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REVIEW

What is (Still not) Known of the Mechanism by Which Electroporation Mediates Gene Transfer and Expression in Cells and Tissues

Jean-Michel Escoffre · Thomas Portet · Luc Wasungu · Justin Teissié · David Dean · Marie-Pierre Rols

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Abstract Cell membranes can be transiently permeabilized under application of electric pulses. This treatment allows hydrophilic therapeutic molecules, such as anticancer drugs and DNA, to enter into cells and tissues. This process, called *electropermeabilization* or *electroporation*, has been rapidly developed over the last decade to deliver genes to tissues and organs, but there is a general agreement that very little is known about what is really occurring during membrane electropermeabilization. It is well accepted that the entry of small molecules, such as anticancer drugs, occurs mostly through simple diffusion after the pulse while the entry of macromolecules, such as DNA, occurs through a multistep mechanism involving the electrophoretically driven interaction of the DNA molecule with the destabilized membrane during the pulse and then its passage across the membrane. Therefore, successful DNA electrotransfer into cells depends not only on cell permeabilization but also on the way plasmid DNA interacts with the plasma membrane and, once into the cytoplasm, migrates towards the nucleus. The focus of this review is to describe the different aspects of what is known of the mechanism of membrane permeabilization and associated gene transfer and, by doing so, what are the actual limits of the DNA delivery into cells.

Keywords Gene transfer · Gene expression · Membrane · Electric field · Electroporation · Electropermeabilization

Introduction

The administration of naked nucleic acids into cells and tissues can be considered as the simplest and safest method for gene delivery [1]. However, one drawback of the method for gene therapy is the low efficiency of gene expression. Therefore, different strategies have been developed for years in this field, based on the use of virus as biological vectors and on the development of chemical or physical methods. The common aspect of these methods is to transfer DNA into cells via cell membrane modification. Virus and chemical vectors fuse with the plasma membrane and/or are endocytosed. Physical approaches transiently destabilize the membrane creating leaky structures or "pores". One of the most successful, but indeed the only one, clinical trials of gene therapy has been obtained with virus for the treatment of severe combined immunodeficiency disease, but this clinical trial had to be suspended because the virus caused leukaemia due to the insertion of the gene near an oncogene [2–4]. Therefore, there is still nowadays a challenging need to develop an efficient and safe method for gene transfer.

The use of electric pulses as a safe tool to deliver therapeutic molecules to tissues and organs has been

Jean-Michel Escoffre and Thomas Portet have contributed equally to this work.

J.-M. Escoffre · T. Portet · L. Wasungu · J. Teissié · M.-P. Rols (✉)
CNRS, IPBS (Institut de Pharmacologie et de Biologie Structurale), 205, Route de Narbonne, 31077 Toulouse, France
e-mail: rols@ipbs.fr

J.-M. Escoffre · T. Portet · L. Wasungu · J. Teissié · M.-P. Rols
Université de Toulouse, UPS, IPBS, 31077 Toulouse, France

T. Portet · D. Dean
Laboratoire de Physique Théorique - CNRS UMR 5152, IRSAMC, Université Paul Sabatier, 118 Route de Narbonne, 31062 Toulouse cedex 4, France

rapidly developed over the last decade. The method refers to the transient increase in the permeability of cell membranes when submitted to electric field pulses. This process is commonly known as *electropermeabilization* or *electroporation* [5]. Hydrophilic molecules that are otherwise non-permeant, such as the highly toxic drug bleomycin, can gain direct access to the cytosol of cells. A cancer treatment modality, *electrochemotherapy (ECT)*, has emerged [6–8]. It is successfully used in clinical trials for cancer treatment [9–12]. Beside drugs, electropermeabilization can be used to deliver a wide range of potentially therapeutic agents including proteins, oligonucleotides, RNA and DNA [13]. Nowadays, it represents one of the most widespread techniques used in molecular genetics. In vivo gene electrotransfer is of special interest since it is the most efficient non-viral strategy for gene delivery and also because of its low cost, easiness of realization and safety. The most widely targeted tissue is skeletal muscle. The strategy is not only promising for the treatment of muscle disorders, but also for the systemic secretion of therapeutic proteins. Vaccination and oncological gene therapy are other fields of application [14]. *Electrogene-therapy (EGT)* is relevant in a variety of researches and clinical settings including cancer therapy, modulation of pathogenic immune responses, delivery of therapeutic proteins and drugs [15]. This, together with the capacity to deliver large DNA constructs, greatly expands the research and clinical applications of in vivo DNA electrotransfer [16–22].

However, the mechanisms underlying cell membrane permeabilization and associated gene transfer are not completely understood. This is of high importance for in vitro use in terms of efficiency but also for in vivo use in terms of security. The successful electrotransfer of plasmid DNA into cells depends on the way the cell membrane has been permeabilized, reversibly or not, and on the way DNA interacts with the membrane and is transported from the plasma membrane towards the nuclear envelope. The focus of this review is to describe the different aspects of what is known (and still not known) on the mechanism of membrane electropermeabilization and associated gene transfer and by doing so, reviewing what are the actual limits for DNA delivery into cells.

Basics of Cell Membrane Electropermeabilization

Membrane Electropermeabilization or Membrane Electroporation?

As its name suggests, the basic theory of *electroporation* is that the electric field causes the formation of pores in the membrane [23, 24]. Theoretical models have been proposed to explain the mechanism of this reversible membrane electropermeabilization and its potentiality to allow the access of non-permeant molecules inside the cells [25]. The formation of pores is supported by a thermodynamic approach because it allows the reduction of the electrostatic energy of the system. It is hindered by the line tension needed to create the associated transbilayer hole (assumed to be a cylinder). It is thus only at a critical value of the field strength (energy threshold) that their formation is possible. Roughly speaking, the poration process is an activated one, similar to nucleation, where the surface tension term favours pores formation while the line tension tends to suppress it. The effect of the electric field is to effectively increase the surface tension and thus lower the activation barrier [26].

It is widely accepted however that the standard theory of electroporation has limits [27]. These pores have never been observed. Large post pulse pores, arising from primary pores have indeed been detected in red blood cells pulsed under hypoosmotic conditions [28]. An alternative theory, which is less familiar because of conflicts with experimental results, is based on an electromechanical instability where the electric field compresses the membrane. Molecular dynamics and coarse grain simulations are the newest approaches. Computer simulations at the microscopic length scale are becoming possible, however the complexity and more importantly the size of the system that can be simulated are still rather limited [29]. Molecular dynamics has suggested that electropores (in fact water leaks) could be generated but they have been obtained only under field conditions larger than those experimentally required to induce reversible membrane permeabilization [30]. The geometry of the membrane and its mechanical properties such as surface to volume ratio, rigidity and composition, for example the role played by microdomains, must be taken into account as well as the surface tension and line tension associated with the pores. Indeed, only a few experimental data concerning the molecular changes involved in membrane electropermeabilization have been reported. ^{31}P NMR studies performed both on model membranes and on mammalian cells suggested a reorganization of the polar head group region of the phospholipids leading to a weakening of the hydration layer [31, 32]. Transbilayer reorientation of phospholipid probes has been reported in the human erythrocyte membrane suggesting an increase in the flip-flop of phospholipids as a direct consequence of electropermeabilization [33].

Altogether, and even if the term electroporation is commonly used among biologists, the term electropermeabilization should be preferred in order to prevent any molecular description of the phenomenon.

Membrane Electropermeabilization is Controlled by the Transmembrane Voltage

It has been known for more than 30 years that, as far as membrane permeabilization is concerned, the key effect of electric field on cells is a position-dependent change in the resting transmembrane potential difference $\Delta\Psi_o$ of their plasma membrane. If we model the cell membrane as a thin spherical dielectric shell, the electrically induced potential difference $\Delta\Psi_E$, which is the difference between the potential inside the cell Ψ_{in} and the potential outside the cell Ψ_{out}, at a point M on the cell surface is given by:

$$\Delta\Psi_E(t) = \Psi_{in} - \Psi_{out} = -g(\lambda)rE\cos\theta(M)\left[1 - e^{-t/\tau}\right] \quad (1)$$

where t is the time after the onset of the electric pulse, g depends on the conductivities λ of the membrane, of the cytoplasm and of the extracellular medium, r is the radius of the cell, E the field strength, θ(M) the angle between the normal to the membrane at the position M and the direction of the field and τ is the membrane charging time [34]. The field-induced potential difference is added to the resting potential [35, 36]

$$\Delta\Psi = \Delta\Psi_o + \Delta\Psi_E. \quad (2)$$

Being dependent on the angular parameter θ, the field effect is position-dependent on the cell surface. Therefore, the side of the cell facing the anode is going to be hyperpolarized while the side of the cell facing the cathode is depolarized (Fig. 1a). This theoretical prediction has been experimentally validated by using a voltage-sensitive fluorescent dye [37]. The transmembrane potential difference of a cell exposed to an electric field is therefore a critical parameter for successful cell permeabilization, whatever the size of the cell, its shape or orientation [38, 39]. It defines the sites (location, extend) where molecules uptake can take place.

Membrane Electropermeabilization is Controlled by Electric Field Parameters

Permeabilization indeed occurs only on the part of the cell membrane where potential difference has been brought at its critical value [36, 40]. This value has been evaluated in the order of 200–300 mV independently of the cell type [41, 42]. Permeabilization is therefore controlled by the field strength. This means that field intensity E larger than a critical value, Ep, must be applied. E_p is dependent on the size of the target cells. It ranges from values close to 200 V/cm in the case of large cells such as myotubes to 1–2 kV/cm in the case of bacteria [41]. Large cells are therefore more sensitive to lower field strengths than smaller ones. Electric field values have therefore to be adapted to each cell line in order not to affect their viability. The field strength triggers permeabilization: when E is larger than E_p, it controls the area of the cell surface which is affected [43]. From Eq. 1, it is clear that for field intensities close to E_p, permeabilization is only present for θ values close to 0 or π. Under that condition, only the localized parts of the membrane surface facing the electrodes are affected. However, within these permeabilized cell caps, the extent of permeabilization is not a function of the field strength [42, 44, 45], whereas, it is controlled by the number and duration of electric pulses [45]. So, membrane permeabilization only occurs for electric field values E higher than the threshold value E_p, whatever the pulse number and the pulse duration. Increasing E, above

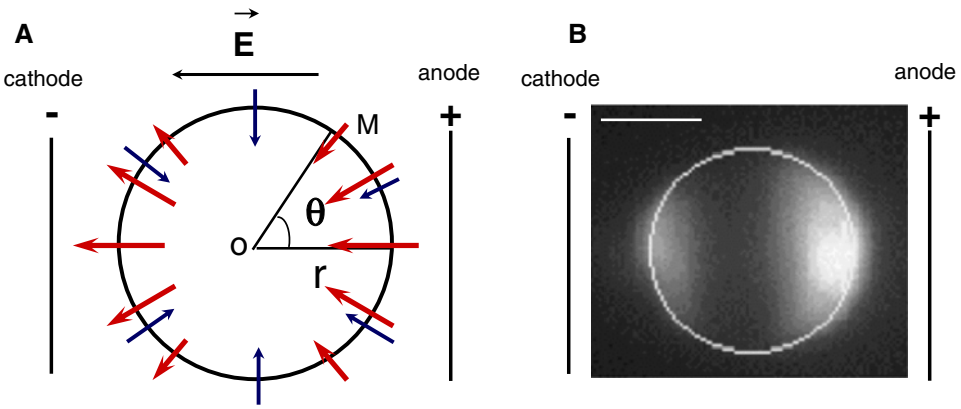

Fig. 1 Physical principle of electropermeabilization. **a** The plasma membrane is the site of native transmembrane potential difference, $\Delta\Psi 0$ (*Blue arrow*). If we consider the cell like a sphere and the plasma membrane like a dielectric spherical shell, when we apply an electric pulse, an induced transmembrane voltage $\Delta\Psi E$ is created (*Red arrow*). Being dependent on the angular parameter θ, the $\Delta\Psi E$ effect is position-dependent on the cell surface; therefore, the side of the cell facing the anode is hyperpolarized while the side of the cell facing the cathode is depolarized. **b** The localization and asymmetry of electropermeabilization can be detected by propidium iodide uptake in a CHO cell submitted to a train of 10 pulses, 5 ms, 1 Hz at 0.7 kV/cm. Scale bar: 10 μm

E_p, leads to an increase in the area where permeabilization takes place and, in that particular area, the extent of permeabilization is determined by the number and duration of electric pulses.

This electro-induced permeabilization of the cell membrane can be quantified in terms of the flow F_S of molecules S diffusing through the plasma membrane. Fick's law and experimental data obtained in the case of the release of ATP from mammalian cells allowed to establish that:

$$F_S(t) = P_S x(N,T) A/2\left(1 - E_p/E\right)\Delta S\, e^{-k(N,T)t} \quad (3)$$

where P_S is the permeation coefficient of the molecule S across the membrane, x is a function which depends on the pulse number N and the pulse duration T, it represents the probability of permeabilization ($0 < x < 1$), A is the cell surface, E is the applied electric field intensity, E_p the threshold for permeabilization, ΔS is the concentration difference of S between cell and external medium, k is the time constant of the resealing process and t is the time after the pulse [44]. Such a concept leads to the notion of *membrane domains* involved in electropermeabilization: *macrodomains* where permeabilization can take place, which area is determined by the pulse intensity according to: $A/2\ (1 - E_p/E)$, and, within that macrodomains, *microdomains (defects, permeant structures)* where permeabilization actually takes place, which density depends on the pulse number and on the pulse duration according to the x function. The molecular characteristics of these domains in terms of lipid and proteins composition, structures and dynamics remain an open question.

Membrane Electropermeabilization is a Fast and Localized Process

The use of videomicroscopy allowed visualization of the permeabilization phenomenon at the single cell level. Propidium iodide, a fluorescent non-permeant molecule, can be used as a probe for small molecules. Its uptake in the cytoplasm is a fast process that can be detected during the seconds following electric pulses application. In less than 1 min, it appears at the nucleus level. Moreover, exchange across the pulsed cell membrane is not homogeneous on the whole cell membrane. It occurs at the sides of the cells facing the electrodes on an asymmetrical way (Fig. 1a). It is more pronounced at the anode-facing side of the cells than at the cathode one, i.e. in the hyperpolarized area than in the depolarized one [46], in agreement with the above theoretical considerations and Eqs. 1 and 2.

Electropermeabilization can therefore be described as a 3-step process in respect with electric field (EF):

(i) Before EF: membrane acts as a barrier that prevents the free exchange of hydrophilic molecules between cell cytoplasm and external medium.
(ii) During EF: when reaching a threshold value, the transmembrane potential increase induces the formation of local *Transient Permeable Structures* facing the electrodes that allow the exchange of molecules.
(iii) After EF: resealing is occurring. Membrane permeability to small molecules is present with a lifetime ranging from seconds to minutes depending on EF conditions and on the temperature [44, 47]. After resealing, the uptaken solutes are sequestered inside the treated cell.

However, in the case of larger molecules (MW above 4 kDa), direct transfer to the cytoplasm is only observed if macromolecules (proteins, DNA) are present during the electric pulse. Proteins added after the permeabilizing electric field can indeed enter the cell by macropinocytosis [48, 49]. Gene expression is only obtained when DNA is present during application of the pulses. Therefore, the mechanism of macromolecules uptake is different from the one observed for small molecules.

Basics of Plasmid DNA Electrotransfer and Expression

Theoretical Considerations

Although the first pioneering report on plasmid DNA electrotransfer in cells has been published more than 20 years ago by E. Neumann [23], the mechanisms that mediate DNA electrotransfer remain to be elucidated. Different scenario can be proposed [50]. The simplest one is that the membrane is permeabilized, then the DNA is pushed through the *putative electropores* by the electrophoretic effect, which may be necessary to overcome the problem of passing a charge into the relatively low dielectric medium of the membrane. Another possibility is that the electric field leads to an aggregation of ion pumps which open and permit the passage of the DNA. Finally, another scenario is that the DNA forms a charged vesicle which is internalized by the cell in an endocytic process.

IIn the context of studies on model membranes, DNA interactions with lipid bilayers have indeed been studied. DNA injection by a micropipette, to a part of a giant unilamellar vesicle, resulted in membrane topology transformations which can be monitored using phase contrast microscope [51, 52]. DNA-induced endocytosis was observed in the absence of any electric field. A possible mechanism for DNA/lipid membrane interaction is DNA encapsulation within an inverted micelle included in the lipid membrane. High-molecular mass DNA that was

efficiently taken up by large unilamellar vesicles exposed to a short pulse of electric field (0.1–1 ms) with an intensity as high as 12.5 kV/cm indicated that DNA was taken up as a result of the electrostimulated formation of endosome-like vesicles rather than via field-induced membrane pores [53]. Other data report that electrotransfer of DNA through lipid bilayer could be mediated by transient complexes between DNA and the lipids in the pore edges of elongated, electropercolated hydrophilic pore zones [54]. Moreover, the association of DNA with a lipid bilayer greatly facilitates the transport of small ions. This suggests a locally conductive DNA/lipid interaction zone where parts of the DNA strand may be transiently inserted in the bilayer, leaving other parts of the DNA probably protruding out from the outer surface of the bilayer. DNA is not only transiently inserted in, but also actually electrophoretically pulled through, the permeabilized zones onto the other membrane side leaving finally the bilayer structure basically intact [55].

DNA Electrotransfer Depends on Membrane Permeabilization but also on DNA Electrophoresis

Electrotransfection, i.e. plasmid DNA electrotransfer into cells and then its expression, is detected for electric field values leading to plasma membrane permeabilization. Millisecond pulses are generally required to obtain efficient gene expression i.e. keeping intact the cell viability, by limiting the electric field intensities required when short pulses are used [47, 56, 57]. Nevertheless electrotransfection can be obtained with short strong pulses [23]. Transfection threshold values are the same as those for cell permeabilization [58].

The transfection efficiency TE obeys the following equation:

$$\mathrm{TE} = KNT^{2.3}\left(1 - E_p/E\right)f(\mathrm{ADN}) \quad (4)$$

where K is a constant. The dependence on the plasmid concentration f(ADN) is rather complex and it is observed that high levels of plasmids are toxic [59]. The effect of pulse duration appears to be crucial. Pulse duration appears to be a key parameter for efficient gene expression in cells and tissues [23, 60].

Electrically induced DNA uptake by cells is a vectorial process with the same direction as DNA electrophoresis in an external electric field [61, 62]. The transfection efficiency is significantly higher in cell monolayers facing the cathode compared to those exposed to field pulses of the reverse direction. This is due to contribution of the electrophoresis to the translocation of the polyanionic plasmid DNA across the electropermeabilized cell membrane [63]. The polarity of the electric field has therefore a direct effect on transfection. Adherent cells facing the cathode exhibit much higher transfection yield as well as gene expression than cells facing the anode [63]. This dependance of the transfection efficiency on the direction of the field might be due to the involvement of the electrophoretic force in the translocation of the negatively charged DNA molecule [61]. While cell permeabilization is only slightly affected by reversing the polarity of the electric pulses or by changing the orientation of pulses, transfection level increases are observed. These last effects are due to an increase in the cell membrane area where DNA interacts [64].

The use of a two-pulse technique allowed the separation of the two effects provided by an electric field: membrane electropermeabilization and DNA electrophoresis. The first pulse (high voltage, μs time range duration) creates permeabilization efficiently. The second pulse of much lower amplitude, but substantially longer (ms time range), does not cause permeabilization and transfection by itself but enhances TE in muscles by about one order of magnitude. In vivo, the effect of electrophoresis is not completely understood. A recent publication indicates that electrophoresis may not be instrumental in electro-gene transfer [65]. But several studies from the group of Mir show the benefit of the combination of short high-voltage and long low-voltage pulses allowing to evidence the necessity of association of cell electropermeabilization and convenient electrophoretic transport of DNA towards and/or across the permeabilized membrane within the tissue [66–68]. DNA electrotransfer has indeed been achieved in tibialis cranialis muscles of C57BL/6 mice by using such long but low-intensity pulses [69, 70]. Other data performed in the skin also demonstrate that the combination of such electric pulses is an efficient protocol to enhance DNA expression [71].

DNA Electrotransfer is a Multistep and Localized Process

Fluorescent plasmids indeed allow to monitor the interaction of nucleic acids with field treated cells. DNA molecules, negatively charged, migrate when submitted to an electric field. In low electric field regime, the DNA simply flows around the membrane towards the anode. However beyond a critical permeabilizing field value ($E_c > E_p$) the DNA interacts with the permeabilized membrane, but only at the pole facing the cathode (as opposed to the case for small molecules that are observed to enter the cells through both poles) [46]. Clusters or aggregates of DNA are formed, but once the field is turned off the growth of these clusters is stopped. The DNA/membrane interaction is not homogeneously distributed on the permeabilized areas facing the cathode (macrodomains) but is present into membrane *competent sites* whose size

range from 0.1 to 0.5 μm. These observations are consistent with a process where plasmids interact with electropermeabilized part of the cell surface, following their interfacial electrophoretically driven insertion [5, 72]. Due to the good correlation between visualization of DNA insertion in the membrane and gene expression, these results are consistent with a multistep process of DNA electrotransfer (Fig. 2):

(i) Before EF: membrane acts as a barrier that prevents the entrance of plasmid DNA into the cell.
(ii) During EF, the plasma membrane is permeabilized on the sides of the cell facing the electrodes and the negatively charged DNA migrates electrophoretically towards the plasma membrane on the cathode side where it is inserted in particular domains.
(iii) After EF, a translocation of the plasmid to the cytoplasm takes place leading to gene expression. This second step, including plasmid DNA diffusion in the cytosol and its passage through the nuclear pores, remains rather poorly understood.

New directions of research are needed to characterize membranes domains involved in molecule electrotransfer. DNA transfer occurs through *competent domains* present in the electropermeabilized cell membrane. Their size is in the same range of order than the so-called rafts domains. Lipid rafts are plasma membrane microdomains enriched in sphingolipids and cholesterol. These domains have been suggested to serve as platforms for various cellular events, such as signalling and membrane trafficking [73, 74]. One can wonder if they are involved in DNA electrotransfer.

DNA Expression from DNA Electrotransfer is Hindered by Internal Membrane

If the first steps of gene electrotransfection, i.e. migration of the plasmid DNA towards the electropermeabilized plasma membrane and its interaction with it, are starting to be described and therefore represent guidelines to improve gene electrotransfer, the successful expression of the plasmid depends on its subsequent migration into the cell. Therefore, diffusional properties of plasmid DNA, metabolic instability of plasmid DNA in cells and tissues as well as plasmid DNA nuclear translocation represent cell limiting factors that have to be considered [75].

The cytoplasm is composed of a network of microfilament and microtubule systems, and a variety of subcellular organelles bathing in the cytosol. The mesh-like structure of the cytoskeleton, the presence of organelles and the high protein concentration impose an intensive molecular crowding of the cytoplasm which limits the diffusion of large sized macromolecules. The translational mobility of macromolecule smaller than 500–750 kDa is only 3–4 fold slower than in water, but it is markedly impeded for larger molecules [76]. Mobility of plasmid DNA is negligible in the cytoplasm of microinjected myotubes [77]. But, successful in vivo DNA expression can be obtained by electric fields. These discordant results might be explained by the disassembly of the cytoskeleton network that may occur

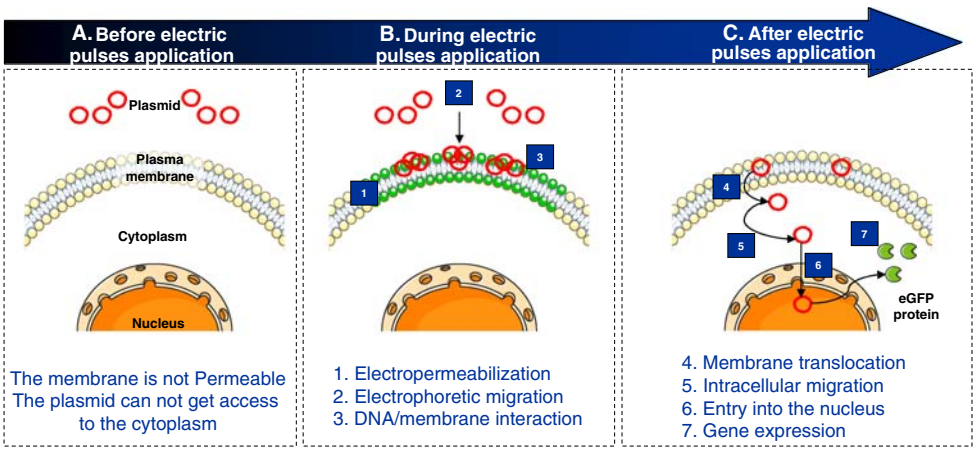

Fig. 2 DNA electrotransfection: Electrotransfection mechanism is a multistep process. **a** Before electric pulses application: DNA molecules are labelled with fluorescent marker, rhodamine. DNA molecules are incubated with CHO cells. No natural adsorption of DNA molecules on plasma membrane is observed. **b** During electric pulses application: Plasma membrane is electropermeabilized (step 1; in *green*). DNA molecules undergo the electrophoretic migration (step 2) and interact with permeabilized membrane. DNA aggregates are formed (step 3). This interaction takes place only on the membrane facing the cathode. **c** After electric pulses application: DNA translocation into the cytoplasm occurs 30 min after application of electric pulses (step 4). Then, DNA molecules migrate into the cytoplasm (step 5). About 2 h after electric pulses application, DNA molecules are present at nucleus level (step 6). Finally, 24 h after electrotransfection, eGFP expression is detected under fluorescence microscopy (step 7)

during electropermeabilization [78] and reinforces the idea that the cytoplasm constitutes a diffusional barrier to gene transfer.

Stability of plasmid DNA can be quantitatively assessed by microinjection. Such experiments revealed that 50% of the DNA is eliminated in 12 h from HeLa and COS cells and in 4 h from myotubes [79]. Cytosolic elimination of plasmid DNA cannot be attributed to the only cell division, since degradation is observed in cell cycle arrested cells. In the case of tissues, radiolabelled plasmid indeed progressively leave muscles and is degraded as soon as 5 min after plasmid injection, with or without electrotransfer. While a major part of plasmid DNA is rapidly cleared and degraded, the electrotransferable pool of plasmid DNA represents a very small part of the amount injected and belongs to another compartment where it is protected from endogenous DNAses [80].

Finally, beside cytoskeleton, the nuclear envelope represents the last physical obstacle to the expression of the plasmid DNA. The inefficient nuclear uptake of plasmid DNA from the cytoplasm was recognized more than 20 years ago. While molecules smaller than 40 kDa can diffuse through the nuclear pore complexes, larger molecules must carry a specific targeting signal, the nuclear localization sequence to traverse. The significant size of plasmid DNA (2–10 MDa) makes it unlikely that the nuclear entry occurs by passive diffusion. Dividing cells are highly transfectable compared to quiescent ones, suggesting that DNA enter the nucleus upon the disassembly of the nuclear envelope during mitosis. Cell synchronization indeed affects gene delivery by electric field [81–83], reinforcing the statement that the melting of the nuclear membrane facilitates direct access of plasmid DNA to the nucleus.

Therefore, clear limits of efficient gene expression by electric pulses are due to cytoplasm crowding and transfer through nuclear envelope. New challenges are to overcome these limiting steps. The dense latticework of the cytoskeleton impedes free diffusion of DNA. However, since transfections do work, there must be mechanisms by which DNA circumvents cytoplasmic obstacles. One possibility is that plasmids become cargo on cytoskeletal motors, much like viruses do, and move to the nucleus in a directed fashion. Electrotransfered plasmid DNA, containing specific sequences, like most viruses, could then utilize the microtubule network and its associated motor proteins to traffic through the cytoplasm to the nucleus [84, 85].

Another alternative could come from nanosecond pulsed electric fields. New findings indeed indicate that very short (10–300 ns) but high pulses (up to 300 kV/cm) extend classical electropermeabilization to include events that primarily affect intracellular structures and functions. As the pulse duration is decreased below the plasma membrane charging time constant (see Eq. 1), plasma membrane effects decrease and intracellular effects predominate [86, 87]. When used in conjugation with classical electropermeabilization, nanopulses can increase gene expression. The idea is to perform classical membrane permeabilization allowing plasmid DNA electrotransfer, and then 30 min later to permeabilize specifically the nuclear envelope by using short nano pulse EF. By this way, it will become possible not only to electropermeabilize cells but also to electromanipulate them.

Conclusions

Clear differences of processes by which molecules of different sizes translocate across the electropermeabilized membrane have been observed. While small soluble molecules could rather freely cross the permeabilized membrane for a time much longer than the duration of the electric pulse application, DNA transfer involves complex steps including interaction with the permeabilized membrane and migration into the cytosol. If the effects of the electric field parameters are about to be elucidated (pulse strength higher than a threshold value, long pulse duration for efficient gene expression), the associated destabilization of the membrane which is a stress for the cells and may affect the cell viability has still to be clearly described. Moreover, it becomes evident that extracellular as well as intracellular barriers compromise the transfection efficiency. Clearly, DNA electrotransfer through an electropermeabilized cell membrane is much more complex than the passage of a thread through the eye of a needle. The challenge for electro-mediated gene therapy is to pinpoint the rate limiting steps in this complex process and implement strategies to overcome the barriers encountered by therapeutic plasmid DNA.

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