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Electropermeabilization, a physical method for the delivery of therapeutic molecules into cells

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Abstract

Electropermeabilization designates the use of short high-voltage pulses to overcome the barrier of the cell membrane. A position-dependent reversible local membrane permeabilization is induced leading to an exchange of hydrophilic molecules across the membrane. This permeabilized state can be used to load cells with therapeutic molecules. In the case of small molecules, such as anticancer drugs, transfer occurs through simple diffusion. In the case of DNA, transfer occurs through a multi-step mechanism, a process that involves the electrophoretically driven association of the DNA molecule with the destabilised membrane and then its passage.

Keywords: Electric field; Electroporation; Electrotransfection; Electrochemotherapy; Electrogenotherapy

1. Introduction

Cell membrane acts as a barrier that hinders the free diffusion of molecules between cell cytoplasm and external medium. However, the permeability of membranes can be transiently increased when external electric field pulses are applied [1–3].

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. Nevertheless, the molecular definition of the "Transient Permeable Structures" is not yet known [4]. One of the longest accepted theories is based on the generation of electropores, leading to the term electroporation. However, these pores have never been observed. Large pores, arising from primary pores have indeed been detected in cells pulsed under hypoosmotic conditions [5]. More recently, molecular dynamics has suggested that electropores could be generated but, they have been obtained under field conditions larger than those experimentally required to induce reversible membrane permeabilization [6]. Only a few experimental data concerning the molecular changes involved in membrane electropermeabilization have been reported. ^{31}P NMR studies performed on mammalian cells suggested a reorganization of the polar head group region of the phospholipids leading to a weakening of the hydration layer. This was thought to explain the electric field induced fusogenicity of these cells [7]. Therefore, while the term electroporation is commonly used among biologists, the term electropermeabilization should be preferred in order to prevent any molecular description of the phenomenon.

The use of electric pulses to deliver therapeutic molecules to tissues and organs has been rapidly developed over the last decade. A new cancer treatment modality, electrochemotherapy, has emerged [8–10]. Trains of short (100 μs) high voltage (1.3 kV/cm) electric pulses are applied directly on the tumour following drug injection. Molecules that are otherwise non-permeant can gain direct access to the cytosol of cells. Highly cytotoxic molecules such as the hydrophilic drugs bleomycin and cisplatin have been successfully used in clinical trials for cancer treatment [11–14]. Successive treatments can be performed with a 2-week interval in the case of human cutaneous and subcutaneous malignant tumours [13] as in the case large animal tumours such as cat sarcoma [15] and horse sarcoid [16].

Beside drugs, electrotransfer can be used to deliver a wide range of potentially therapeutic agents including proteins, oligonucleotides, RNA and DNA [17,18]. The most widely targeted tissue for DNA transfer is skeletal muscle [19,20]. This strategy is promising for the systemic secretion of therapeutic

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proteins [21]. Vaccination and oncology gene therapy are also major fields of application of electrotransfer [22]. This, together with the capacity to deliver very large DNA constructs, greatly expands the research and clinical applications of in vivo DNA electrotransfer [23–27]. But the safe and efficient use of this physical method for clinical purposes requires the knowledge of the mechanism underlying that phenomenon of electropermeabilization.

Up to these last years, almost all investigations related to membrane electropermeabilization process, have been performed on cell populations. No clear evidence of the molecular mechanism has been obtained at the single-cell level. Gene transfer was always evaluated by the associated gene expression. Difficulties are due to the molecular definition of the events underlying membrane permeabilization and DNA transfer on a so complex system as a living cell. Therefore, there is a general agreement that very little is known at the molecular level about what is really occurring during membrane electropermeabilization [4]. With the development of cell imaging, it becomes now possible to visualize the membrane regions where the transfer of external molecules takes place.

The focus of this article is to make a short report on what is known on the processes supporting the electrically mediated membrane permeabilization and the DNA transfer in mammalian cells.

2. Experimental procedures

All experimental procedures have been described elsewhere and therefore are only briefly described here [28–31].

Chinese hamster ovary (CHO) cells were used. The WTT clone was selected for its ability to grow in suspension or plated on Petri dishes. They were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% foetal calf serum. Their ability to grow on a support after being maintained in suspension is direct evidence of their viability.

Electropulsation was operated by using a CNRS cell electropulsator (Jouan, France) which delivered square-wave electric pulses. An oscilloscope (Enertec, France) monitored pulse shape. A uniform electric field was generated between the electrodes connected to the voltage generator.

Penetration of a non-permeant fluorescent dye, propidium iodide, into cells was used to monitor permeabilization. Cells in suspension were centrifuged and resuspended in the pulsing medium (a low ionic content isoosmotic buffer). 10 pulses with controlled duration, at a frequency of 1 Hz, were applied at a given electric field intensity at room temperature. For plated cells, culture medium was removed and replaced by the same buffer described above. Gene transfer could be visualized by using plasmids labelled with a fluorescent dye (Toto-1). Gene expression could be visualized by using plasmids coding for the green fluorescent protein.

For microscopic observations, electropulsation chambers were designed using two stainless-steel parallel rods put on a microscope glass coverslip chamber. The chamber was placed on the stage of an inverted digitized videomicroscope (Leica DMIRB, Germany).

3. Results and discussion

3.1. Membrane permeabilization

It is known for more than 30 years that the exposure of cells to an electric field, a process called Electro-Pulsation, induces a position dependent change in their resting transmembrane potential difference $\Delta\Psi_o$. 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:

$$\Delta\Psi_E = \Psi_{in} - \Psi_{out} = -fg(\lambda)rE\cos\theta(M) \quad (1)$$

where f is related to the shape of the cell, 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 and $\theta(M)$ the angle between the normal to the membrane at the position M and the direction of the field [32]. The field induced potential difference is added to the resting potential [33,34]:

$$\Delta\Psi = \Delta\Psi_0 + \Delta\Psi_E \quad (2)$$

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. This theoretical prediction has been experimentally verified by using a voltage sensitive fluorescent dye [35]. The transmembrane potential on a cell exposed to an electric field is therefore a critical parameter for successful cell permeabilization. Theoretical and experimental results show that permeabilization is not only a function of electric field intensity and cell size but also of cell shape and orientation [36].

A general agreement is that electropermeabilization can be described as a 3-step-process by respect with Electro-Pulsation (Fig. 1):

(i) before EP: membrane acts as a barrier that prevents the free exchange of hydrophilic molecules between cell cytoplasm and external medium.
(ii) during EP: when reaching a threshold value (see below), the transmembrane potential increase induces the formation of local "Transient Permeable Structures", or "TPS", that allows the exchange of molecules.
(iii) after EP: resealing is occurring. Membrane permeability to small molecules is present with a lifetime ranging from seconds to minutes depending on EP conditions and on the temperature [37,38]. After resealing, the uptaken solutes are sequestered inside the treated cell.

Permeabilization indeed occurs only on the part of the membrane where potential difference has been brought at its critical value [34,39]. This value has been evaluated to be of the order of 200–300 mV whatever the cell type [40,41]. Permeabilization is therefore controlled by the field strength. This means that field intensity E larger than a critical value, Ep, must be applied. Ep is dependent on the size of the target cells. It ranges from values closed to 100 V/cm in the case of large cells such as muscle cells to 1–2 kV/cm in the case of bacteria [40]. Large cells are therefore more sensitive to lower field strengths than small ones. Electric field values have therefore to be adapted to each cell lines in order not to affect their viability. The field strength triggers permeabilization: when E is larger than Ep, it controls the area of the cell surface which is affected [42]. From Eq. (1), it is clear that for field intensities closed to

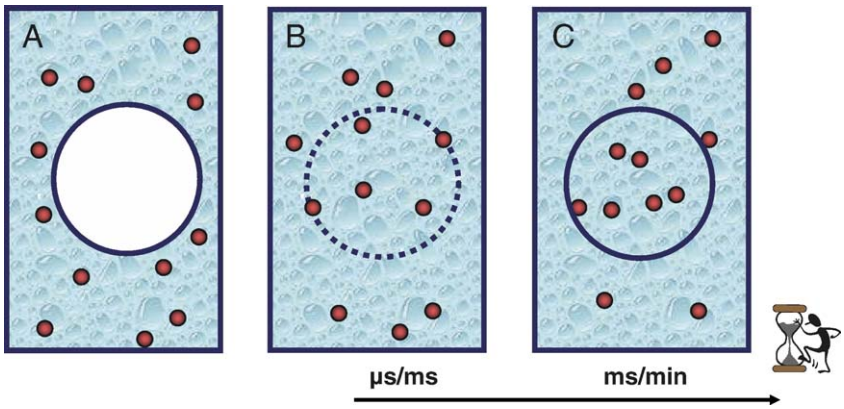

Fig. 1. Scheme for electropulsation. (A) Before pulse application, membrane is not permeable. (B) During pulse application, ranging from few µs to few ms, entrance of a large variety of molecules as well as an efflux of cell soluble compounds occurs. (C) After pulse application, membrane permeability can be maintained for small size molecules from few sec to several min. After cell membrane resealing, the uptaken molecules are sequestered into the pulsed cells.

*E*p, permeabilization is only present for θ values closed 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 function of the field strength [37,41,43]. The density and/or size of TPS, i.e., the extent of permeabilization, are indeed controlled by the pulse number and duration [43]. 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 *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 pulse number and duration (Fig. 2).

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

$$Fs(t) = Ps \times (N,T)A/2(1-Ep/E)\Delta S e^{-k(N,T)t} \quad (3)$$

where Ps 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*, *x* 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 [37]. Such a concept leads to the notion of "membrane domains" involved in electropermeabilization: "macrodomains" where permeabilization can takes place, which area is determined by the pulse intensity according to: A/2 $(1-Ep/E)$, and, within that macrodomains, "microdomains" where permeabilization actually can take place, corresponding to the so-called Transient Permeable Structures which number and size depends on the pulse number and on the pulse duration according to the *x* function. It remains to elucidate the molecular characteristics of these domains in terms of lipid composition, organisation, asymmetry and dynamics.

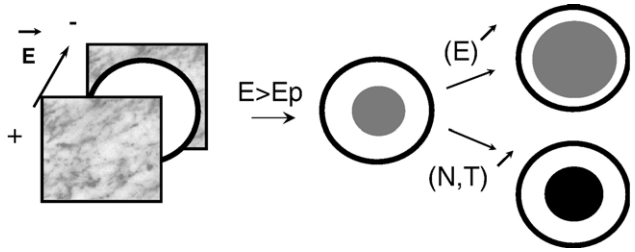

Fig. 2. Effect of the electric field parameters on membrane permeabilization. The electric field *E* leads to the induction of a transmembrane potential difference $\Delta\Psi_E$ which surrimposes to the resting one $\Delta\Psi_o$. Cell is therefore hyperpolarized at the anode facing side, while depolarized at the cathode one. The grey area represents the area where the resulting potential is higher than the threshold value, so where permeabilization is present when $E>Ep$. Increasing *E* above *E*p leads to an increase in this area. At a constant *E* value, increasing the pulse number *N* or the pulse duration *T* does not lead to any increase of that area but leads to an increase in permeabilization efficiency as shown here by a darker grey color. Grey squares represent the electrodes.

The use of videomicroscopy allows to analyse the permeabilization phenomenon at the single cell level. Propidium iodide 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 Electro-Pulsation. Less than one min later, it appears at the nuclei 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. 3). 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, in agreement with the above theoretical considerations [28] and Eqs. (1) and (2).

3.2. Gene transfer

Direct transfer of large molecules to the cytoplasm is observed but only if macromolecules (proteins, DNA) are present during the permeabilizing electric pulses. Proteins added after the permeabilizing electric field can enter the cell via endocytosis like processes [44,45]. Gene expression is obtained after applying electric pulses to a mixture of cell and DNA. No transfected cells are detected in the absence of electric

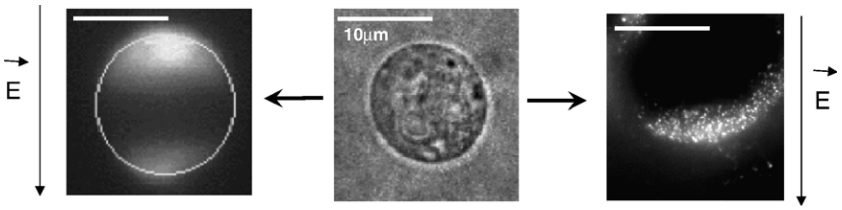

Fig. 3. Visualization of the electro-mediated membrane permeabilization and gene transfer. Micrographs of a CHO cell pulsed in a buffer containing propidium iodide or a fluorescent-labelled plasmid. 8 pulses of 5 ms at 0.8 kV/cm were applied. Center, phase contrast imaging. Left: entrance of propidium iodide observed 1 s following electropulsation. Right: fluorescence imaging of DNA transfer observed 1 min following pulsation.

field, in absence of DNA or when DNA is added after the pulses. Therefore, the mechanism is different from the one observed for small molecules. However, electrotransfection is only detected for electric field values leading to cell membrane permeabilization.

The other main difference between the electrotransfer of small size molecules and macromolecules is the duration of the pulses. Milliseconds pulses are required to obtain efficient gene expression with a good cell viability to limit electric field intensities required when short pulses are used [38,46,47]. Under those conditions, transfection threshold values are the same as whose for cell permeabilization. DNA is a large molecule, the apparent size of the TPS allowing its transfer through the membrane must be sufficient enough. This is obtained by applying pulses of low intensity but long pulse duration. Under that optimized conditions, cell viability is preserved even if an ATP leakage happens during electropermeabilization. ATP level controls gene electrotransfer and expression in mammalian cells [48]. The same efflux of intracellular ATP is obtained by applying pulses of low intensity and long pulse duration or high intensity with short duration, i.e., by either strongly affecting a small area of cell membrane or by slightly affecting a larger area.

Electrically induced DNA uptake by cells is a fast process involving DNA electrophoresis [49]. In that context, pulse duration also appears to be a key parameter for efficient gene expression in tissues. Electrotransformation has been obtained with short and strong pulses in pioneering experiments [50,51] but it appears less safer. DNA electrotransfer in tibialis cranialis muscles of C57BL/6 mice has been achieved by using long but low intensity pulses [19,20]. Moreover, the combination of short high-voltage and long low-voltage pulses allowed to evidence the necessity of association of cell electropermeabilization and convenient electrophoretic transport of DNA toward and/or across the permeabilized membrane within the tissue [52–54].

In the context of studies on model membranes, DNA interactions with lipid bilayers have 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 [55,56]. DNA-induced endocytosis was observed in the absence of any electric field. A possible mechanism for DNA/lipid membrane interaction was DNA encapsulation within an inverted micelle included in the lipid membrane. High molecular mass DNA 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 [57].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 [58]. Moreover, the association of DNA with a lipid bilayer greatly facilitates the transport of small ions. That suggested 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 was 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 [59].

In the case of cells in culture, using fluorescent plasmids allowed to monitor the interaction of nucleic acids with membrane at the single cell level. No free diffusion of plasmid into the cytoplasm was detected. DNA molecules, negatively charged, migrated towards the anode when submitted to an electric field. Plasmids interacted with the cell surface side facing the cathode where it is accumulated by the field associated electrophoretic drag only for $E > E$p, i.e., for electric field values leading the membrane to be permeabilized [28]. The DNA/membrane interaction is not homogeneously distributed in the permeabilized areas facing the cathode but is present into membrane "microdomains" which size ranges from 0.1 to 0.5 μm (Fig. 3). It remains to know if they correspond to the Transient Permeable Structures.

These observations are consistent with a process where plasmids interact with electropermeabilized part of the cell surface due to their interfacial electrophoretic accumulation. This result is consistent with a multi step process of DNA transfer [60]:

(i) during EP, plasma membrane is permeabilized facing the two electrodes and DNA migrates electrophoretically towards the plasma membrane facing the cathode side where it interacts,
(ii) after EP, a translocation of the plasmid to the cytoplasm follows. Expression is detected after 2 h over several days.

Such kind of localized process led to the development of experimental strategies aimed to increase amount of DNA

interacting with the permeabilized membrane and therefore gene expression. 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. Plasmids only interact with the electropermeabilized side of the cell facing the cathode. When changing both the pulse polarity and their direction, by a 90° rotation of the electrodes, DNA interacts with the whole membrane cell surface. This was associated with an increase in gene expression [31]. Such kinds of experimental protocols have also been successfully used in vivo to increase electropermeabilization in the case of electrochemotherapy and gene expression [30,61].

4. Conclusion

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 membrane. 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 destabilisation of the membrane which is a stress for the cells and may affect the cell viability has still to be clearly described.

New directions of research are needed to characterize membranes domains involved in molecules electrotransfer. DNA transfer occurs through “micro 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 [62,63]. One can wonder if they are involved in DNA electrotransfer [64].

The use of electric pulses to transfect cells is nowadays extended in vivo on several tissue types, the most widely targeted tissue being skeletal muscle [11,22,27]. In addition to its potential use in gene therapy, in vivo DNA electrotransfer is also, because of its simplicity, a powerful laboratory tool to study in vivo gene expression and function in a given tissue [21]. But, other studies will also be necessary to understand the cascade of events triggered by electropermeabilization at the cell and tissue levels where new constraints coming from tissues organisation are present, such as the inhomogeneity of the electric field strength and the intercellular distribution of DNA [65].

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