Mechanistic Description of Membrane Electropermeabilization

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Abstract

Classical cell membrane electropermeabilization of cell membrane is the result of the delivery of electric field pulses on cells. The electric field pulse lasts from submicro- to several milliseconds. The electric field intensity is large enough to induce a dramatic structural local alteration of the cell membrane organization. This results in an enhanced permeabilization of the target cell membrane for molecules otherwise poorly transportable. This structural alteration is indeed a complex process, and its molecular characterization remains an intense field of investigations. The new transient organization of the cell membrane supports a massive transport due to electrophoretic forces and diffusion-driven gradients.

This chapter describes the fast events inducing electropermeabilization or the immediate consequences of the field-induced alteration of the membrane and cellular organization. The methods suited to monitor these fast events are critically described as they are key factors in the accuracy of the informations. Three steps are present in cell membrane electropermeabilization: trigger, expansion, and stabilization. The experimental results are discussed in terms of structural information on the new transient membrane organization. Most informations are related to the massive enhanced molecular transport across the membrane and its modulation by the electric field pulse delivery.

Keywords

Membrane • Transmembrane Voltage • Transport • Membrane Structure

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Introduction

This chapter describes the events occurring along classical electropermeabilization (electropermeabilization). This is what is observed with a field range of $0 < E < 10$ kV/cm for mammalian cells (diameters between 10 and 30 μm) and pulse durations larger than submicros (therefore classical electropermeabilization included some experiments called nsPEF (nanosecond pulsed electric field) when the duration is larger than 100 ns) (Deng et al. 2003; Son et al. 2014). This is obtained with pulse generators with square wave profile rising time larger than 0.1 μs.

Electric pulses when delivered to a cell suspension or a tissue result in a membrane permeabilization (electropermeabilization). Classical electroperme-abilization results in an enhanced transport of poorly permeable ions and molecules

across the membrane and in a change in its electrical conductivity. Electropermeabilization can be reversible if the pulsing parameters are selected in a proper way. This reversibility is called resealing (resealing). This means that an enhanced membrane permeabilization is transiently induced while preserving the cell viability. More drastic pulsing conditions can make this permeability irreversible (cell lysis would result) (Rems and Miklavcic 2016). This chapter is an attempt to make a state of the art in the field by pointing out the most recent observations. More classical information can be read in the works of the leading groups in the field during the last 40 years (see "Cross-References" at the end of the chapter).

The Electric Field Induces a Transmembrane Voltage (TMV)

When a cell is subjected to an external electric field, the cell (where the plasma membrane can be considered as a dielectric shell) can be considered as a spherical capacitor, where the redistribution of electrophoretically driven charged ions in the electrolytes surrounding the membrane, i.e., electric current, leads to an induced transmembrane voltage (TMV) (transmembrane voltage). During the electric field application (electropulsation) (electropulsation) on the cell, an induced transmembrane voltage (ΔVi) is created which is locally associated with the dielectric properties of the plasma membrane. Using a physical model based on a thin, weakly conductive shell (the membrane, conductivity λm), full of an internal conductive medium (the cytoplasm, conductivity λi), and bathed in an external conductive medium (conductivity λe), solution of Laplace's differential equation gives ΔVi as:

$$\Delta \mathrm{Vi}(\mathrm{M},\mathrm{E},\mathrm{t}) = -\mathrm{f}\ \mathrm{g}(\lambda)\mathrm{rE}\cos\theta(\mathrm{M})\left(1-\exp(-\mathrm{t}/\tau\mathrm{m}\right) \qquad \text{(Eq.1)}$$

where M is the point on the cell that is considered, t is the time after electropulsation is turned on, f is a factor depending on the cell geometry (for a sphere, f = 1.5), r is the radius of the pulsed cell, E is the electric field strength, and θ(M) is the angle between the direction of the field and the normal of the cell surface in M. g(λ) is related to the different conductivities as (Zimmermann et al. 1947)

$$\begin{aligned} \mathrm{g}(\lambda) = \quad & \left(2\lambda\mathrm{e}\left[2\lambda\mathrm{m}+\lambda\mathrm{i}+(\lambda\mathrm{m}-\lambda\mathrm{i})(\mathrm{r}-\mathrm{d}/\mathrm{r})^3-3\lambda\mathrm{m}(\mathrm{r}-\mathrm{d}/\mathrm{r})\right]\right. \\ & \left./\left[(2\lambda\mathrm{e}+\lambda\mathrm{m})(2\lambda\mathrm{m}+\lambda i)+2(\mathrm{r}-\mathrm{d}/\mathrm{r})^3(\lambda\mathrm{i}-\lambda\mathrm{m})(\lambda\mathrm{m}-\lambda\mathrm{e})\right]\right. \end{aligned} \qquad \text{(Eq.2)}$$

where d is the thickness of the membrane (a few nm). τm is the characteristic time constant of the membrane charging and can be written as (Kinosita and Tsong 1977):

$$\tau\mathrm{m} = \mathrm{rCm}(2\lambda\mathrm{e}+\lambda\mathrm{i})/(2\lambda\mathrm{e}\lambda\mathrm{i}) \qquad \text{(Eq.3)}$$

where Cm (0.5–1.0 μF/cm2) is the specific membrane capacitance. τm is calculated to be in the submicrosecond time range for mammalian cells. It is strongly dependent on the buffer composition as the internal composition is fixed by the cell metabolism.

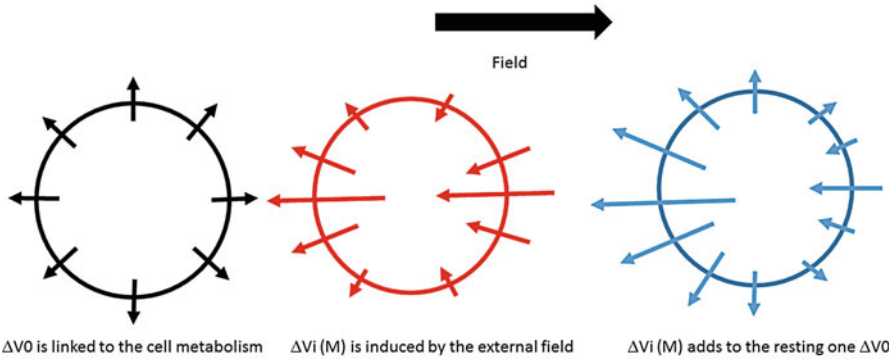

Fig. 1 Modulation of the TMV of a spherical cell by an applied external field. The TMV point from negative to positive and their lengths are proportional to the voltage. The large arrow on the top represents the electric field lines. On the left, a cell is pictured with its homogeneous resting voltage; the middle is indicative of the field-induced TMV that is highly position dependent along the cell surface. On the right, the resulting modulation of the TMV when the field is present, a highly complex position-dependent TMV, is observed

If the plasma membrane is considered to be a pure spherical dielectric ($\lambda m = 0$), we obtain $g(\lambda) = 1$. In these conditions and when the steady state is reached ($t >> \tau m$) as for millisecond pulses, Eq. 1 simplifies to:

$$\Delta Vi(M, E) = -1.5rE\cos\theta(M) \qquad \text{(Eq.4)}$$

When living cells are electropulsed, ΔVi adds to the resting one $\Delta V0$. This gives an additive effect on one side of the cell and a subtractive one on the opposite. The electric field effect is strongly position dependent (Fig. 1).

The Resulting TMV Induces Membrane Permeabilization (Electropermeabilization)

When the new membrane electric potential difference ΔVm ($\Delta V0 + \Delta Vi$) locally reaches a critical value (ΔVc) (nowadays estimated between 0.25 and 0.6 V for living cells) (Rems and Miklavcic 2016), a local alteration of the membrane structure leads to membrane permeabilization. Molecular processes supporting classical electropermeabilization (electropermeabilization) remain poorly understood (Teissie et al. 2005) in spite of more than 40 years of effort (Neumann and Rosenheck 1972). The membrane is called "porous." There is a general agreement that it cannot be described by the occurrence of reversible hydrophilic holes, so-called pores ("electroporation") (electroporation), in the lipid bilayer. A membrane of a cell is a more complex system. It is an out of equilibrium complex from a thermodynamic point of view. It forms a complex with the extracellular matrix and the cytoskeleton. Its transmembrane voltage is dependent on the cellular energy reserves.

If at any point ΔVm gets high enough to induce sufficient number of conductive defects, the defects allow enhanced ionic transport through the membrane and increase the membrane conductivity by several orders of magnitude. This in turn partially discharges the membrane and thereby reduces ΔVm during the pulse. In such case, the membrane conductivity has to be considered as a function of ΔVm, which will be in more details described in other chapters (see "Cross-References" at the end of the chapter).

Electropermeabilization is described as a local event in the cell surface.

Membrane permeabilization is associated by an enhanced transport of water-soluble small molecules (that are of a low molecular weight, say less than 2 kD) and ions. It is a time-dependent process, and its kinetics can be observed by monitoring the electrical conductivity change in the membrane or the change in the transport parameters. It can be assayed by the inflow of dyes (trypan blue, propidium iodide, and similar) by observation under the microscope or with a flow cytometer. Permeabilization can then be quantified by two parameters: the number of cells in the population where the uptake can be detected and the amount of uploaded dyes (i.e., the distribution of fluorescence intensity in each single cell in the population).

To follow the onset of electropermeabilization which occurs during the pulse delivery, the observation is on the submillisecond time range. But in most experiments, it results in an enhanced transport (transport) during the post-pulse step that is observed. But this is affected by the resealing (see "Cross-References" at the end of the chapter). Different biophysical approaches monitor the transport as mentioned above. They are shortly described in a first part of this chapter. They are used to analyze the processes and their control by biophysical and physiological parameters.

A lot of studies have been developed on pure lipid assemblies (planar lipid membrane, liposomes). Processes on such physical models are relevant of soft matter physics and would not be considered in the present chapter. Only events occurring in living cell membranes will be approached.

Computer-assisted simulations of transvoltage effect on lipid bilayers are not considered relevant of classical electropermeabilization (different pulse duration, much higher equivalent field strength).

Methods Used in Detecting and Follow-up of Membrane Electropermeabilization

Conductance Changes

Direct Assays by Electrical Measurements

By measuring the voltage and the current flowing between the electrodes, one gets access to the conductance of the suspension (conductance). Under steady-state condition, the conductance of a diluted intact cell suspension can be considered controlled by the conductance of the external solution. An ohmic behavior is observed when a voltage pulse is delivered on the cell suspension (as long as the Joule heating is small).

When electropermeabilization appears, two events are present:

(i) The dielectric shell has conductive defects that increase during the pulse
(ii) There is leak of the internal (cytoplasmic) solution

Both events result in an increase in the cell suspension conductivity. This is a very fast process that can be detected on the submicrosecond time scale after correcting from the interfacial electrochemical reactions on the electrodes (Kinosita and Tsong 1979).

Indirect Approaches by Fluorescence

Membrane-embedded fluorescent probes have their emission controlled by the transmembrane voltage by a direct effect of the transmembrane field (Hibino et al. 1991). The probe emission change linked to the alteration of the membrane conductance (that affects the transmembrane field) is very fast and closely follows the change in conductance. A very fast detection is needed where short (ns to μs) laser flashes triggered at delayed times after the onset of the field pulses are used to stimulate the emission (Frey et al. 2006; Hibino et al. 1991).

Transport Assayed by Fluorescence

Membrane permeabilization is associated to an enhanced diffusion-driven transport of water-soluble molecules and ions following the physical laws of diffusion (Fick's equation) (diffusion). This is a time-dependent process, and its kinetics can be observed by monitoring the inflow of a fluorescent molecules. In most cases, molecules such as propidium iodide are used as their fluorescence emission is strongly enhanced in the cytoplasm due to their binding to nucleic acids. Transport is assayed online with time resolution down to the us (Pucihar et al. 2008) or imaging on the ms range as shown by video imaging in the late 1990s (Table 1).

In most cases, it is the post-pulse transport that is assayed as it is a result of the structural alterations present during the pulse delivery (see "Cross-References").

Table 1 Synthetic view of the different techniques which are used to investigate membrane electropermeabilization (strength and limits, main results)

Methods	Temporal resolution	Spatial resolution	Molecular information
Conductance	Fast (microsecond)	Low (on population)	average size of defects
Patch camp	Slow (second)	Low (on single cell)	molecular definition of defects
Fluorescence	Fast (microsecond)	1 μm on single cell	transport
NMR	very slow (hours)	Average information	tilt of lipid headgroups
SEM	very slow	High (but fixed sample)	ultrastructural defects
AFM	very slow	High	ultrastructural defects

Permeabilization during the Pulse Delivery Depends on the External Field Strength and Pulse Duration

Conductance Changes

Electrical Measurements

Conductance changes during the pulse can be observed on cell suspension only when the field is larger than a threshold (critical value Ec) (Kinosita and Tsong 1979) (conductance). The increase is time dependent during the delivery of the pulse. Its magnitude is dependent on the electric field intensity. The relative conductivity changes during the application of a rectangular field pulse between 0.75 and 1.2 kV/cm, respectively, with a duration of 1 ms to a mammalian cell dense suspension (pellet) in a low conductivity isosmotic buffer sucrose are also a very fast increase. The conductance increase can be detected within the first μs of the pulse delivery after correcting for the electrodes interfacial contribution to the electrical signal. A plateau in the conductance change is observed for long pulses in conductive buffers.

Imaging Observations

The use of a very fast fluorescence imaging microscopy connected to a pulse generator gives access to the conductance changes (Hibino et al. 1991) (imaging). A voltage-sensitive fluorescent dye was used an indicator of the time-dependent transmembrane change induced by the external electric field on sea urchin eggs. But when the field was over a critical value, the transmembrane voltage kept locally a constant value on a cap facing the electrodes. A high membrane conductance of the order of 1 S/cm2 is therefore present within 2 μs after the onset of the external field. The size of the conductive cap is determined by the strength of the external field. This high local membrane conductance affects the electric field-induced transmembrane voltage as predicted by Eq. 1. A steady value is observed in the cap where the transmembrane conductance is obtained (Hibino et al. 1993). Only 0.5 microseconds after the onset of the rectangular electric pulse, the two sides of the cell facing the electrodes are affected, and a high membrane conductance in the order of 1 S/cm^2 is measured; an asymmetric process is observed; a higher conductance is detected on the positive side. Along the pulse delivery (the external field remaining constant), a further increase in the conductance is detected, reaching the order of 10 mS/cm by 1 ms. The kinetics of the conductance increase is different on the two opposite sides of the cell, being faster on the negative-electrode side; as a result at the end of a 1 ms pulse, the conductance on the negative side is more than twice that on the positive side. Different processes clearly support electropermeabilization on the opposite caps of a pulsed cell.

Induced Transport across the Membrane

The transport of a water-soluble dye (propidium iodide, PI) induced by electro-permeabilization was monitored under an ultrafast fluorescence microscope

(bandwidth larger than 1 MHz) during the electric pulse on a cell population of mammalian cells as well as at the single cell level (Pucihar et al. 2008) (propidium iodide). The transport became detectable as early as 60 μs after the start of the pulse (transport). Transport is delayed as compared to the enhanced membrane conductance. After the delay of 60 μs, the time course of fluorescence during the pulse was approximately linear, suggesting mainly electrophoretic transport of molecules during the pulse delivery lasting up to 1 ms.

Transport through these pathways can occur by different mechanisms during and after a pulse. To determine the time scale of transport and the mechanism(s) by which it occurs, efflux of a fluorescent molecule, calcein, across erythrocyte ghost membranes was measured with a fluorescence microscope photometer with a fast (millisecond) time resolution during and after electric pulses that lasted several milliseconds (Prausnitz et al. 1995). Only a reduced leak was observed during the pulse, and most of the efflux from the ghost was after the pulse. Transport caused by electropermeabilization occurred predominantly by electrophoresis and/or electroosmosis during a pulse, although transport occurred in part or almost completely by diffusion within milliseconds to seconds after the pulse (electrophoresis, electroosmosis).

On a shorter time scale (microsecond), the course of electropermeabilization was analyzed during the electric field application using a rapid single cell fluorescent imaging system. Interaction and penetration of propidium iodide, with and across cell membrane, was asymmetrical during electropermeabilization. Localized enhancement of the dye fluorescence was observed during the pulsation on the cell surface and in the cytoplasm. But this was observed only when the amplitude of the external field was higher than a critical value. Specific staining of a limited anode-facing cell volume was observed during the first milliseconds when the pulse was applied. The membrane fluorescence level increased linearly during the pulse delivery, whereas the geometry of the staining was unchanged. The fluorescence in the cap increased linearly during the pulse delivery (lasting up to 20 ms), as expected from an electrophoretic drift. The fraction of the membrane on which structural alterations occurred, Ap, was defined by the electric field strength E. It was a linear function of the reciprocal of the electric field strength.

$$\mathrm{Ap} = \mathrm{A0}(1 - \mathrm{Ep}/\mathrm{E}) \qquad \text{(Eq.5)}$$

where Ep is the critical field strength needed to trigger membrane electropermeabilization.

The size of the cap where permeabilization is present increased with an increase of the field strength as soon as the field was larger than the critical field Ep (Fig. 2).

The density of defects supporting the transport across the permeabilized membrane cap was governed by the pulse duration (Fig. 3).

Electropermeabilization is a localized but asymmetrical process (electropermeabilization). Transport was not the same on the two cell sides. The membrane defects (defects) are created unequally on the two opposing sides of the cell during the pulse, implying a vectorial effect of the electric field on the membrane.

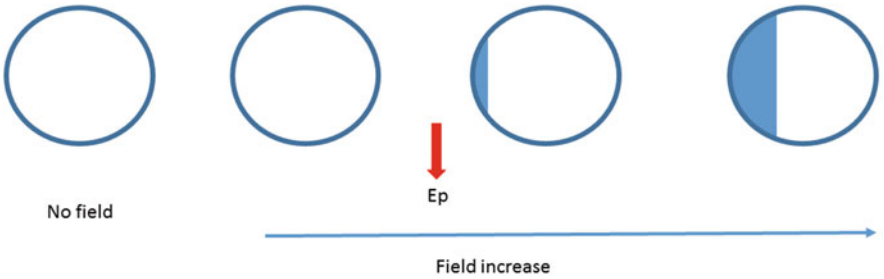

Fig. 2 Effect of the field strength on the size of the electropermeabilized cap. Cells are pictured as a sphere. Field pulses are delivered at a constant pulse duration. No permeabilization is present before the pulse delivery (*left side* of the cartoon). Low field has no effect on the membrane organization. As soon as the field induces a permeabilizing TMV, i.e., the field strength is larger than Ep, a cap is brought to the "permeabilized state" (a *gray* cap on the cartoon). The size of this permeabilized cap increases with a further increase of the field strength

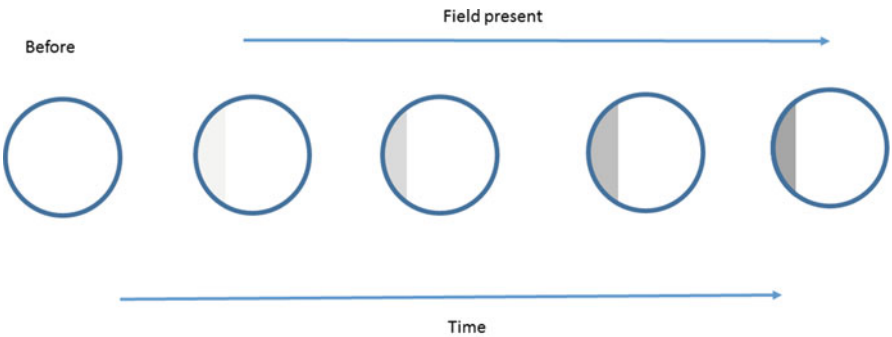

Fig. 3 Effect of the pulse duration on membrane electropermeabilization. Cells are pictured as a sphere. No permeabilization is present before the pulse delivery (*left side* of the cartoon). As soon as the field induces a permeabilizing TMV, a cap is brought to the "permeabilized state" (the gray cap on the cartoon) (trigger step). As long as the field is present with a constant strength, an increase in the defects supporting the permeabilization occurs shown by *darker* levels in the *gray* levels of the cap. This is called the expansion step

Exchanges of calcium ions through electropermeabilized membrane of Chinese hamster ovary cells were found to be asymmetrical. Entry of calcium ions during a millisecond pulse occurred on the anode-facing cell hemisphere. The exchanges during the pulse were mostly by an electrophoretic drift. The membrane regions stained by propidium iodide uptake were the same as those where calcium exchanges occurred. Positively charged species are electrophoretically pushed in the cell cytoplasm on the anode-facing cell hemisphere. Electropermeabilization is facilitating the electrophoretic movement of any kind of small species across a membrane cap controlled by the field strength during the pulse delivery.

Water transport was detected resulting in huge osmotic swelling of the cell when using ms pulses (a high level of permeabilization) (swelling). This is easily prevented by adding large water-soluble additives in the pulsing buffer (such as sucrose or polyethylene glycol, PEG).

Permeabilization as Described by its Post-Pulse Consequences

Most studies are not on the events during the pulse but on the observations of cells that had just been pulsed. The consequence is that it is not the direct effect of the onset of electropermeabilization that is observed but its synergy with the resealing process that follows. What is then observed is the percentage that have been permeabilized and the level of the inflow of the reporter molecules (Canatella et al. 2001; Pucihar et al. 2008). The last parameter is indeed strongly controlled by the resealing. A more relevant approach of the onset of permeabilization during the pulse is to observe the subpopulation of permeabilized cells in the total population of pulsed cells. One limit is of course the sensibility of the assay to detect permeabilized cells. Results were observed to be in many cases different by using different assays. The inflow is under the control of the permeability coefficient of the reporter molecule (roughly dependent on the size). The number of molecules that can be detected inside the cytoplasm is controlled by the method of detection. For example, detection is obtained under less stringent conditions when using PI penetration (fluorescence detection) rather than trypan blue labeling (colorimetric method) (detection).

Control of Cell Membrane Electropermeabilization by the Electrical Parameters

Field Strength

The electric field strength of the pulses delivered on the cell suspension was observed to be a critical parameter to trigger cell membrane permeabilization (detected in most cases by the uptake of a fluorescent dye). Transport is indeed controlled by the field strength E. Fluorescent-positive cells (i.e., permeabilized) induced by the pulse are detected only when the intensity of the pulse is larger than a critical threshold (Ep) (note: in a cell population, a percentage of cells has a leaky membrane where the dye uptake spontaneously takes place. They are observed in the control cells. This must be corrected from the observed permeabilization). For a given cell (size r) and a given pulse duration (T), Ep induces the permeabilization on a cap with a critical size, across which transport can be detected. An accurate definition should be Ep(r,T).

A fairly linear relationship is observed between permeabilization and the reciprocal of E (Eq. 5). This supports the online observation that a cap on the cell surface is brought to the permeabilized state by the pulse with a size controlled by the field. This cap is where the critical permeabilizing TMV was reached along the pulse. At high field, all cells are permeabilized, as Ep(r) is obtained for all values of r in the population.

Pulse Duration

When the field strength of the delivered pulses is larger than this critical strength Ep, it is observed that the percentage of cells detected as permeabilized is increased with an increase in the pulse duration. Transport is controlled by the pulse duration T (duration). This increase is detected to level off at a plateau value close to 100% (all cells are detected to be permeabilized when long pulses are delivered).

Interestingly, the critical parameter Ep is observed to decrease with an increase in the pulse duration. A linear relationship is observed between Ep(T) and the reciprocal of the pulse duration (T). This gives access by extrapolation to a limiting value of Ep (for an infinite duration). This may result from several factors such as:

- The density of defects increases with the pulse duration in the cap where the critical TMV is triggered (defects). The inflow increases as a consequence and detection is obtained with a very limited permeabilized cap.
- Due to the electrophoretic drift of membrane proteins, a microdomain is created that is more sensitive to the field-induced TMP.
- Due to the electromechanical stress on the cell, membrane stretching occurs, and the cap facing the electrode has a forced curvature making it more fragile.
- The transport by the electrophoretic forces during the pulse is high enough to give a detectable accumulation of the reporter dye.

Repetition of Pulses

In most studies, not only a single pulse is delivered to the cells, but a train of pulses is applied where two complementary parameters are to take into account: the number of successive pulses and delay between each single pulse (frequency) (delay).

Conductance measurements give a direct access of the effect of the repetitive pulses. It is observed that the membrane conductance is further increased by the repetitive pulses but that a partial resealing occurs during the delay (Kinosita and Tsong 1979). More conducting defects are induced along a repetition of pulses.

The local defects are considered part of the structural membrane reorganization induced by the external field. They remain present for a few seconds after each single pulse. Their density is dependent on the number of pulses. Cumulative effects are shown by an enhanced transport and are observed when repeated pulses are applied. Transport therefore increases with the number of pulses. More cells are then detected as permeabilized. Again a plateau level is detected when a large number of pulses are delivered. Indeed, this brings a result where all cells are permeabilized.

Delay between Pulses

The delay plays a role in the final state of pulsed cells. This is a complex process that is not clearly explained at the present state of our knowledge (so-called electrosensitization) (electrosensitization).

At a constant number of pulses/pulse duration product, transfer of molecules is strongly affected by the time between pulses. Permeabilization is observed to be higher when a low number of long pulses are delivered with a long delay rather than a repetition of short pulses.

Orientation of Pulsing

The electric field being a vector has a given orientation (perpendicular to the electrodes when they are plane parallel). Cell electropermeabilization is triggered by an electric field; its characteristics should depend on its vectorial properties. The direction of the field is easily changed by moving the position of the parallel electrodes relative to fixed cells (either plated or anchored to a dish). Four different pulse sequences were compared: normal (pulses all in one given direction), crossed (pulses delivered in two perpendicular directions), inverted (pulses with opposite polarities), and crossed-inverted (pulses delivered in two perpendicular directions with opposite polarities). Under non-saturating conditions, the normal condition was observed to be the less effective conditions, while the highest effect was obtained under the crossed-inverted one. Permeabilized caps are formed only on one side of the cell under the less effective conditions (field strength close to Ep), while they appear on four different places on the cell surface under the most effective setting.

Multiple studies have shown that delayed bipolar (BP) electric pulses in the microsecond range are more effective at permeabilizing cells as compared to monopolar (MP) pulse equivalents. To study permeabilization effectiveness, MP or BP (with no delay in the inversion) pulses were delivered to single Chinese hamster ovary (CHO) cells, and the response of propidium iodide (PI) or calcium uptake was measured by confocal microscopy. Results showed that BP pulses were less effective at increasing intracellular calcium concentration or PI uptake than MP pulses. Flow cytometry analysis of CHO cells after exposure (at 15 min) revealed that to achieve positive PI transport, ten times more BP pulses were required than MP pulses. Overall, unlike longer pulse exposures, BP nsPEF exposures proved far less effective at both membrane permeabilization than MP nsPEF (Ibey et al. 2014). The uptake of calcium ions was decreased by more than an order of magnitude by the delivery of a second one of opposite polarity immediately after a first pulse. This effect reflects the vectorial character of the electrophoretic transport of ions through the electropermeabilized caps in the membrane during the two parts of the bipolar pulse. This observation supports the conclusion that electrophoresis is the dominant transport mechanism during the pulse delivery (where permeabilization is created), rather than diffusion that is driven by the concentration difference. By delivering the second pulse with a delay after the first one, the effect of reverse electrophoresis disappears (electrophoresis). This delay is very short (in the hundred microseconds range) and illustrative of a very fast recovery of the membrane perturbation supporting the electrophoretic transport.

Biochemical Definition of Permeabilized Structures

Lipids

Lipid unilamellar vesicles (LUV) (vesicles), physical models of cells but composed of pure lipids, are quite suitable systems as their size is close to the one of cells. Their molecular packings are similar to what one expects to be present in the plasma membrane. When submitted to short (where stretching is avoided) high field pulses,

LUV are transiently permeabilized. Leakage of trapped sucrose can be detected when the field strength is larger than a critical value (Ep). No global and permanent damages to the vesicle bilayer are detected. Electropermeabilization is therefore obtained in a lipid assembly. Lipid domains when present in cell membrane are putative target of electropermeabilization.

Proteins

Electropermeabilization of red blood cells was supported by the associated cell osmotic swelling. It was observed that in a low ionic medium, at least 35% of the conductive defects were related to the opening of Na+/K+ ATPase channels. This conclusion was supported by the partial blockage of the membrane conductance generated by the externally applied electric field pulse by a specific inhibitor, ouabain. A similar inhibition of the conductance was obtained when using a specific cross-linking reagent, Cu++ – phenanthroline, of the ATPase. Interestingly this effect was not observed in a high ionic medium as expected for the effect of the cross-linker. A large fraction of the voltage-induced defects that occurred are still unidentified. The effects of large magnitude transmembrane voltage pulses on voltage-gated Na and K channel behavior were studied in frog skeletal muscle membrane of isolated fibers (Chen and Lee 1994). A 4 ms transmembrane potential pulse of 600 mV reduces both Na and K channel conductivities and the ionic selectivity of the K channels against Na + ions. This reflects an electro-conformational damage to ionic channels in parallel to the induction of defects in the lipid bilayer. Classical effectors such as tetrodotoxin (TTX) and tetra-ethylammonium (TEA) binding effects were unaltered. By the whole-cell patch-clamp method investigation on neuronal cells, currents of voltage-gated (VG) Ca(2+) and Na(+) channels (I(Ca) and I(Na)) in cultured GH3 and NG108 cells were observed to be inhibited when cells were pulsed with submicrosecond fields at or above 1.5–2 kV/cm. The field pulses caused prolonged inhibition of I(Ca) and I(Na). A “leak” current (I(leak)) was present indicative that other field targets were present in the plasma membrane.

Structural Arrangements

Biphotonic Microscopy

Molecular center asymmetry is required for the creation of second harmonic generation (SHG) (second harmonic generation) signals. It makes this spectroscopy a powerful technique for visualizing changes in interfacial layers in the plasma membrane of biological cells. Lipophilic SHG probes, such as Di-4-ANEPPDHQ (Di-4)(pyridinium, 4-(2-(6-(dibutylamino)-2-naphthalenyl)ethenyl)-1-(3-sulfopropyl)-, hydroxide), can detect instantaneous/immediate perturbations in the plasma membrane. Rapid changes in membrane symmetry could be detected using SHG (Moen et al. 2014). Following pulsed electric fields exposure, an

instantaneous drop of ~50% in SHG signal was detected only from the anodic pole of the cell. This dramatic loss in SHG signal reflects a local dramatic perturbation in the interfacial nature of the membrane.

Nuclear Magnetic Resonance (NMR) Spectroscopy

31P NMR spectroscopy gives structural informations on the lipid polar head region (nuclear magnetic resonance). Chinese hamster ovary (CHO) cells were reversibly permeabilized by submitting them to short, high-intensity, square wave pulses (1.8 kV/cm, 0.1 ms). Due to these pulsing conditions (high field, short duration), a large cap was electropermeabilized covering the majority of the cell surface, but the population viability was preserved. It was taken advantage from the observation that cells remained in a permeable state without loss of viability for several hours at 4 °**C**. A differential method in the NMR pulse sequence was used in 1987 to analyze the phospholipid head groups and to get rid of the signals coming from other phosphorus groups. Control cells displayed the classical bilayer signature. A new anisotropic peak with respect to control cells was observed on 31P NMR spectroscopic analysis of the phospholipid components. This peak is only present when the cells are permeable, and normal anisotropy is recovered after resealing. This field-induced anisotropic peak was located downfield from the main peak associated to the phospholipids when organized in bilayers, but its localization peak is very different from the one of a hexagonal phase. This new transient signature associated to the electropermeabilized state can be explained by a new erected orientation of the lipid polar heads or by a random distribution of their orientation. This reorganization of the polar head group region results in a weakening of the hydration layer.

Electron Microscopy

Ultrastructural changes of the plasma membrane were detected just after the pulse delivery by scanning electron microscopy (scanning electron microscopy). Cells were chemically fixed a few seconds after their exposure to electric pulses in order to investigate the ultrastructural changes associated with the reversible permeabilization and observed by electron microscopy. By scanning electron microscopy, numerous microvilli and blebs were observed immediately after application of the field. Blebbing was facilitated under hypoosmolar conditions suggesting that they were assisted by the osmotic swelling induced by the membrane permeabilization (Gass and Chernomordik 1990). The appearance of osmotic pressure-dependent "blebs" was indicative of local weakening of the plasma membrane.

Fluorescence Microscopy

Studies using fluorescence microscopy with 1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-sn-glycero-3-phosphocholine (C6-NBD-PC),

a phospholipid analog, monitored phospholipid scrambling of mammalian cells (fluorescence microscopy). Millisecond permeabilizing pulses induced membrane disorganization by increasing the translocation of phosphatidylcholines according to an ATP-independent process. These pulses resulted in a rapid phospholipid flip/flop within less than 1 s and were exclusively restricted to the regions of the permeabilized membrane. The electrically mediated local membrane disorganization was not associated with a loss of cell viability. The occurrence of such a flip-flop supports the existence of direct interactions between the movement of membrane zwitterionic phospholipids and the electric field.

Lateral Mobility of Membranes

Lateral movement in each layer of the plasma membrane is present in all cell membranes. They are measured by using fluorescence recovery after photodegradation (FRAP). Electropermeabilization by ms long pulses affects the fast lateral mobility of a glycosylphosphatidylinositol (GPI)-anchored protein (Rae-1). The mobile fraction is indicative that 10–20% of the membrane surface is affected by defects under these conditions (where only a moderate field intensity was used to preserve the cell viability). These structures, which support the membrane permeability, propagate rapidly ($<$1 min) over the cell surface after the pulse.

Atomic Force Microscopy

Atomic force microscopy (AFM) directly visualized the consequences of electropermeabilization in terms of membrane reorganization (atomic force microscopy). The classical approach is to obtain morphological changes, and transient rippling of membrane surface was detected in the minutes following the pulse delivery. A more sophisticated approach gave information on the changes in nanorheology as one can locally measure the membrane elasticity. Permeabilizing pulses lasting several milliseconds with field intensity preserving the cell viability were delivered on CHO cells. The morphology of cells was preserved. A decrease in membrane elasticity by 40% was measured (Chopinet et al. 2013). This was observed both on chemically fixed and living CHO cells. This observation supports the occurrence of a modification affecting the entire cell surface suggesting that a cytoskeleton (a key feature in membrane stiffness) destabilization took place. A puzzling conclusion was that the mechanical change was homogeneous on the cell surface in the few minutes following the pulse delivery with no correlation with the part of the membrane that was detected as electropermeabilized by the fluorescence assay.

Interfacial Modifications

The organization of the membrane water interface gives rise to a set of different forces that are called hydration forces (Leikin et al. 1993). They are responsible for the non-spontaneous fusion of two cells when their respective membranes are

brought in close contact. A strong repulsion is detected when the intermembrane space is reduced to a few nanometers. They are described as resulting from a local organization of the interfacial water. Water dipoles are sensitive to the local electric field, and as a result, a regular organization of the water takes place. The associated dipolar fields on the two membranes are in opposite directions during the close approach. When cells are electropermeabilized, these forces vanished, and a spontaneous fusion is detected (Sowers 1986). This is observed during the pulse delivery but remains present in the few seconds following the pulse delivery. This is indicative that the structures supporting these repulsive forces, i.e., the regular organization of interfacial water molecules, have been altered under the electropermeabilization processes. This interfacial modification is restricted to the electropermeabilized part during and in the few seconds following the pulse delivery.

Modes of Transport

Transport during the onset of electropermeabilization (when the external field is present) occurs by electrophoretic forces acting on charged molecules (Prausnitz et al. 1995) (transport). Indirect evidences (transdermal delivery) are indicative that the transport of neutral molecules (mannitol) during the pulse delivery is very low. Different patterns are observed depending on the size of the molecule that is transferred.

For small molecules (using PI as a model), observed during the very first milliseconds during the pulse, the field strength controlled the part of the cell surface where the permeabilized state was detected. These caps are where transport occurs. After a short delay, a linear increase in fluorescence is observed during the pulse illustrative of an electrophoretic drift across the electropermeabilized cap of the cell membrane. After the pulse, transport occurs by diffusion.

Small interfering ribonucleic acids (siRNA) are 20–22 bp oligonucleotides that can achieve a transient silencing in protein expression. Electrotransfer of siRNA is a very efficient method for its introduction in the cytoplasm to obtain silencing. Electrotransferred siRNA passed through the plasma membrane of cells only during the pulse leading to electropermeabilization. The siRNA, being negatively charged, migrated against the field direction by electrophoresis and penetrated the cytoplasm (Paganin-Gioanni et al. 2011). The transfer results from the electrophoretic drift in the buffer and across the electropermeabilized cap of the cell membrane. This conclusion was further supported by quantification of the intracellular siRNA-associated fluorescence that is a linear function of the number of successive pulses in a train and by the observation that inversion of the electric field polarity led to a penetration of the siRNA on opposite sides of the cell in the cytoplasm. After the pulse delivery, a free diffusion is present in the cytoplasm but no further entry (electrophoresis).

In the case of deoxyribonucleic acid plasmid (pDNA), the field pushes the oligonucleotides (a charged polymer) toward the membrane where their interaction on the electropermeabilized part of the cell membrane results in the formation of

long-lived localized spots. This pDNA/membrane interaction occurs on the side where they are accumulated by the electric field-associated electrophoretic drag. This local accumulation, present only during the pulse, is therefore controlled by the pulse duration and by the number of pulses. No transfer occurs during the pulse. Translocation within the cytoplasm was another step that happened several minutes after the pulse train.

Modulation of Electropermeabilization by Physicochemical Factors

Temperature

Electropermeabilization is a collective structural change in the membrane organization and therefore is temperature dependent. Results on the temperature effect during pulse application on cell membrane permeabilization were determined in two different cell lines: V-79 and B16F-1 (Kanduser et al. 2008). Cells were trypsinized to be brought in suspension. The cell membrane electropermeabilization was determined by the toxicity following the uptake of bleomycin (clonogenic assay). Viability was preserved only on non-permeabilized cells due to the high cytotoxicity due to bleomycin uptake. A train of eight rectangular pulses lasting 0.1 ms were delivered at 1 Hz with increasing amplitudes between 500 V/cm and 900 V/cm. The cells were incubated at the experimental temperature and pulsed, and immediately after the pulse application, the treated cell suspension was maintained at room temperature in order to allow cell membrane resealing. The main conclusion was that chilling of cell suspension, during the pulse train delivery from physiological temperature (of 37 °C) to 4 °**C**, led to a lower percentage of cell membrane permeabilization (a higher cell survival). The differences were the highest under the more drastic conditions where a large number of cells were affected by the electrical treatment (electric pulse amplitude of 900 V/cm). A loss of membrane dynamics (obtained at low temperature) makes cell membrane less sensitive to electropermeabilization.

Buffer Osmotic Pressure

The effects of pulsing buffer osmotic pressure on electric field-induced permeabilization were analyzed on Chinese hamster ovary cells growing either in monolayers or in suspension. Osmotic pressure has no effect on the induction step of permeabilization, but its increase was shown to inhibit the expansion step for pulses lasting 0.1 ms (osmotic pressure).

When cells were subjected to long (ms) square wave pulses, a condition mediating a high gene transfer, no effect on cell permeabilization for a small molecule such as propidium iodide was observed. A cell swelling is detected under such long pulse conditions. Its magnitude is controlled by the osmotic pressure. But the use of a

hypoosmolar buffer during pulsation allows more efficient loading of cells with macromolecules such as beta-galactosidase, a tetrameric protein. This is indicative that the swelling was associated with an increase in the structural changes during the pulse.

Buffer Ionic Strength

The impact of external medium conductivity on the efficiency of the reversible permeabilization caused by pulsed electric fields is complex. Of course a trivial effect is the Joule heating during the pulse delivery that increases with the conductivity. Temperature effects on electropermeabilization are therefore present.

Increasing the ionic strength of the pulsing medium results in an increase in sieving of the permeabilizing electrodefects (expansion step). For permeabilization linked to the delivery of one single pulse of 0.1 ms, media of lower conductivity induced more efficient permeabilization to bleomycin independently of the medium composition (Silve et al. 2016). Treatment of cells with trypsin or pronase before application of the pulses abolishes the ionic modulation of both electropermeabilization. A similar rate of expansion of permeabilization is obtained whatever the ionic content of the pulsing buffer. The proteolytic manipulation of the extracellular matrix modulates the process of electropermeabilization suggesting its control by surface membrane proteins. It stresses that the conductivity of the medium affects permeabilization by several ways. As the energy delivered by pulses increases with the ionic content of the buffer, electropermeabilization cannot be related to the energy delivered to the cells.

Nucleotides

The structure of a biological membrane is dependent on the energy reserves in the cytoplasm, which are controlled by the nucleotide level. Permeabilization is associated to a leak of adenosine triphosphate (ATP). The permeabilization by ms long pulses efficiency remains unaffected by the ATP and adenosine diphosphate (ADP) cytoplasmic levels.

Cytoskeleton

The cytoskeleton is an internal framework of a eukaryotic cell, built by a dynamic network of protein filaments that provide structural support and drive the movement of the cell internal components. Three categories (microfilaments, intermediate filaments, and microtubules) are present. Colchicine, known to depolymerize the microtubules in CHO cells, induced no modification on the first two steps of the electropermeabilization process, creation and expansion (microtubules). Disruption of the actin cytoskeleton structures was reported as one of the characteristic effects of

submicrosecond-duration pulsed electric field (nsPEF) in both mammalian and plant cells (Pakhomov et al. 2014). CHO cells modified to get expression of a fluorescent protein (mApple)-tagged actin were used to analyze if nsPEF modifies the cell actin by a direct action (during the pulse delivery) or as a result of the events following cell membrane permeabilization (actin). A train of repetitive high field short pulses resulted in cell membrane electropermeabilization (YO-PRO®-1 dye (Thermo Fisher) uptake) and swelling. Actin filaments were replaced by dimmer and uniform distribution of diffuse actin. To block the PEF-induced swelling, the pulsing buffer was isoosmotically supplemented with a solute (sucrose), which remains unpermeant along membrane electropermeabilization. Disintegration of the actin cytoskeleton was a result of cell swelling, which, in turn, was caused by cell electropermeabilization and the resulting transmembrane diffusion of solutes which led to the osmotic imbalance.

Membrane Order

Cell electropermeabilization resulting from the delivery of pulses lasting 0.1 ms is detected only under more stringent conditions when cells have been treated by ethanol; lysolecithin is observed to facilitate cell electropermeabilization. More precisely, these molecules that modify membrane order and the related membrane fluidity, when used in concentrations compatible with cell viability, are shown to affect the expansion step, not the critical field that triggers electropermeabilization. Electropermeabilization is inducing a transition in the membrane organization. Membrane order is modulating the energy barrier needed to evoke the slow step in this membrane transition which occurs when cells are submitted to a field larger than a characteristic threshold (expansion step). Less order would increase the magnitude of this energy barrier; more order would decrease it.

When millisecond square wave pulses are delivered, no effect was observed on cell permeabilization for small-sized molecules when cells were pretreated by lysolecithin. Only little change on electroloading of proteins such as R-phycoerythrin was obtained in the presence of ethanol. Cell membrane fluidity, which is controlled by the membrane order alterations, does not have significant effect on reversible electroporation (Kanduser et al. 2006).

Conclusions

Cell membrane electropermeabilization is a local effect in the cell surface. The size of the permeabilized area is controlled by the field strength. This means that permeabilization was triggered locally as soon as the induced transmembrane voltage reached a critical permeabilizing threshold (0.2–0.6 V). Defects supporting the transmembrane transport are induced (defects). The density of defects (membrane conductance, transport efficiency) within the cap was controlled by the pulse duration in a complex way. The behavior of the permeabilized caps was different on the

two opposite cell sides facing the electrodes. The onset of the membrane structural changes associated to the increase in conductance was in the submicrosecond range. Transport was delayed after the membrane conductance changes. Osmotic swelling is very large with long (ms) pulses. The local membrane alteration induces a collective reorganization of the cell. The molecular organization of the conductive defects remains poorly characterized, but they result in an interfacial reorganization.

The critical value of the field-inducing permeabilization is under the control of the size of the targeted cell. In a mixed population (such as blood), the field effect can therefore be targeted on the subpopulation of large cells. Electropermeabilization and resulting electroloading of phagocytes was obtained on a whole blood sample without hemolysis of the smaller red blood cells (electropermeabilization). Furthermore, leucocyte membrane integrity was recovered and was maintained for at least 24 h. This was one more convincing evidence that the driving force to induce electropermeabilization is the field-induced transmembrane potential. This factor is under the control of the cell size. It is delayed from the onset of the pulse delivery by the membrane charging time by less than 1 μs in a conductive buffer. The triggering of permeabilizing (conductive) defects in the pulsed cell membrane is very fast (less than 1 μs) as observed from the conductance experiments, when corrected for the electrolytic artifacts on the electrode surface.

The onset of electropermeabilization follows the onset of the external field pulse. It can be described in three main steps:

1. "Induction step." The electric field induced the membrane voltage increase which results/creates local defects (may be due to kinks in the lipid chains) when it reached a critical value (about 200 mV). A mechanical stress was present with a magnitude that depended on the buffer composition. This is a very fast event (submicrosecond range) under the control of the external electric field.
2. "Expansion step." The conducting structural defects expanded as long as the electric field was present and with a strength larger than a critical value (observed during the induction). Again, an electromechanical stress remained present mainly with pulses in the ms time range.
3. "Stabilization step." As soon as the field intensity was lower than the threshold value, a relaxation process was taking place within a few milliseconds, which brought the membrane to the permeabilized state for small molecules.

Cross-References

- ▶ Critical Electric Field and Transmembrane Voltage for Lipid Pore Formation in Experiments
- ▶ Different Approaches Used in Modeling of Cell Membrane Electroporation
- ▶ Different Cell Viability Assays Following Electroporation In Vitro
- ▶ Fluorescent Indicators of Membrane Permeabilization Due to Electroporation
- ▶ Measurement of Molecular Transport after Electropermeabilization
- ▶ Membrane Permeabilization Lifetime in Experiments

- ▶ Modeling Transport Across the Electroporated Membrane
- ▶ Molecular Transmembrane Transport with Giant Unilamellar Vesicles (GUVs)
- ▶ Patch Clamp in Use of Electroporation Mechanisms Studies
- ▶ Phospholipid Head Group Dipoles and Electropore Formation
- ▶ Single Cell Electrical Characterization Techniques
- ▶ Transmembrane Voltage Induced by Applied Electric Fields

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