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Setting optimal parameters for in vitro electrotransfection of B16F1, SA1, LPB, SCK, L929 and CHO cells using predefined exponentially decaying electric pulses

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Abstract

To achieve the maximal introduction of plasmid DNA into cells and, at the same time, to prevent undesirable cell deaths, electrotransfection conditions should be determined for every single cell type individually. In the present study, we determined the optimal electrotransfection parameters for in vitro transfection of B16F1, SA1, LPB, SCK, L929 and CHO cells. Some of these varying parameters were electric field strength, number of applied pulses and their duration, osmolarity of electroporation buffer, plasmid DNA concentration and temperature at which the electroporation was carried out. The maximal transfection rates at optimal electrotransfection parameters in B16F1, SA1, LPB, SCK, L929 and CHO were 85%, 40%, 60%, 1%, 40% and 65%, respectively. The obtained results confirmed that the electroporation is a useful procedure for an in vitro transfection of the majority of mammalian cells. The method, if optimized, may generate reproducibly high proportion of transfected cells among the cell types that are sensitive to electric field action. Thus, the determined parameters could serve for the subsequent implementations of this method.

Keywords: Transfection; Electroporation; Tumor cells

1. Introduction

The electroporation is a process where the electric field causes a highly transient permeability of the cell membrane and the subsequent formation of membrane pores [1–4]. If the membrane is permeable, it allows different kinds of small and large molecules to be introduced into the cytoplasm, although, in its normal state, the cell plasma membrane represents a formidable barrier for their transfer. Among the first authors who demonstrated that electroporation could be used for the introduction of DNA into mammalian cells were Neumann et al. [5] and Wong and Neumann [6]. They transfected the mouse fibroblasts with linear or circular plasmid DNA containing the herpes simplex thymidine kinase (TK) gene and clearly showed that the introduced DNA was expressed. The subsequent results with other types of cells provided further evidence that the electroporation is an effective procedure for the in vitro introduction of DNA into various cells [5,7,8], thereby becoming also an important method for the transfer of genes (and other molecules) in numerous cases. Though the electroporation is widely used in biotechnology and medicine, the molecular mechanisms have not been definitely explained. In general, the mechanisms could be a reflection of combined effects of electrophoresis, osmotic processes and direct electric field action on the cell membrane and DNA molecules. Nowadays, the electroporation is also being used over and over again for introducing the DNA, proteins or drugs into the cells in vivo. There are some reports of successful in vivo DNA transfer into the cells of malignant melanoma as well as normal liver, skin and skeletal muscle cells [9–14], as well as of a rapidly developing method, the so-called electrochemotherapy, intended for the transfer of chemotherapeutical drugs directly into the tumor cells [15,16].

Although electroporation in vitro is effective in a wide variety of cells, each type of them requires slightly different electroporation conditions. Among the main parameters that importantly affect the electroporation effectiveness are electric field strength, number of pulses, duration of pulse and

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the frequency applied [17–19]. Other parameters affecting the electroporation are temperature, conductivity of the poration medium, molecular size of the transferred molecules, shape of the target cells, charge of the cell membrane as well as the cell density [17–20]. Thus, the optimal electroporation parameters must be determined empirically for each cell line severally. In the present study, we determined the effect of different parameters on the in vitro transfection in B16F1, SA1, LPB, SCK, CHO and L929 cells. The transfection efficacy was assessed by counting the cells expressing the reporter β-galactosidase plasmid, while the cell survival was determined using the clonogenic assay.

2. Experimental

2.1. Materials

2.1.1. Cell culture

Mouse B-16 melanoma (clone F1) cells (American Type Culture Collection—ATCC, Rockville, MD, USA), mouse fibrosarcoma SA1 cells (Jackson Laboratory, Bar Harbor, ME, USA), mouse mammary adenocarcinoma cells—SCK (kindly provided by Chang W. Song, University of Minnesota, Minneapolis, MN, USA), murine fibrosarcoma cells—LPB, clonal derivative of TBL.Cl2 cells (kindly provided by Lluis M. Mir, Institute Gustave Roussy, France), mouse fibroblast cells—L929 (Instituto Zooprofilatticco Sperimentale della Lombardia e dell'Emilia, Brescia, Italy) and Chinese hamster ovary cells—CHO (ATCC) were grown in Eagle's minimal essential medium (EMEM) supplemented with 10% of fetal calf serum—FCS (Sigma, St. Louis, MO, USA), 100 units/ml of penicillin (Pfizer, New York, NY, USA), 100 mg/ml of streptomycin (Pfizer) and 11 mg/ml of gentamycin (Invenex, Chagrin Falls, OH, USA). Cell lines were cultured in petri dishes (Costar, Badhoevedorp, The Netherlands) with a diameter of 60 mm, in a CO_2 incubator at 37 °C and at humidity of 5%. Electroporation was performed using the cells that have already passed several growth cycles and were in the exponential growth phase.

2.1.2. β-Galactosidase vector

The 6.821-kb large plasmid pSV β-galactosidase, that directs the synthesis of β-galactosidase enzyme under the control of the SV40 early promotor, was used in the experiments (Promega, Madison, WI, USA). It was grown in *Escherichia coli* strain TOP10F′ or DH5α and purified using the Endo Free Plasmid Maxi Kit (Qiagen, Chatsworth, CA, USA).

2.2. Setting the parameters of electroporation on the electroporator

For the study, the Eppendorf's Multiporator® 4308 10096 (Eppendorf-Netheler-Hinz, Hamburg, Germany) was used. The multiporator enables a transient transfection of various adherent and suspension cell lines by producing exponentially diminishing and electronically controlled electric pulses. Such a pulse is defined by a peak voltage and the time constant, i.e. the time at which the voltage has dropped to 1/e of the initial value. Since the pulse of multiporator is electronically regulated every 5 μs, an ideal form of pulse curve can be achieved. The parameters will not vary with the conductivity of the sample. The multiporator enables multiple pulsing from 1 to 99 pulses with 1-min interval and a time constant between 15 and 500 μs in increments of 5 μs. To perform the electroporation of mammalian cells, the eukaryotic module that generates the pulse voltage between 20 and 1200 V was applied.

2.2.1. Electroporation buffer

The optimal osmolarity of the low electrical conductivity electroporation buffer was achieved by mixing hypoosmolar and isoosmolar buffers (Eppendorf-Netheler-Hinz) for each cell line separately. In Table 1, we present the main characteristics of the buffers. The mixture that allows maximal water absorption by the cells (maximal swelling and cell diameter) and causes less then 10% cell lysis was considered as optimal. According to the manufacturer's electroporator protocol, the electrotransfection procedure should be completed in maximum 30 min. So, the cell viability was determined after 30 min of incubation in different buffer mixtures using trypan blue. After the determination of the diameter of the cells in the optimal hypoosmolar buffer, the corresponding electric field strength was calculated. The diameter of the cells was determined under the microscope's ocular micrometer scale, which was previously calibrated against an objective micrometer scale (with the spacing value of 10 μm).

The precision of the measurements at a magnification of the microscope of 10×40 was ± 1 μm.

2.2.2. Electric field strength

The parameters (peak voltage), which have been set, are applicable. The course of the curve is calculated internally, so that the course of the discharge curve (e-function) is maintained according to the parameters, which have been entered. The formula for estimating the critical field strength

Table 1
Composition of electroporation buffers [21]

	Hypoosmolar electroporation buffer	Isoosmolar electroporation buffer
KCl	25 mM	25 mM
KH_2PO_4	0.3 mM	0.3 mM
K_2HPO_4	0.85 mM	0.85 mM
myo-Inositol	90 mOsm/kg	280 mOsm/kg
pH value	7.2 ± 0.1	7.2 ± 0.1
Conductivity at 25 °C	3.5 mS/cm ± 10%	3.5 mS/cm ± 10%

that allows a membrane breakthrough is based on Laplace equation:

$$E_c = U_c/1.5 \times R$$

where E_c is the critical electrical field strength (V/cm), U_c is the critical breakdown voltage (V)—1 V at room temperature and 2 V at 4 °C [21], and R cell radius (cm).

The voltage required for the multiporator was calculated as a function of the electrical field strength and the distance between the electrodes in the cuvette:

$$U = E_c \times D$$

U is the voltage required for the multiporator (V) and D the distance between electrodes (cm).

The calculated voltage was supposed to be the minimum value at which the membrane could be permeated. To determine the optimal pulse voltage, a series of experiments at the minimum and at gradually increasing voltages were carried out (Table 2). The voltage set on the multiporator corresponds to the initial voltage (V_0) of the discharge curve ($V_t = V_0 \times e^{-t/\tau}$). The time constant ($\tau$) is the time required for the voltage to decrease to the value V_0/e (=approximately 37% of the initial voltage).

2.2.3. Pulse number and their duration

Electroporation was carried out with one, two, three or four pulses and with the pulse duration of 70 and 100 μs.

2.3. Gene transfer procedure

Adherently growing cells with identical passage numbers were harvested in the exponential growth phase. Following trypsinization, the cells were resuspended in the growth medium (to inactivate trypsin) and centrifuged (at room temperature and 1500 rpm for 5 min). The electroporation was performed in the plastic cuvettes with the volume of 400 μl. The distance between the electrodes in the cuvette was 2 mm. In one electroporation cuvette, we electroporated 400 μl of cell suspension with the cell density of 5×10^5–1.5×10^6 cells/ml. The electroporation buffer contained the mixture of hypoosmolar and isoosmolar buffers with different amounts of the plasmid DNA (2.5, 5 and 10 μg/ml). The β-galactosidase plasmid DNA was ultra pure without endotoxin contamination ($A_{260}/A_{280} = 1.8$). The electroporation was performed at 4 °C or room temperature with Eppendorf's Multiporator (Eppendorf-Netheler-Hinz). After electroporation, the cells were kept in the electroporation cuvette for 10 min at room temperature and than transferred onto 6-well microtiter plates. Forty-eight hours after electroporation, the transient expression (β-galactosidase assay) and the number of viable cells were determined (clonogenic assay).

Table 2
Applied electroporation field strength and voltage required for its achievement

Cell line	B16F1	SA1	LPB	L929	CHO	SCK
Electrical field strength (V/cm)	0–1200	0–1200	0–1600	0–1350	0–1050	0–6000
Applied voltage (V)	0–240	0–240	0–320	0–270	0–210	0–1200

The field strength was increased by 100 V/cm.

2.4. β-Galactosidase assay

β-Galactosidase gene expression in transfected cells was followed by X-gal staining procedure [22]. The transfection efficacy was determined by counting a total number of 200 adherently growing electroporated cells in representative areas of the plate under the magnification of 20 and by determining the percentage of blue stained cells in the counted areas (transient expression of the reporter gene exclusively among the viable cells).

2.5. Trypan blue staining

The permeability of the electroporated cells was verified by the electroporation with trypan blue dye. The stock solution of trypan blue dye was prepared by dissolving of 400 mg of trypan blue in 100 ml of PBS buffer. The cells were electroporated under the same conditions as described previously, yet the plasmid DNA was replaced with 10 μl of the trypan blue stock solution. The electric field strength for experiments was being varied from 0 to 6000 V/cm with the increments of 100 V/cm. The percentage of colored cells was determined 24 h after the electroporation.

2.6. Clonogenic assay

The survival rate of the electroporated cells was determined by clonogenic assay. The transfected and untreated ($E = 0$ V/cm) control cells (300 cells of each) were plated in petri dishes with a diameter of 60 mm, each containing 4 ml of EMEM supplemented with 10% of FCS. After 7 days, the colonies were fixed and stained with crystal violet diluted in absolute ethanol (Sigma) and counted. Abortive colonies were not counted. The survival of cells treated with electric pulses was presented as survival rate—i.e. the ratio between the number of formed colonies in treated groups and the number of colonies in the untreated groups (considered as 100%).

3. Results

In order to optimize the in vitro process of electrotransfection in B16F1, SA1, LPB, SCK, CHO and L929 cells, some parameters were being varied: electric field strength, pulse number and duration, electroporation buffer, temperature, cell growth medium, DNA amount and cell

Table 3
Determination of the electroporation buffer optimal osmolarity, diameter of the cells in isoosmolar as well as in optimal hypoosmolar electroporation buffer mixture, the transfection rate at calculated critical field strength of pulses by all combinations of their number and duration for each cell line

Cell line	Determined optimal mixture of electroporation buffer (isoosmolar/ hypoosmolar buffer)	Osmolarity of the optimal electroporation buffer (mOsm/kg)	Diameter of the cell in optimal electroporation buffer osmolarity (μm) (AM ± SD)	Diameter of the cell in isoosmolar electroporation buffer (μm) (AM ± SD)	Calculated critical field strength (V/cm)	Calculated critical voltage (V)[a]	Duration of pulse (μs)	Number of pulses	Transfection rate (%) in the optimal electroporation buffer[b]
B16F1	30/70%	147	20 ± 3	14 ± 2	650	130	70, 100	2–4	57–85
SA1	30/70%	147	19 ± 4	13 ± 3	700	140	70, 100	2–4	15–20
LPB	80/20%	242	19 ± 3	13 ± 3	700	140	70, 100	3	5–60
L929	20/80%	128	19 ± 2	15 ± 2	700	140	70, 100	3	10–13
CHO	40/60%	166	19 ± 3	14 ± 3	700	140	70, 100	3	60–63
SCK	30/70%	147	20 ± 1	14 ± 2	650	130	70, 100	3	1

There were at least three sets of experiments performed for each electroporation condition in every cell line under study.
[a] The critical voltage was calculated by using critical electric field strength and the distance between the electrodes in the cuvette.
[b] Corresponding to different number of pulses and their duration.

density. The effect of varying parameters was followed by determining the expression of the transferred β-galactosidase gene and the survival rate of the electrotransfected cells.

3.1. Setting the osmolarity of the electroporation buffer, determining the cell diameter in the electroporation buffer and setting the temperature and cell medium

To determine the most appropriate buffer osmolar concentration needed for electroporation, we tested the viability of the cells in the mixtures of hypoosmolar and isoosmolar buffers. The determined optimal buffer osmolar concentration (that enabled a maximum cell stretch and caused at the same time minimum cell deaths) for B16F1, SA1, SCK cells was 147 mOsm/kg, and for LPB, L929 and CHO cells 242, 128 and 166 mOsm/kg, respectively (Table 3). The corresponding cell diameters were 20 μm for B16F1 and SCK cells and 19 μm for SA1, LPB, L929 and CHO cells. The cell diameters were used for the calculation of the initial electric field strength (Table 3).

The transfection rates as well as survival of the electrotransfected cells were not significantly different when the electroporation was done at 4 °C or at room temperature. The results were quite similar when different cell growth media were used: neither EMEM with phenol red nor RPMI without phenol red influenced the transfection rates or survival of the electrotransfected cells. Therefore, additional electroporation procedures were carried out at room temperature using EMEM with phenol red as a growth medium.

3.2. Setting the amount of β-galactosidase vector and cell density

Considering the fact that the transfection rate was determined as a percentage of cells expressing *β*-galactosidase reporter gene, we tested the influence of different plasmid DNA concentrations on the proportion of electroporated B16F1 cells expressing this gene. Under equal electroporation conditions (electric field strength = 650 V/cm, number of pulses = 3, pulse duration = 70 *μ*s), 2.5, 5 or 10 *μ*g/ml of ultra pure plasmid DNA were added to the cells and the expression rate was determined. The highest percentage of cells expressing the reporter gene was found when 5 *μ*g of DNA per ml was used (85%) (Fig. 1). Almost the same results were obtained with 2.5 *μ*g DNA/ml, where 75% of cells expressed reporter gene, while the electroporation carried out at the concentration of 10 *μ*g plasmid DNA/ml yielded only 55% cells expressing reporter gene. Besides, different concentrations of plasmid DNA did not affect the cell survival during and after the electroporation; so, their survival was somewhere around 95% in all tested cases. It is also worth mentioning that the cell density of 1×10^6 cells/ml (recommended also by the producer) allowed the best transfection results (data not shown).

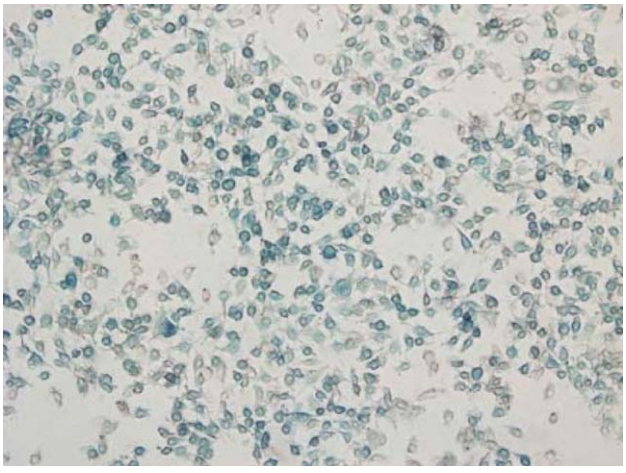

Fig. 1. B16F1 cells transfected in vitro with β-galactosidase reporter gene by the electroporation method (plasmid DNA concentration was 5 μg/ml). In transfected cells, the transient expression of reporter gene resulted in the production of β-galactosidase, which then changed the color of X-gal substrate to blue.

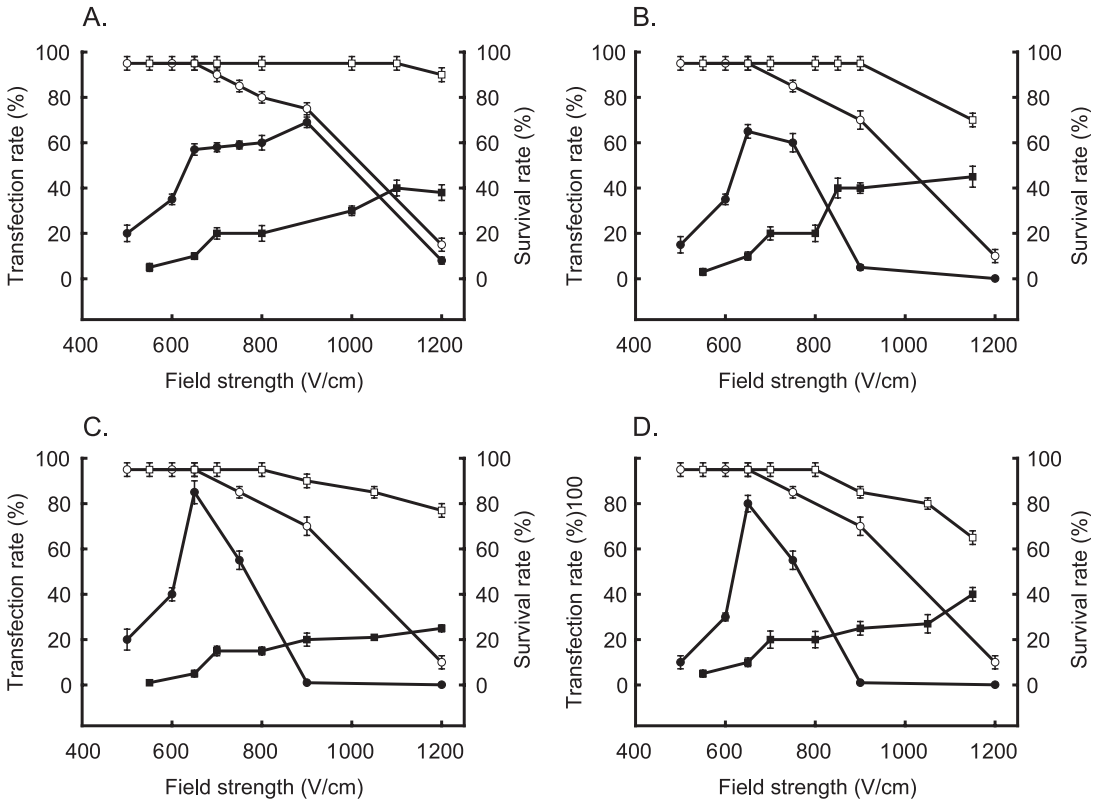

Fig. 2. Transfection and survival rates of B16F1 and SA1 cells when the number of electric pulses, their duration and electric field strength were varied: (A) two pulses with the duration of 100 μs; (B) three pulses with the duration of 100 μs; (C) three pulses with the duration of 70 μs; (D) four pulses with the duration of 70 μs. Plasmid DNA concentration was 5 μg/ml. Dotted line represents the mean value of at least three independent experiments. (●) Transfection rate of B16F1 cells, (○) survival rate of B16F1 cells, (■) transfection rate of SA1 cells, (□) survival rate of SA1 cells.

3.3. Setting the optimal electric field strength of pulse and the number and duration of pulses

The electrotransfection and survival rates of electroporated cells were determined in respect to different electric-field strengths, number of field pulses and pulse duration. One, two, three or four electric pulses with the duration of 70 or 100 μs, repeatedly occurring at 1-min interval and at a field strength of 0–6000 V/cm, were used in our experiments. The plasmid DNA concentration was 5 μg/ml in all experiments. The results of gene expression and consequently relative plasmid DNA uptake by the electroporated cells as well as the survival rates of the very same cells are presented in Figs. 2–4. As it can be seen from the presented results, the transfection efficiency and survival patterns in SA1, LPB, L929 as well as in CHO cells after the electro-

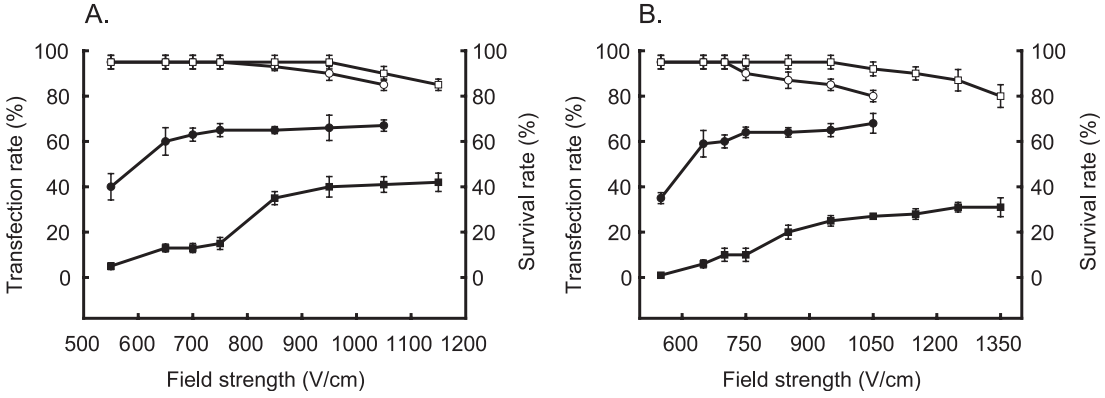

Fig. 3. Transfection and survival rates of CHO and L929 cells when the number of electric pulses, their duration, as well as electric field strength were varied: (A) three pulses with the duration of 100 μs; (B) three pulses with the duration of 70 μs. Plasmid DNA concentration was 5 μg/ml. Dotted line represents the mean value of at least three independent experiments. (●) Transfection rate of CHO cells, (○) survival rate of CHO cells, (■) transfection rate of L929 cells, (□) survival rate of L929 cells.

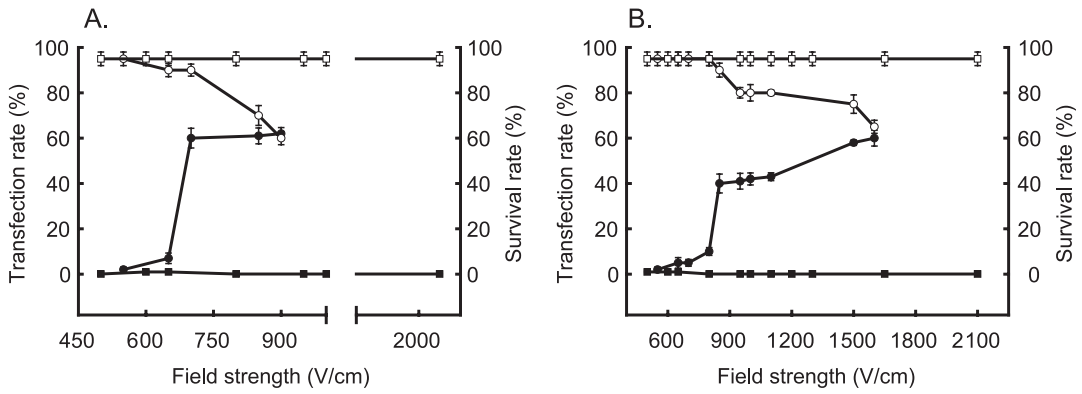

Fig. 4. Transfection and survival rates of LPB and SCK cells when the number of electric pulses, their duration, as well as electric field strength were varied: (A) three pulses with the duration of 100 μs; (B) three pulses with the duration of 70 μs. Plasmid DNA concentration was 5 μg/ml. Dotted line represents the mean value of at least three independent experiments. (●) Transfection rate of LPB cells, (○) survival rate of LPB cells, (■) transfection rate of SCK cells, (□) survival rate of SCK cells.

transfection were very similar (Figs. 2–4). Namely, the increase of the electric field strength was followed by the increase of the proportion of cells expressing reporter gene and diminishing of the number of viable cells. The results were slightly different with B16F1 cells that had an extremely narrow range of electric field strengths in which an optimum transfection efficacy and surviving were achieved (Fig. 2). An additional increase in the electric field strength was followed by a rapid decrease in the proportion of cells expressing reporter gene as well as by a decrease of the number of viable cells. In all of these cell lines, the transfection rate was substantially lower when the transfection procedure was carried out using electric field strength below the critical one. The transfection was unsuccessful and also no unspecific coloration was observed in the control cells that were incubated with the same amount of plasmid DNA (5 μg/ml), but without electric field treatment ($E = 0$ V/cm).

The only cell line in which no coloration of the cytoplasm could be detected after the electroporation in the presence of control plasmid DNA was SCK. We were unable to transfect these cells even when the applied electric field was enormously increased (6000 V/cm) (Fig. 4). As the electric field did not support the cell transfection, also the cell survival remained stable (comparable to the one in control cells that were not exposed to the electric field), but only up to 2500 V/cm. Further increase in the electric field strength above 2500 V/cm resulted in a diminished survival of the tested cells (at 2600 V/cm the cell survival was reduced to 70% and at 6000 V/cm to merely 1%). Therefore, additional experiments with trypan blue dye were carried out to confirm whether the electropermeabilization of these cells actually did or did not occur. Surprisingly, no dye penetration was detected even after 6000 V/cm had been applied to the cells, which speaks for the fact that the membranes of SCK cells remained completely undamaged.

The variation of pulse number revealed that the transfection efficiency is improved by applying more than one electric pulse, but in majority of cells, not more than four. Actually, by applying only one pulse, no transfection at all was observed, while the best transfection results were obtained using three pulses (except in SCK cells) (Figs. 2–4) (Table 4). Additional parameter that affected the transfection efficiency and survival rates of electroporated

Table 4
Determination of the optimal field strength of pulses, their optimal duration and number, as well as the maximal transfection rate at optimal electroporation conditions for each cell line

Cell line	Optimal field strength (V/cm)	Applied voltage (V)	Duration of pulse (μs)	Number of pulses	Maximal transfection rate (%) in electroporation buffer with optimal osmolarity[a]	Maximal transfection rate (%) in isoosmolar electroporation buffer
B16F1	650	130	70	3	85	30
SA1	850	170	100	3	40	2
SA1	1100	220	100	2	40	2
LPB	700	140	100	3	60	15
L929	950	190	100	3	40	10
CHO	750	150	70	3	65	20
SCK	650[b]	130	70	3	1	1
SCK	650[b]	130	100	3	1	1

There were three sets of experiments performed for each electroporation condition in every cell line under study.
[a] The electroporation buffer and its optimal osmolarity are described in Table 3.
[b] The calculated critical field strength.

cells was the pulse duration. This influence was expressed differently, depending on the cell type. LPB cells were the most sensitive to this parameter. The survival rate was 20% higher in the cells treated with 70 μs durable pulses than in the cells treated with 100 μs durable pulses (electric field strength 850 V/cm). Under the same conditions, the transfection rate was 21% lower in the cells treated with 70 μs durable pulses (Fig. 4). The same tendency was observed in CHO, L929 and partially in SA1 cells, while no effect of the pulses of different duration was noted in B16F1 and SCK cells. The optimal electrotransfection conditions for the tested cells are summarized in Table 4.

4. Discussion

The need for the transfer of DNA into mammalian cells arose from our research work in the field of classic and genetically modified tumor vaccines [23–27]. The creation of genetically modified tumor vaccines urged the optimization of appropriate vector systems for introducing DNA into the target cells. The procedure that we were seeking was supposed to enable an efficient transfection of a large number of cells (primarily in vitro), to be simple, controllable and without undesired side effects to the cells. Although the viral vectors (*Retro*, *Adeno*, *Adeno-associated*) are superior to plasmid-based vectors in their DNA delivering efficiency [28–30], we considered the non-viral methods as more appropriate ones. The reasons lay primarily in securing high standards of safety and controllability of the plasmid-based vectors and also in the intention to restore only a transient expression of the transferred genes. The first methods that we optimized were the receptor-mediated gene transfer and the method of a biolistic gun [31]. Since the receptor-mediated gene transfer proved to be superior to biolistic gun for in vitro gene transfer, it became the method of choice for the transfection of mammalian cells in our laboratory. However, the method is quite expensive and requires well-trained technicians to achieve the optimal transfection efficiency. On the other hand, the method of electrotransfection was reported to be successful for both in vitro and in vivo gene transfer by different authors [5,8,10–14,32]. The mechanisms that allow electrotransfection of mammalian cell with plasmid DNA are not yet clearly defined, but they seem to be relatively cell unspecific (that was a prerequisite for our additional work). Based on some early presumptions, the DNA is moved into the cells with electrophoretic forces in the applied electric field. This theory was proposed by Andreason and Evans [33] and rests on their observations that a significant increase in transfection yield was achieved when the cells were treated by low-voltage pulses after the first short electroporative pulse. The next evidence that DNA is moving under the action of electrophoretic forces came from the experiments by Klenchin et al. [34] and Sukharev et al. [35] who did not only prove that DNA was passing the cell membrane during the electric field pulse, but also extended the theory by suggesting that electric field had a double action, first on the cell membrane (by forming the membrane pores), and second, on the DNA molecules susceptible to orientation and deformation. According to this model, the electric pulses are responsible for DNA transfer through the cell membrane and mechanical interaction with the membrane pores, as well as for simultaneous pore expansion. Although this theory set the fundamental principles of electrotransfection, there was still a substantial gap in understanding the molecular mechanisms of the DNA transfer into the cell. One more step forward in understanding the electrotransfection was made by Teissie, Rols and Neumann. This group of authors and their co-workers demonstrated experimentally and theoretically that, when the pulses with a duration that was longer than the critical value were applied, the plasmid DNA formed a stable complex with the electropermeabilized membrane; the introduction of the DNA into the cell was controlled by membrane enzymatic activities (necessary for passing of the molecules through the membrane) [9,18,36–38]. Therefore, in the process of electrotransfection, two main activities are involved: the membrane poration and DNA transport through the cell membrane. The poration kinetics includes four different steps: (i) electric field induction of local defects on the cell membrane (poration), (ii) size expansion of defects (as long as the electric field is present), (iii) stabilization of electropermeabilized membrane and (iv) resealing of the membrane [38]. The DNA transport occurs partially under the direct action of electrophoretic forces and partially through enzymatic cell membrane transportation.

With no intention to study the mechanism involved in the process of electrotransfection, the present study was aimed to optimize the conditions for electrotransfection of a larger number of different mammalian cells in vitro. The electroporation parameters were optimized on Eppendorf's device for six laboratory cell lines and the yielded results of transfection efficiency as well as of cell survival were surprisingly good. Except in SCK cells, where the electric current could not affect the cell membrane (even under high electric field strength—6000 V/cm), the transfection efficiency in other five lines was at least comparable to the transfection efficiency of receptor-mediated gene transfer, or in some cases, even to the transduction efficiency of viral vectors [28–31,39,40]. The comparison of the transfection efficiency achieved in our study (40–85% of viable cells, except in SCK) with the ones in other electrotransfection studies was not simple since many authors presented the level of transfected gene activity (e.g. for gene encoding Green Florescence Protein, or for gene encoding Luciferase) instead of presenting the percentage of transfected cells [9,37,41]. To our knowledge, some of the authors also cited the percentage of transfected cells that varied from 1% to 20% in case of in vitro electrotransfection with β-Gal [37,42,43].

According to our findings, the determination of appropriate electroporation buffer and respective cell diameter were of extreme importance. As indicated by Sukhorukov et al. [44], the electropermeability of the cell membrane is easily achieved when the electroporation is carried out in the buffer with the osmolarity just above the critical one for the particular type of cells. Similar observations were described by Golzio et al. [45] who showed that the electroloading of β-galactosidase was decreased in a hyperosmotic and increased in hypoosmotic buffer. They concluded that the membrane undulation and hydration forces controlled repulsive forces preventing protein transfer across the electropermeabilized membrane during the pulse. On the other hand, Neumann et al. [36] theoretically proved that electroporation more frequently occurs in the strongly curved parts of the membranes than in its planar parts. This is probably not in collision with the described results since the basic molecular membrane structure is not affected by cell swelling and the fragility of primarily strongly curved membrane parts in the electric field just becomes intensified due to the hydration forces and a more profound membrane exposure to electric forces. In other words, this means that the optimal electric field strength should be calculated with regard to the maximum cell diameter when the cells are maximally swollen. In the majority of cell lines, the electric field strength calculated in this way was quite close to the optimum and only fine-tuning was needed to achieve a compromise between the optimal transfection rate and optimal survival of the electroporated cells. However, there was a difference between the cells: B16F1 demonstrated a sharp field strength optimum for the cell survival and transfection rate (at 650 V/cm) (Fig. 2), while in SA1, LPB, L929 and CHO cells, the higher the field strength, the better the transfection and the lower the survival rate (Figs. 2–4).

The determined optimal number of two to three pulses was constant for all of the cells tested. This is in accordance with the above described theories that, with the first pulse, the external electric field initializes the formation of membrane pores and that the second pulse is required to transfer DNA by electrophoretic forces and enzymatic cell membrane transportation. Actually, we believe that the second pulse is required to trigger the electrophoretic processes that also include electroosmosis and diffusion, and to support the endocytosis that is predominately dependent on the active cell transmembrane transportation. The pulses applied in our study were exponentially diminishing and, considering the transfection efficiency, two of them delivered enough energy to transport DNA into the large number of cells. An additional increase in the pulse number resulted rather in increased cell damage than in increased transfection efficiency. Thus, instead of delivering a few square wave pulses with the usual frequency of 1 Hz [9,37], we delivered just two exponentially diminishing pulses. The pulse voltage is readjusted every 5 μs. After the time constant, the pulse voltage has dropped down to 37% of its initial value. The interval of 1 min between these two pulses was determined by the manufacturer of the electroporator and is intended to offer the electroporated cells some time to regenerate certain essential membrane structures and functions [21]. Although 1-min period might seem to be too long, Teissié et al. [38] provided the data that at least 50% of cells are still permeabilized 6 min after the initiation of permeabilization. One of the latest article by Golzio et al. [46] describes the plasmid interaction with the electropermeabilized part of the membrane and formation of plasmid-membrane aggregates that remain stable at the membrane up to 10 min after the pulses of a longer duration than the critical value have been applied. Considering these data, the prolonged time interval between the pulses should have no negative effect on the final electrotransfection result.

The number of electric pulses used in other studies (range 1–10 pulse) was different from the number in our study (range 1–4 pulses) [9,35,37,41,42]. The most commonly used electric field strengths for electroporation of mammalian cells were between 0.2 and 2.0 kV/cm with the pulse duration of 1–100 μs or even 1–10 ms. The determined electric field strength in our study was just within the ranges used in other studies, while the pulse duration (as well as the number of pulses) was different due to the fact that we used exponentially diminishing pulses and other authors square wave ones.

Supposing that the first pulsation of the cell membrane resulted from the formation of numerous pores, it could then be expected that the increase of DNA concentration should be followed by the increased transfection efficiency. As a matter of fact, the increase of DNA concentration in our experiments had a negative influence on the transfection efficiency. Namely, the optimal concentration for the electrotransfection of 1×10^6 cells was 2.5–5 μg/ml of DNA, which is comparable to the concentrations used by some authors [37], but at least 10 times lower than those reported by other authors [47]. However, a saturation effect of plasmid DNA has already been described previously [11,41]. Furthermore, we observed no significant difference in assessing the effect of the temperature on the electrotransfection efficacy at 4 °C or at room temperature. This is slightly different from what Rols et al. [43] described when they compared the electrotransfection efficacy of CHO cells with β-galactosidase reporter gene at 4 °C with the one at 21 °C. Applying 10 square wave pulses with the duration of 5 ms at 600 V/cm, they accomplished to transfect 9% of cells at 4 °C and 6% at 21 °C. We assume that such a difference was not observed in our experiments primarily because we did not preincubate the cells at 4 °C before the electrotransfection procedure (like it was done by Rols et al.). In our experiments, the cells were just electrotransfected at the indicated temperatures and, when the procedure was finished, the cells were incubated at 37 °C.

The question why the electric current did not affect the SCK cells is interesting, but in this article we could not provide the answer to it. The basic set of experiments

included the detection of expression of reporter gene in transfected cells and the clonogenic assay for the determination of cell survival. The results of these experiments showed a completely equal survival of the cells exposed to the electric field strength up to 2500 V/cm and of untreated control cells, as well as a complete absence of characteristic blue coloration of the substrate in the presence of β-galactosidase. The coloration could be absent because (1) the plasmid DNA did not enter the cells or (2) because the expression of the control gene was suppressed. Considering the fact that the survival of the treated SCK cells was unaffected (when the applied electric field strength was below 2500 V/cm), we presumed that the SCK cell membranes were not permeabilized. To confirm this hypothesis, an additional set of experiments was performed with trypan blue that should be internalized by the cells when their membrane is electropermeabilized. In the range of electric field strengths that were applied in our experiments (0–6000 V/cm), we could detect no dye internalization even though the cell survival assessed by clonogenic assay was reduced (as compared to control) following the treatment with the electric field strengths above 2500 V/cm. On one hand, this speaks for an unexpected cell membrane resistance (of SCK cells) to the electric field action and, on the other, that certain processes in the cells caused by high electric field inhibited cellular proliferation.

In conclusion, we have to accentuate that the main goal of this study was to determine the basic parameters for electrotransfection of various cell lines and to optimize the procedure to become useful for the transfection of a larger number of cells than demanded in clinical experiments. The achieved average transfection, especially in B16F1 cells (85%), was absolutely above our expectations and comparable to the values obtained by many other well established procedures. Unfortunately, we were not able to transfect all of the tested cell types meaning that this physical transfection method is not as unspecific as we supposed and, thereby, not suitable for all types of mammalian cells. Finally, it should be mentioned that the intensity of gene expression in the electrotransfected cells was slightly lower than in the cells transfected by the receptor-mediated gene transfer (even in cases where the percentage of transfected cells was higher), which is most likely due to the physical damage of the cell membrane and physiological processes in the cytoplasm. This speculation could be supported by the experimental results of other authors that confirmed the leakage of the important energy-supplying molecules, such as cAMP, ATP and GTP from electroporated cells. These molecules are known to play an important role in the organization of the cytoskeleton as well as of the membrane structure components. Additionally, it was proved that electropermeabilization could induce the generation of reactive oxygen species that affect the electropermeabilized cells [48,49]. Therefore, even when the plasmid DNA is successfully loaded into the cell cytoplasm, the gene expression and translation could be affected.

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