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Short communication

Effect of Mg ions on efficiency of gene electrotransfer and on cell electropermeabilization

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ABSTRACT

Gene electrotransfer is a promising nonviral method that enables DNA to be transferred into living cells with electric pulses. However, there are many parameters that determine gene electrotransfer efficiency. One of the steps involved in gene electrotransfer is interaction of DNA with the cell membrane. Divalent cations in the electroporative media can influence the anchoring of DNA to the cell membrane and by that gene electrotransfer efficiency. Here we report the effect of different concentrations of Mg^{2+} on electropermeabilization for small molecule (propidium iodide), gene electrotransfer and viability of the cells. We also used TOTO-1 dye to visualize DNA-cell membrane interaction for different [Mg]. For this purpose, we used different electroporative media with increasing [Mg]. Our study shows that higher [Mg] lead to higher electropermeabilization for propidium iodide and higher viability, while causing lower gene electrotransfer efficiency. Because we observed higher TOTO-1 labeled DNA at cell surface when using higher [Mg], we suggest that Mg^{2+} ions can bind DNA at cell surface at such strength that cannot pass into the cell during application of electric pulses, which can lead to lower gene transfection. There may also be other mechanisms involved, since there are many steps of gene electrotransfer on which Mg^{2+} ions can have an effect on. Our results also imply that membrane permeability changes are not sufficient for an efficient gene electrotransfer.

1. Introduction

Exposing cells to short intense electric pulses increases permeability of cell membrane. It has been shown on lipid membrane model that electric field hastily reorients lipid heads in the membrane and that the number of local spots with fluid conformation increases [1]. The phenomenon is shown as electroporation or electropermeabilization. It has been used for many years to introduce small molecules that otherwise cannot pass the cell membrane into cells [2–4]. In 1982 Neumann with colleagues first achieved successful transfection of a foreign gene into eukaryotic cells with electric pulses [5,6]. Even though today gene electrotransfer is widely used to transfect all types of cells and represents a safer alternative to viral vectors, the processes underlying transfer of genetic material through cell membrane and into the cell are still not completely understood [7,8].

Different mechanisms of electropermeabilization for small molecules have been reported [9–14]. In addition, gene electrotransfer mechanisms are also available in the literature which suggests that several steps are involved in gene electrotransfer [10,13,15–21]: (a) formation of a complex between DNA and cell membrane, (b) translocation of DNA across the permeabilized membrane, (c) transfer of DNA from cytoplasm into nucleus and (d) gene expression.

The main obstacle in gene electrotransfer of mammalian cells is its low efficiency, which depends not only on permeability changes of cell membrane but also on the way DNA interacts with the membrane and migrates towards the nucleus. Although several studies showed that different parameters (e.g. cell type, temperature, parameters of electric pulses, ions in electroporative media) have influence on the efficiency of uptake of small molecules as well as on gene electrotransfer [10,22–39], it was also suggested that one of the key parameters which affect the process of DNA interaction with the cell membrane is the concentration of ions in media [15,17,40]. It was suggested that especially divalent cations (such as Ca^{2+} or Mg^{2+}) may have important impact on forming a complex between DNA and the cell membrane during application of electrical pulses, which can lead to the improvement of gene electrotransfer [17,29,40]. Namely, since DNA is negatively charged polyelectrolyte, divalent cations can bridge the DNA with negatively charged cell membrane during application of electric pulses. This hypothesis was supported by the study of anionic unilamellar vesicles, where DNA adsorption to vesicle membrane was greatly enhanced with increasing concentration of divalent cations such as Ca^{2+} ions [41,42].

Up to now only few researchers have experimentally investigated the effect of different ions on gene electrotransfer efficiency in vitro or in vivo [5,6,17,28,29,32,40], however their results are contradictory.

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In one of the in vitro studies researches observed increased gene electrotransfer efficiency for increased [Mg] from 1 to 8 mM [17]. However, this is not in agreement with Neumann et al. [5,6] study. This can be a consequence of small DNA concentration used by Xie and Tsong [17], which resulted in a relatively low percentage of transfection (max. 9%) and therefore higher [Mg] could have a positive effect at such low plasmid concentration.

In in vivo studies the influence of a wide range of ionic composition in electroporative media on gene electrotransfer was also analyzed. It was demonstrated that gene expression decreased for higher concentrations of ions in used media [28]. Further some researchers showed that higher concentration of divalent cations limits plasmid DNA entrance into the cell during electroporation [32]. They proposed that higher concentration of divalent cations alters stability and physical properties of DNA molecules. However, in another in vivo study it was shown, that gene electrotransfer efficiency in mice is improved by increasing the concentrations of ions in initial injected media [29].

Hence, we report here a study of the influence of different [Mg] in electroporative media on electropermeabilization for propidium iodide (PI), cell viability and gene electrotransfer. The main objective of our study was to understand the role of Mg^{2+} ions on different steps of gene electrotransfer by analyzing separately effects of Mg^{2+} ions on electropermeabilization for PI, viability and transfection.

2. Materials and methods

2.1. Cell culture

Chinese hamster ovary cells (CHO-K1) were grown in Ham's tissue culture medium for mammalian cells with 10% fetal bovine serum (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) at 37 °C in a humidified 5% CO_2 atmosphere in the incubator (Kambič, Slovenia).

Electroporation was performed on 24 hour old cell culture in different electroporative media.

2.2. Plasmid DNA

Plasmid pEGFP-N_1 (Clontech Laboratories Inc., Mountain View, CA, USA) encoding green fluorescent protein (GFP) was amplified in DH5α strain of Escherichia coli and isolated with HiSpeed Plasmid Maxi Kit (Qiagen, Hilden, Germany). Plasmid DNA concentration was spectrophotometrically determined at 260 nm and confirmed by gel electrophoresis.

2.3. Electropermeabilization for propidium iodide

To evaluate electropermeabilization for propidium iodide of CHO cells in different electroporative media with different concentration of Mg^{2+} ([Mg] = 1, 4, 10 and 50 mM), propidium iodide (PI) was used. PI is a small molecule which enters a cell, if the membrane of the cell is permeabilized [43]. All electroporative media were isoosmolar (10 mM phosphate buffer, NaH_2PO_4/Na_2HPO_4, pH = 7.4), where media with [Mg] = 1 mM represents standard electroporative media.

Cell suspension was prepared by 0.25% trypsin/EDTA solution (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany), centrifuged for 5 min at 1000 rpm (180×*g*) at 4 °C (Sigma, Germany) and resuspended in different electroporative media to a cell density of $\rho = 2.5 \times 10^6$ cells/ml. Because of easier survey of the results we used only electroporative media with [Mg] = 1 mM, 4 mM, 10 mM and 50 mM. For electroporation, cuvettes with built in aluminium electrodes were used (Eppendorf, Hamburg, Germany).

The volume of the cells placed in cuvette was 200 µl (for each electric pulse parameter). Immediately before electric pulse application, 2 µl of 0.15 mM PI was added to the media. Final concentration of PI in a sample was 10 µg/ml.

Samples were then exposed to electric pulses to deliver PI into the cells using Cliniporator™ (IGEA s.r.l., Carpi, Modena, Italy) pulse generator. A train of four rectangular pulses with duration of 200 µs and repetition frequency 1 Hz was applied. The applied voltages were 240 V, 400 V and 560 V which resulted in 0.6 kV/cm, 1.0 kV/cm and 1.4 kV/cm *E*, respectively. Applied electric field is defined by

$$E = U(\text{appl}) / d, \tag{1}$$

where *U*(appl) denotes applied voltage and *d* electrode distance (*d* = 4 mm). To achieve saturation of fluorescence of PI in cells we also exposed cells to *E* = 1.8 kV/cm. No electric pulses were applied to cells in control.

After pulses were applied, cells were incubated for 3 min at room temperature (22 °C) and then centrifuged for 5 min at 1000 rpm (180×*g*) at 4 °C to remove extracellular PI that did not enter the cells. 200 µl of fresh media was added and the uptake of PI was evaluated with spectrofluorometer (Tecan infinite M200, Tecan Austria GmbH) at 617 nm. The permeabilization (uptake of PI) is defined as:

$$\text{Permeabilization } (\%) = \frac{F_{(\text{PI,E})} - F_{(\text{PI,E}=0)}}{F_{(\text{PI,max})} - F_{(\text{PI,E}=0)}} \cdot 100, \tag{2}$$

where $F_{(\text{PI, E})}$ denotes fluorescence intensity of cells subjected to electric pulses, $F_{(\text{PI, E}=0)}$ fluorescence intensity of cells at *E* = 0, i.e. cells in control, and $F_{(\text{PI, max})}$ fluorescence intensity of cells at *E* = 1.8 kV/cm, i.e. where saturation fluorescence is achieved.

2.4. Cell viability

Cell viability was evaluated with crystal violet dye elution method (CVDE) as previously described [44]. After exposing cells to electric pulses with plasmid DNA in concentration 40 µg/ml, the cells were plated in multiwells at a cell density of $\rho = 1 \times 10^5$ cells/ml and grown for 24 h in cell culture medium at 37 °C in a humidified 5% CO_2 atmosphere in the incubator. Because of easier survey of the results we used only electroporative media with [Mg] = 1, 4, 10 and 50 mM.

After 24 h samples were stained with 0.1% crystal violet (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) solution in sodium phosphate media (10 mM phosphate buffer, NaH_2PO_4/Na_2HPO_4, pH = 7.4) for 30 min at room temperature (22 °C). After incubation, crystal violet was removed and cells were washed with sodium phosphate media and lysed with 10% acetic acid. The same staining protocol with crystal violet was performed also in wells without cells (background wells). CVDE is a simple assay that evaluates cell density by staining DNA. After elimination of the excess dye, the absorbance at 540 nm was measured with spectrofluorometer (Tecan infinite M200, Tecan Austria GmbH), which is proportional to the amount of viable cells in the well.

The experimental fraction of cells stained with crystal violet (CV) is defined as:

$$f(\text{CV}) = \frac{A_{(\text{CV,E})} - A_{(\text{CV,bg})}}{A_{(\text{CV,E}=0)} - A_{(\text{CV,bg})}}, \tag{3}$$

where $A_{(\text{CV, E})}$ denotes absorbance of cells subjected to electric pulses, $A_{(\text{CV, bg})}$ background absorbance and $A_{(\text{CV, E}=0)}$ absorbance of cells at *E* = 0, i.e. that of non-pulsed cells.

The fraction of stained cells equals cell viability, therefore *f*(CV) = *f*(viability) and:

$$\text{Viability } (\%) = f(\text{CV}) \cdot 100. \tag{4}$$

2.5. Gene electrotransfer

Electroporation was performed on CHO cells that were in the exponential growth phase. Cell suspension was prepared in the same