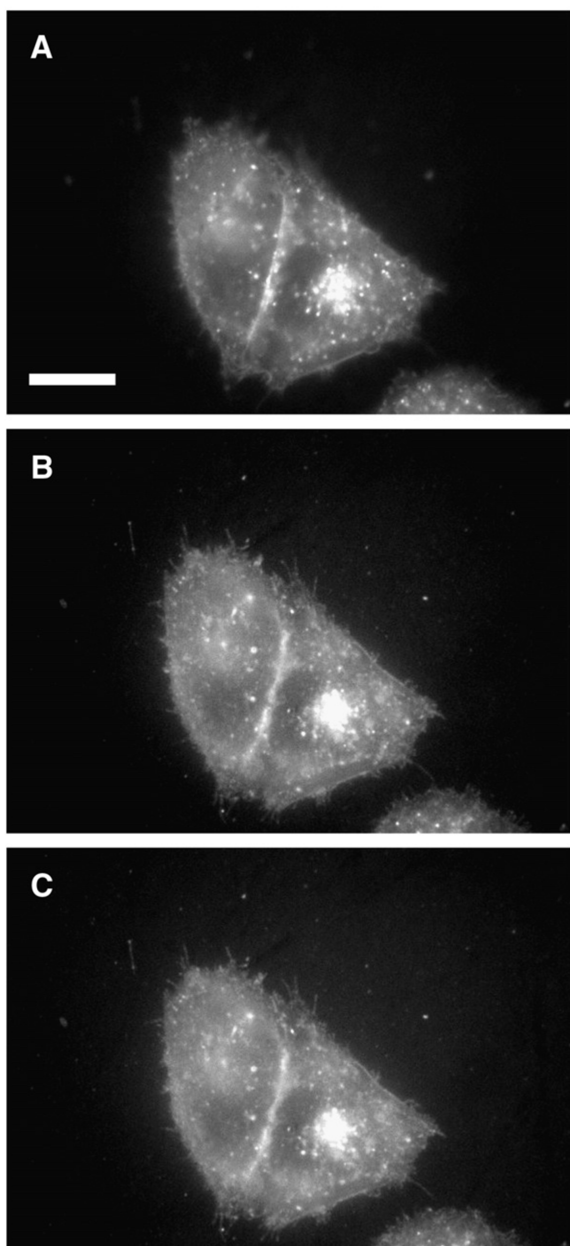

Fig. 2. Time dependent visualization of CHO cells stained with membrane dye FM 1-43FX. Cells were kept at 22 °C, no electric field was applied. Images were recorded (A) 5 min, (B) 15 min, and (C) 30 min after staining. The bar in panel A represents 10 μm.

3.3. Short-term observations of electro-endocytosis

To analyze the role of electro-stimulated endocytosis in the transfer of plasmid DNA during gene electrotransfer we monitored the endocytotic processes before and after the exposure of cells to electric pulses. Cells were exposed to pulses with the amplitude either below ($E=0.2$ kV/cm) or above ($E=1$ kV/cm) the threshold value for gene electrotransfer.

In Fig. 4 observation of endocytosis in cells exposed to electric fields below (panels A and C) and above (panels B and D) the threshold value is presented. Fluorescence images of cells were recorded immediately before and 10 min after the pulse delivery. When comparing the images below and above the threshold electric field (Fig. 4C and D) the only observable difference is higher intracellular staining (intracellular structures become more fluorescent) of cells exposed to $E>E_c$. No additional formation of endocytotic vesicles provoked by the electric field was found either at $E<E_c$ or $E>E_c$. Increased intracellular fluorescence

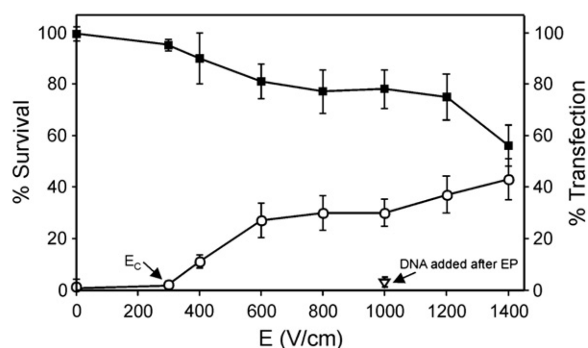

Fig. 3. Transfection efficiency determined as the percentage of fluorescence cells expressing GFP (circles) and the percentage of survival of CHO cells (squares) (see M&M section), E_c indicates the threshold electric field for gene electrotransfer. Cells were exposed to a train of four electric pulses with 200 μs duration and 1 Hz repetition frequency for different applied electric field strengths [33]. Data are presented as means ± standard errors of five independent experiments.

inside cells exposed to pulses with $E>E_c$ can be explained with increased staining of internal cell membranes due to additional influx of residual dye into the cell interior after electroporation. Since FM 1-43FX is not a plasma membrane specific dye, this explains the observed response in Fig. 4D. This experiment was performed several times on different days and Fig. 4 presents a typical response of cells to electric pulses.

To avoid undesired staining of intercellular membranes due to dye influx we designed another experimental protocol (protocol B — see Section 2.3 Monitoring of endocytosis) where cell staining was performed tens of minutes after electroporation when resealing has already been completed. This protocol also allowed us to observe possible long-term response of cells to electric pulses.

3.4. Long-term observations of electro-endocytosis

Cells were first exposed to electric fields with amplitudes either below or above E_c, then incubated for 20 min, and finally stained with FM 1-43FX (protocol B). The pulsing protocol was the same as the protocol used in short-term observations of electro-endocytosis (4×200 μs, 1 Hz).

The process of endocytotic vesicle formation was observed up to 2 h after pulse delivery. Even during this long time interval, no significant increase in formation of endocytotic vesicles was observed (Fig. 5) for cells exposed to electric fields below, as well as for cells exposed to electric fields above the threshold value for gene electrotransfer (Fig. 5). Adding the dye after the pulses prevented the undesirable intracellular staining, which was observed previously (see Fig. 4D).

In additional experiment we stained the cells before the pulse application (protocol A) and performed long-term observations of endocytosis. In this experiment cells were observed for 2 h after pulse delivery and again no significant increase in endocytosis was observed (results not shown).

Altogether, our observations of endocytosis indicate that exposure of cells to electric fields does not stimulate the formation of endocytotic vesicles (Figs. 4 and 5) regardless of the field strength. Since the efficiency of gene electrotransfer strongly depends on the electric field (Fig. 3) our results suggest that electro-endocytosis is not the dominant mechanism responsible for DNA electrotransfer.

4. Discussion

Gene electrotransfer is gaining increasing interest for transfer of foreign genes *in vitro* and especially *in vivo*, however, the mechanisms are still not fully understood [3–11]. Several steps involved in DNA electrotransfer were identified so far: S_1 electroporation of the cell membrane, S_2 interaction of DNA with the cell membrane (formation of DNA-membrane

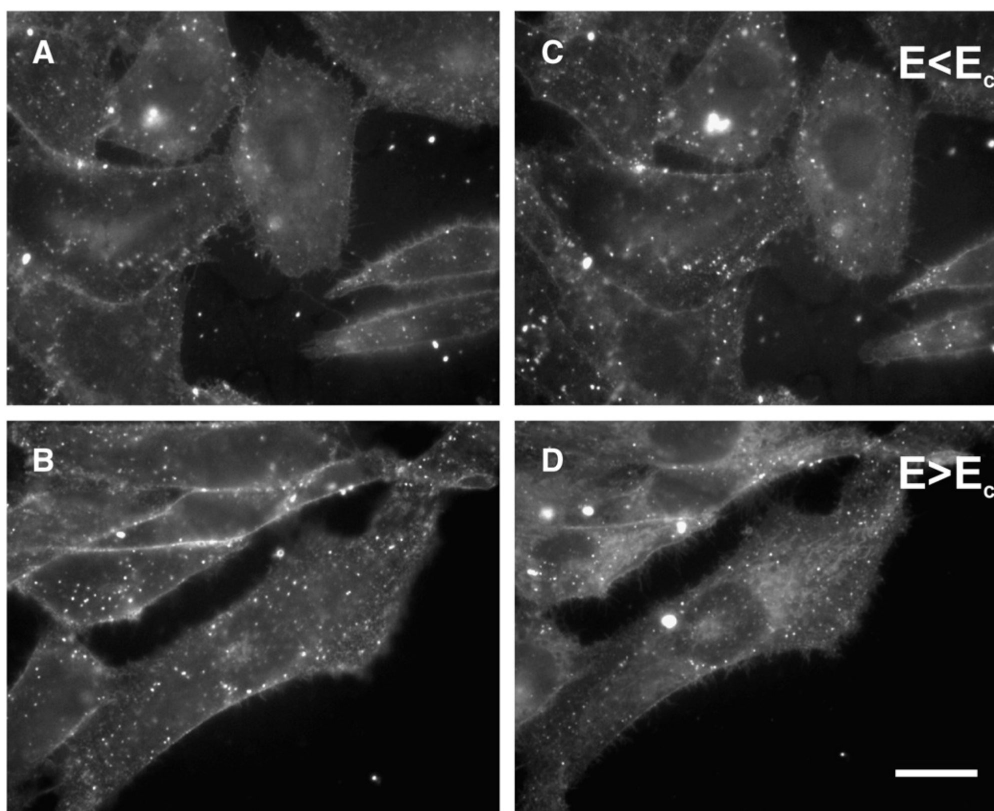

Fig. 4. Short-term observations of endocytosis in cells exposed to electric fields below the threshold value for gene electrotransfer, $E<E_c$, $E=0.2$ kV/cm (top row), and above the threshold value $E>E_c$, $E=1$ kV/cm (bottom row). Panels A and B are fluorescence images of cells immediately before the pulses, and C and D are the same cells observed 10 min after pulse delivery. A train of 4×200 µs pulses with repetition frequency of 1 Hz was applied to cells. The bar in panel D represents 10 µm.

complex), S_3 transfer of DNA across the membrane, S_4 transfer of DNA toward and into the nucleus, and S_5 gene expression [24–29]. However, direct visualization of DNA electrotransfer across the cell membrane and into the cytoplasm was not performed yet [3,13,25].

Among different transport mechanisms, electric field stimulated endocytosis was suggested as one of the possible means of DNA transfer into cells during gene electrotransfer [38,43–45]. Since endocytotic uptake of DNA into the cell cytoplasm was never directly observed, we focused our study on visualization of endocytotic process at the membrane level during gene electrotransfer. We performed direct fluorescence microscopic observations of endocytosis by using membrane dye FM 1-43FX, in the absence and presence of electric pulses used in gene electrotransfer. The aim of our study was to determine if endocytosis is the dominant process for DNA transfer into the cell during gene electrotransfer.

4.1. Theoretical analysis of electrotransfection efficiency

In order to investigate the relation of endocytosis with gene electrotransfer efficiency we have to first analyze how transfection efficiency is related to structural changes in the membrane due to cell electroporation (S_1). For this purpose, we analyzed the results of transfection for different electric fields presented in Fig. 3.

First, we can assume that electrotransfer is proportional to the membrane surface which is electroporated, i.e. the part which is exposed to the above threshold electric field. Namely, larger electroporated membrane surface enables more DNA transfer and more transfected cells — N_{GFP} (E). Therefore, for the fraction of transfected cells we can assume that it is proportional to electroporated surface:

$$N_{GFP}(E)/N(E)\propto S_{CN}. \tag{3}$$

Here, k is a constant, S_{CN} is electroporated surface of N cells, $S_{CN}=N\times\le S_C\ge$ and $\le S_C\ge$ is the average electroporated surface of the membrane of a cell. Further, if we take into account the relation between the average electroporated surface of the membrane $\le S_c\ge$ and electric field strength E of a cell [47]:

$$\le S_c\ge\, = \,\le S_0\ge \times (1-E_c/E), \tag{4}$$

Where $\le S_0\ge$ is the average surface of the cell, we can write:

$$S_{CN} = N\le S_0\ge \times (1-E_c/E) \tag{5}$$

Since $N_{GFP}(E)/N(E)\propto S_{CN}$ then it follows:

$$N_{GFP}(E)/N(E)\propto(1-E_c/E)$$

We can therefore analyze the dependence of the fraction of transfected cells (% Transfection/100, see Fig. 3) in terms of Eq. (4). The dependence of $N_{GFP}(E)/N(E)$ on $1/E$ is presented in Fig. 6. A linear curve ($y=k\times x\pm y0$) can be fitted to the experimental data:

$$N_{GFP}(E)/N(E) = \mathrm{k}\times 1/\mathrm{E}\pm \mathrm{y0}, \tag{6}$$

which yields $y0=0.491$ and $k=-0.145$ kV/cm. The threshold electric field E_c can be extracted from the intersection of the fitted curve with the x-axis:

$$\Delta x = \Delta y/k = -y0/k \Rightarrow 1/E_c = -y0/k, \tag{7}$$

from which it follows $E_c=145/0.491$ kV/cm $=0.295$ kV/cm. When whole average surface of a cell is electroporated ($\le S_c\ge=\le S_0\ge$) fraction of transfected cells reaches saturation, which equals approximately $y0=0.491$.

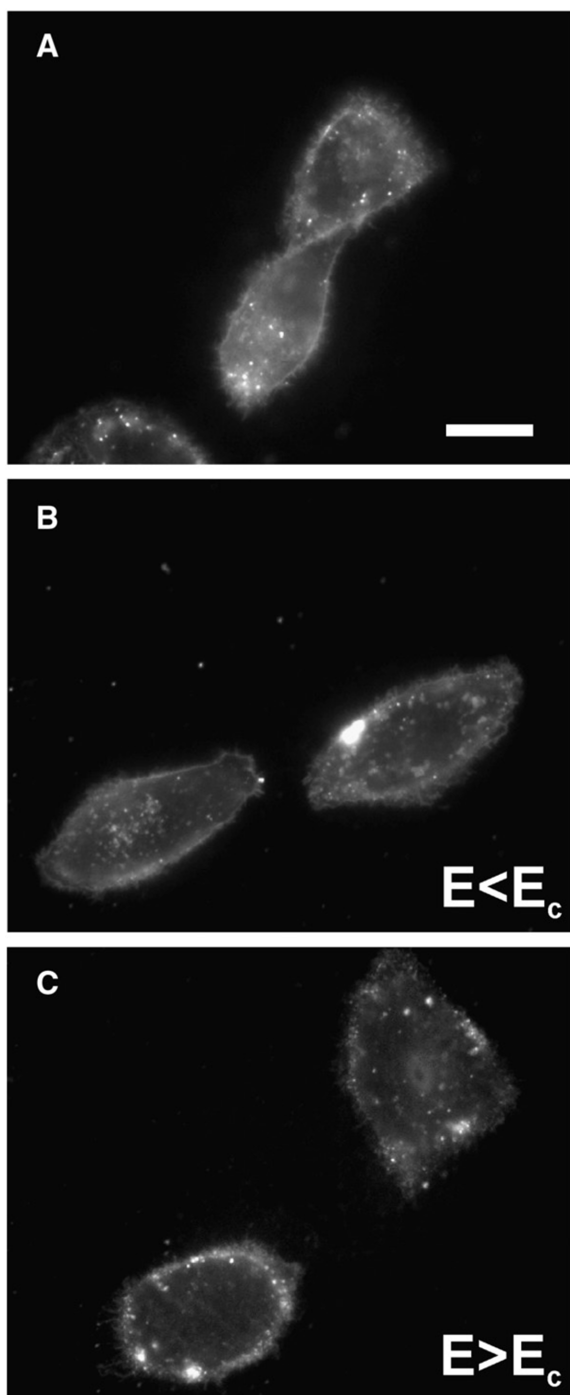

Fig. 5. Long-term observations of endocytosis in cells exposed to electric fields. Cells were stained with FM 1-43FX 20 min after pulse delivery. Fluorescence images where recorded 30 min after EP. (A) Cells not exposed to electric field (control). (B) Cells exposed to $E<E_c$. (C) Cells exposed to $E>E_c$. A train of 4×200 µs pulses, with repetition frequency of 1 Hz was used. The bar in panel A represents 10 µm.

The linear dependency of the fraction of fluorescent cells on $1/E$ demonstrates that electrotransfer efficiency is directly proportional to the electroporated surface of the membrane, which confirms that the first step (S_1) in gene electrotransfer is electroporation of the membrane. This also implicates that the probability of DNA to be transferred into the cell is directly proportional to the surface of the membrane, that is permeabilized, which is also in agreement with other reports [20,21,48]. But, we also have to stress that gene electrotransfer is a multistep process [33,48] and that also other factors, such as the size and mobility of the plasmid in the cytoplasm, as well as physiological state of the treated cells, all influence the final percentage of transfection.

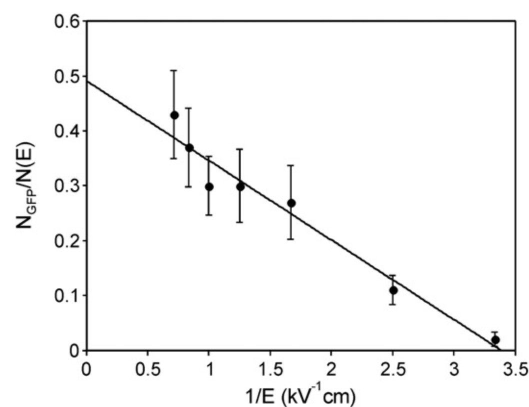

Fig. 6. Dependence of the fraction of fluorescent (GFP positive) cells $N_{GFP}(E)/N(E)$ (obtained from % Transfection in Fig. 3) as a function of $1/E$ (circles). The line represents a linear fit of Eq. 6 to the experimental data.

4.2. Visualization of endocytosis

In the previous subsection we investigated the relation between gene electrotransfer efficiency and electroporation, which is the first step of gene electrotransfer (S_1). This section analyzes the role of endocytosis in the second and the third step of electrotransfer, S_2 — interaction of DNA with the cell membrane (formation of DNA-membrane complex), and S_3 — transfer of DNA across the membrane.

According to our results, no increase in endocytosis was obtained either 10 min after the pulses (Fig. 4), 20 min after the pulses (Fig. 5) or even up to 2 h after the pulses were delivered, regardless of electric field strength. Therefore, endocytosis in our experiments was not stimulated by the electric field. In contrast, results of gene electrotransfer efficiency show (Fig. 3) that below the threshold electric field for gene electrotransfer (0.2 kV/cm) no transfection was detected, while above the threshold field a gradual increase in gene electrotransfer efficiency was obtained [21,22,33]. Therefore, our experiments suggest that electro-stimulated endocytosis is not the dominant mechanism for gene electrotransfer, at least for our experimental protocol (4×200 µs, 1 Hz). However, with different pulsing parameters, e.g. long, low-voltage pulses, the outcome could be different.

Our conclusion that endocytosis is not the dominant mechanism for DNA uptake is also in agreement with our previous study of the effect of high-voltage (HV) and low-voltage (LV) pulses on gene electrotransfer [34]. Namely, HV pulses are used to electroporate cell membranes while LV pulses are used to provide electrophoretic force, which drags DNA toward the cell membrane and thereby increase the formation of DNA-membrane complexes. The hypothesis behind electrically stimulated endocytosis is that DNA-membrane complex, formed during the pulses, provokes membrane invagination and endocytosis. Since LV pulses enhance the formation of DNA-membrane complexes, one would expect that if LV pulses are applied before HV pulses, the increase in electro-endocytotic uptake and consequently of gene transfer would be obtained. But no increase in transfection by using LV+HV combination compared to HV pulses alone was detected [34]. Moreover, the reversed combination of pulses (HV+LV), significantly increased transfection efficiency compared to HV alone or LV+HV combination. If transfer was indeed due to electro-endocytosis, LV+HV pulse combination should be (at least) as efficient as HV+LV pulses. Our previous results [34] and the data presented in this paper suggest that endocytosis is not the dominant mechanism of DNA entry. This is also supported by experimental study of DNA trafficking inside the cell during gene electrotransfer, where it was shown that DNA is actively transported

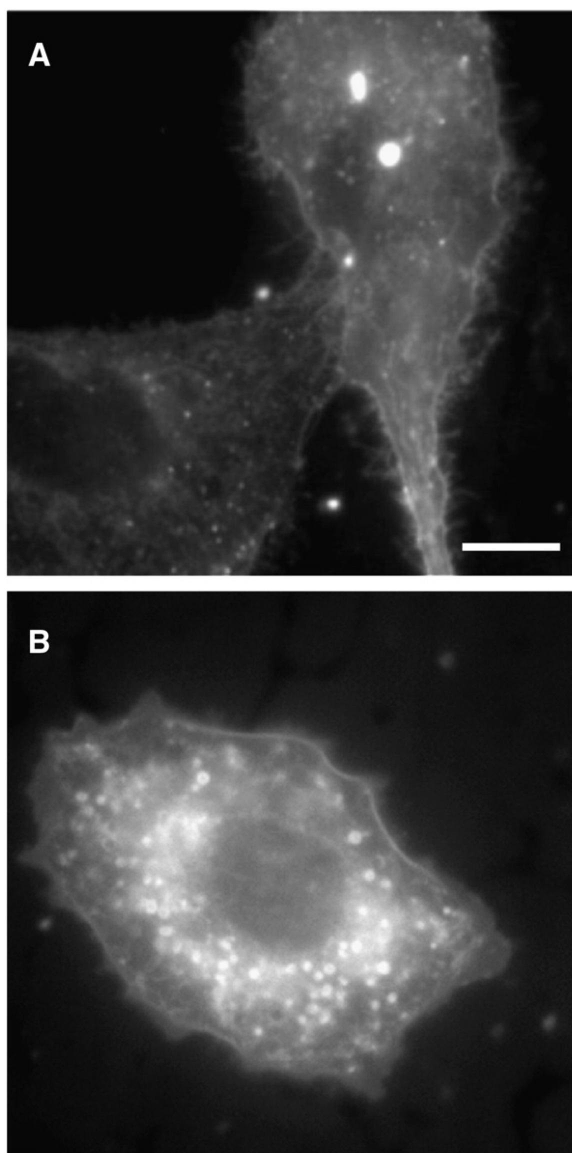

Fig. 7. Observation of intracellular vesiculation in the presence and absence of serum in the medium. Cells were exposed to electric field above the threshold value for gene electrotransfer ($E>E_c$, $E=1$ kV/cm). Cell response if: (A) serum was added to the medium immediately after the pulses, and (B) serum was not added. In both cases, a train of 4×200 μs pulses with 1 Hz repetition frequency was delivered. Cells were kept at 22 °C and images were acquired 10 min after the pulse. The bar in panel A represents 10 μm.

via tubuline fibers toward the nucleus [29], an observation, which is difficult to combine with hypothesis of electro-endocytotic uptake. Instead of electro-endocytotic transfer we propose that DNA is inserted into the electroporated membrane during the electric pulses and later enters the cell by some other mechanism, such as translocation driven by ratchet mechanism and entropic forces [49].

Most of the studies, which suggested endocytosis as a mechanism for DNA entry after exposure to electric pulses, have made this conclusion on the basis of indirect observations only. For example, in the paper of Rols, macropinocytosis was observed after pulse delivery, but it was related only to uptake of proteins and polysacharids and not to DNA electrotransfer [40], while in Šatkauskas et al. [44] no endocytotic marker was used in order to confirm that endocytosis is indeed the dominant mechanism for DNA uptake. The only study where uptake of DNA by stimulated electro-endocytosis was observed was the study by the group of Kornstein [43,45]. They observed massive vesicle like structures in the cytosol following the exposure to electric pulses. However, the pulses used in their study were low voltage and of extremely long duration (trains of pulses lasting up to 10 min) compared to electric pulses typically used for gene electrotransfer (durations up to tens of milliseconds). With the experimental protocol used in our study (4×200 μs, 1 Hz) such vesicles were not observed, although the cells were exposed to relatively strong electric fields. The absence of intracellular vesiculation in our experiments can be explained by the presence of serum in the medium. As reported previously, the electric field induced stress can be considerably reduced by the addition of serum into the medium [50]. Namely, electroporation induces electromechanical as well as osmotic stress [51–54] and addition of serum reduces the stress related effects. It was also shown that mechanical injury of cell membrane causes intracellular vesiculation [55]. To confirm the effect of stress on the intracellular vesiculation, we performed additional experiments were serum was not added to the cells. The intracellular vesiculation was then indeed observed, but only for electric fields above E_c where cell membrane was electroporated (Fig. 7).

The majority of our experiments were performed by adding DNA to cells before the pulses (standard protocol for gene electrotransfer). But we also performed an experiment, where DNA was added after the pulses, and in this case, we did not observe any transfected cells. Therefore, regardless of the mechanism of DNA transfer into the cell, DNA has to be present during the pulse delivery, which is in agreement with other reports [23–25]. The formation of DNA-membrane complex is indeed a crucial step in gene electrotransfer.

In summary, our results show that electro-endocytosis is not the dominant mechanism for gene electrotransfer with short high-voltage pulses. No increase in formation of endocytotic vesicles was observed for pulse parameters resulting in gene electrotransfer. The presented results suggest that DNA enters the cell interior by some other mechanism, most probably by translocation through electroporated cell membrane, but this needs to be confirmed experimentally. The presented study is important for understanding the mechanisms involved in gene electrotransfer as well as for developing new protocols for clinical applications in electrogene therapy and gene vaccination.

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