Electropermeabilization of mammalian cells

Quantitative analysis of the phenomenon

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ABSTRACT Transient membrane permeabilization by application of high electric field intensity pulses on cells (electropermeabilization) depends on several physical parameters associated with the technique (pulse intensity, number, and duration). In the present study, electropermeabilization is studied in terms of flow of diffusing molecules between cells and external medium. Direct quantification of the phenomenon shows that electric field intensity is a critical parameter in the induction of permeabilization. Electric field intensity must be higher than a critical threshold to make the membrane permeable. This critical threshold depends on the cell size. Extent of permeabilization (i.e., the flow rate across the membrane) is then controlled by both pulse number and duration. Increasing electric field intensity above the critical threshold needed for permeabilization results in an increase in membrane area able to be permeabilized but not due to an increase in the specific permeability of the field alterated area. The electroinduced permeabilization is transient and disappears progressively after the application of the electric field pulses. Its life time is under the control of the electric field parameters. The rate constant of the annealing phase is shown to be dependent on both pulse duration and number, but is independent of electric field intensity which creates the permeabilization. The phenomenon is described in terms of membrane organization transition between the natural impermeable state and the electro-induced permeable state, phenomenon only locally induced for electric field intensities above a critical threshold and expanding in relation to both pulse number and duration.

INTRODUCTION

Cell plasma membrane acts as a highly impermeable barrier to penetration of exogenous molecules into the cytoplasm. Only a limited number of compounds can in fact enter the cell via the existence of specific transport systems, with a concomitant expenditure of energy. The transfer of exogenous material into the cell remains a puzzling problem in cell biology.

For a few years, a physical method, using the application of external electric field pulses on cells, called electropulsing, has been developed. It was observed that electric fields larger than a threshold lead membranes to become permeable (Neumann and Rosenheck, 1972). Moreover, under controlled electric field conditions, permeabilization could be reversible, and as such did not affect cell viability (Neumann et al., 1982). Electropulsation is now routinely used in cell biology and biotechnology to introduce plasmids into the host genome for genetic manipulation (electrotransformation, Neumann et al., 1982; Potter, 1988). Another property of the electropulsed membrane is its associated fusogenicity. It was first shown that application of electric field pulses on cells in contact caused them to fuse (Senda et al., 1979; Teissié et al., 1982). More recently, it was observed that fusion occurs if contact between cells was established after pulsation (Sowers, 1986; Teissié and Rols, 1986). Despite the increasing use of the electropulsing phenomenon the molecular mechanisms involved in it remain to be elucidated. The driving force involved in the process is thought to be linked to a change in the transmembrane potential difference. It has been shown, in the case of lipid vesicles, that when the transmembrane potential difference reached a threshold value ~200–300 mV it induced a change in the permeability properties of the membrane (Teissié and Tsong, 1981). ^{31}P NMR studies on mammalian cells have suggested that this long lived alteration in membrane selective permeability should be associated among other facts with a new orientation of the phospholipid headgroups (Lopez et al., 1988).

If one considers a spherical cell with a radius r, the application of an external electric field of intensity E induces a transmembrane potential difference $\Delta\psi$:

$$\Delta\psi = f \cdot r \cdot E \cdot \cos\theta, \quad \text{(Bernhardt and Pauly, 1973)} \quad (1)$$

where θ is the angle between the electric field and the normal vector to the membrane, and f is a factor depending on the geometry and permeability of the cell. Due to

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its dependence on the angle θ, the electro-induced transmembrane potential difference is not homogeneous on the cell surface: being maximal in membrane regions facing the electrodes (i.e., for $|\cos\theta| = 1$), it is equal to zero in orthonormal ones. The validity of this theory was checked by video monitoring of single cells exposed to a uniform electric field (the change in the plasma transmembrane potential difference was observed to be position dependent; Gross et al., 1986; Ehrenberg et al., 1987; Kinosita et al., 1988).

Several theoretical models have been proposed to explain the phenomenon of electropermeabilization. One considers a membrane as a homogeneous fluid which breaks when the electro-induced compressive force is not balanced anymore by the viscoelastic restoring force (Crowley, 1973; Dimitrov, 1984). Another model is based on the existence of natural defects in biological membranes which could grow in size under the application of the electric field (Abidor et al., 1979). However, such theoretical models do not explain experimental observations such as the dependence of electropermeabilization on other electric field controlled parameters such as pulse number and duration, as previously reported by several authors (Rieman et al., 1975; Kinosita and Tsong, 1977, 1979; Schwister and Deuticke, 1985; Rols and Teissié, 1989), or the effect of increasing electric field intensities above the threshold needed for permeabilization.

In this paper, we present a quantitative approach based on the analysis of our experimental results concerning the role of the different electric field parameters (field intensity, pulse number, and duration) on electropermeabilization of mammalian cells. Quantification of the phenomenon was performed by measuring the flow of released compounds from electropulsed cells (ATP) or penetration of normally unpermeant molecules (calcein, trypan blue). Chinese hamster ovary (CHO) cells were chosen because of the large amount of information we had collected on their electropermeabilization, and because of their ability to grow either in suspension or on petri dishes as monolayers (Rols and Teissié, 1989, 1990; Rols et al., 1990).

MATERIALS AND METHODS

Cell culture

CHO cells have been employed in a number of somatic cell genetic laboratories (see Gottesman [1985] for a review). We selected the wild-type CHO cell line (WTT clone) which is not strictly anchorage dependent. It has been adapted for suspension culture at 37°C under gentle agitation (100 rpm) in Eagle's minimum essential medium (MEM 0111; Eurobio, Paris) supplemented with 8% new-born-calf serum (Boehringer, Mannheim, FRG), penicillin (100 units/ml), streptomycin (100 mg/ml), and L-glutamine (0.584 mg/ml). Cells were maintained in the exponential growth phase (4–10 $\times$ 10^5 cells/ml) by daily dilution of the suspension. Cells grown in suspension can be replated readily on Petri dishes (35-mm diam, Nunc, Roskilde, Denmark) and kept at 37°C in a 5% CO_2 incubator (Jouan, Saint-Herblain, France).

Application of electrical pulses to the cells

The cell permeabilization protocol for cells in monolayer or in suspension has been described elsewhere (Teissié and Rols, 1988). Briefly, in the case of cells plated in Petri dishes, the culture medium was discarded and 1 ml of a low ionic content saline buffer: the "pulsing buffer" (10 mM phosphate, 250 mM sucrose, 1 mM $MgCl_2$, pH: 7.2) was added. Two thin stainless-steel parallel electrodes were dipped in the buffer and seated on the bottom of the culture dish; the traces of the electrodes on the dish allowed to separate pulsed cells from the control ones. The electrodes were connected to a voltage generator which gave square-wave electric pulses (CNRS cell electropulser; Jouan, France). The voltage pulses applied to the cells were monitored with an oscilloscope incorporated into the cell electropulser. Cells in suspension were centrifuged for 5 min at 350 *g* (1,000 rpm, C500 centrifuge; Jouan, France) and resuspended in the pulsing medium at a concentration of 10^7 cells per milliliter. 100 μl of the suspension were placed between the electrodes on a culture dish. Voltage pulses were then applied.

Determination of the permeabilization

Electropermeabilization of cells was quantified by two approaches (*a*) penetration of unpermeant dyes, and (*b*) release of intracellular compound (ATP). Penetration of trypan blue (T 0887, 4 mg/ml in the pulsing buffer, Sigma Chemical Co., St. Louis, MO) was used to monitor permeabilization. Cells were pulsed, incubated 5 min at room temperature and then observed under an inverted light microscope (Leitz, FRG). The percentage of permeabilized cells was 100 times the ratio of the number of blue-stained cells to the total number of cells.

Penetration of calcein (C 1075; Sigma Chemical Co.) was performed under the same protocol; the dye concentration was 2.5 mM. After pulsation, cells were washed twice with pulsing buffer and observed under an inverted epifluorescence microscope (Leitz, Wetzlar, FRG). The percentage of permeabilized cells was determined as 100 times the ratio of the number of fluorescent cells to the total number of cells. Fluorescence quantification was performed as follows (Gabriel et al., 1990). Cells were observed under the fluorescence inverted microscope connected to a light intensifying video camera (Lhesa, Cergy-Ponroise, France) and visualized on a B/W monitor (RCA, Lancaster, PA). This video signal was fed to a digitizer (Info'Rop, Toulouse, France) driven by a computer (CPU 68010; Motorola, Tempe, AZ) and stored on line. The software library ("Trimago", Ifremer, France) contained the major routines for digital image processing. Video signals converted into matrix were stored on a hard disk (85 Mo). The gain of the camera was set by manual adjustment to compare images of different cells. Mathematical treatment was used (*a*) to subtract the background level and (*b*) to correct for heterogeneity in incident light level and camera sensitivity by dividing by the illumination map. After such processing, all the pixel values were directly related to the local amount of dye.

ATP release measurements give access to the flow. Release was quantified on line by converting ATP into light through the luciferin-luciferase assay (Lemasters and Hackenbrock, 1979). A custom designed chamber was used. It was built by two stainless-steel parallel strips soldered to the bottom of a Petri dish (width 2 mm, length 20 mm). The volume of this pulsation chamber was 60 mm^3. It was filled with 55 μl of cell suspension in pulsing buffer and 5 μl of an aqueous solution of luciferin-luciferase at 15 mg/ml (L 0633; Sigma Chemical

Co.). Such a system was put in the midst of a very sensitive detection fluorimeter (Teissié, 1979), built in our laboratory and connected to the electropulsator and to a chart recorder. This set up allows the on line detection of the luminescence intensity emitted by the luciferin-luciferase as a function of the release of ATP. It consisted of a rapid increase in signal, followed by a plateau value reached 1 min after pulsing (Fig. 1). Two parameters were quantifying the extent of permeabilization; (*a*) the flow, i.e., dATP/d*t*, (*b*) the maximum release of ATP, i.e., the plateau value.

Permeability can then be quantified by the flow of molecules exchanged between cells and external medium. Two kinds of approaches are used in the following experiments: (*a*) the release of a cytoplasmic compound (ATP) is detected on line by an enzymatic reaction and is directly related to the flow: dATP/d*t*. (*b*) The penetration of external molecules (trypan blue, calcein) detected after a fixed time span of 5 min and which is related to the integration of the flow over 5 min:

$$Q = \int_0^5 F\,(\text{inflow})\,dt. \qquad (2)$$

Determination of reversibility

It was assayed by the trypan blue or calcein test. Cells were pulsed in the "pulsing buffer" which was replaced by the dye containing one after the indicated time span. Cells were incubated 5 min thereafter. Dye penetration was used to monitor the induced permeabilization of the membrane and not to assess the cell viability. The amount of incorporated dye into still permeable cells was quantified when calcein was used by means of the video approach described above.

Determination of electropulsed cell viability

Plated cells were pulsed in the pulsing buffer in absence of dye under the same conditions as for permeabilization assays. They were kept for 5 min at room temperature and the pulsing buffer was discarded and replaced by 2 ml of culture medium. Viability was measured by observing the growth of cells over 48 h (~2 generations) under a phase-contrast inverted light microscope.

Statistical analyses of the data

Computer software from Elsevier-Biosoft (Cambridge, U.K.) (Barlow, 1983) was run on an Apple IIe computer (Les Ulis, France). "Linefit" was used to calculate the correlation coefficient of straight line and "Expofit" the rate coefficient of exponential decay.

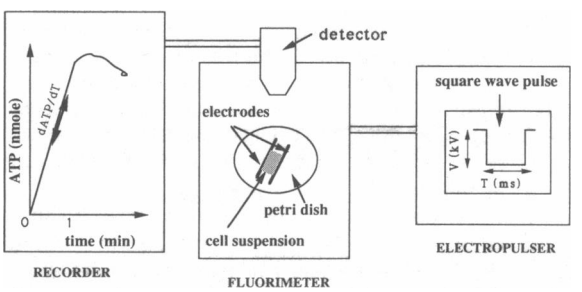

FIGURE 1 Experimental procedure for measuring ATP leakage out of electropulsed cells.

RESULTS

Theory

Membrane permeability to a solute S can be described by the associated flow F according to:

$$F = -D \cdot A \cdot dS/dz, \quad \text{(first law of Fick)} \qquad (3)$$

where D is the diffusion coefficient of S across the membrane, A is the membrane area given to diffusion, and dS/dz is the solute gradient of S across the membrane along the direction z perpendicular to the membrane. In the case of biological membranes, the flow can be expressed as:

$$F = P \cdot A \cdot \Delta S, \qquad (4)$$

where P is the permeability coefficient of the solute S to the membrane and ΔS is the concentration difference of S between cell interior and exterior.

$$P = \alpha \cdot D/X, \qquad (5)$$

where α is the partition coefficient of S between cell membrane and external medium, X is the membrane thickness, P depends on the molecular weight of the solute and on its solubility in the membrane. In case of electropermeabilization, permeability can then be quantified by the flow of molecules exchanged between cells and external medium.

Effect of electric field parameters on cell permeabilization

Cells in suspension were electropulsed with 10 pulses of 100 μs duration. ATP release was used as an indicator of the permeated state of the electropulsed membrane. As shown in Fig. 2, permeabilization only occurred for field

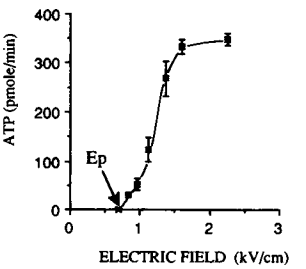

FIGURE 2 Effect of the electric field intensity on ATP release from CHO cells in suspension. Cells were pulsed 10 times, 100 μs duration. Rate of ATP liberated from electropermeabilized cells was measured by the luciferin-luciferase assay.

intensities higher than a threshold value *Ep* equal to 0.7 kV/cm. Above this value, increasing the electric field resulted in an increase in the amount of released ATP. This could be correlated with incorporation of trypan blue or calcein into an increased number of pulsed cells which was detected only for field intensities >0.7 kV/cm (data not shown). Permeabilization of the cell population, i.e., all nuclei stained in blue along the trypan blue assay, was achieved for field intensities close to 1.5 kV/cm (data not shown).

At given field intensity and pulse number, increasing the pulse duration resulted in an increase of the ATP release. As all cells were stained by trypan blue, the proportion of permeabilized cells remained constant. For 10 pulses of 1.6 kV/cm intensity, a plateau was reached for pulse duration >1 ms, (Fig. 3). Similar results were observed in the case of cells pulsed at constant field intensity and pulse duration, and with variable pulse numbers. The increase in ATP outflow was a linear function of the pulse number when <10. A constant value was then obtained for a larger number of pulses (Fig. 4). Similar behaviors were observed with plated cells. Effect of pulse frequency was observed to have no effect on permeabilization results for values up to 0.1 Hz (data not shown).

As previously described, permeabilization extent as a function of field strength was in fact largely dependent on both pulse number and duration. The threshold value *Ep* below which no permeabilization occurs is a function of both pulse number and duration. It decreases by increasing either pulse duration or number until it reaches a threshold that we call the real threshold value written as *Es* (Rols and Teissié, 1989; Rols et al., 1990; Rols and Teissié, 1990). Influence of pulse duration on the threshold value *Ep* for trypan blue penetration in the case of CHO cells in monolayers was studied at a given number of pulses ($N = 10$). When the pulse duration T was below 100 μs, permeabilization of cells was detected for field

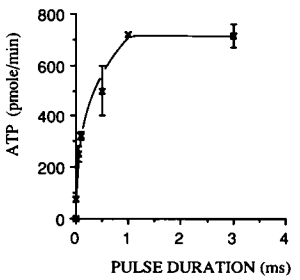

FIGURE 3 Effect of pulse duration on ATP release from CHO cells in suspension. Cells were pulsed 10 times with a pulse intensity equal to 1.6 kV/cm.

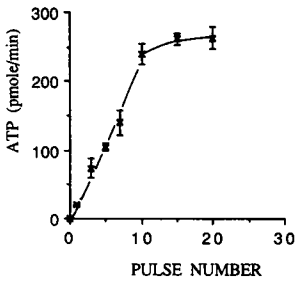

FIGURE 4 Effect of pulse number on ATP released from CHO cells in suspension. Cells were pulsed with durations of 100 μs and with an intensity of 1.6 kV/cm. For N equal or less than 10, the graph is close to a straight line (correlation coefficient of 0.992).

intensities strictly >0.3 kV/cm. Decreasing pulse duration resulted in an increase in *Ep*. As previously reported in the case of red blood cells (Rieman et al., 1975), a hyperbolic relationship between *Ep* and T was obtained. A linear relationship was then found between *Ep* and the reciprocal of T given by:

$$Ep\,(\text{kV/cm}) = 1.5/T\,(\mu\text{s}) + 0.3. \quad \text{(Fig. 5)} \qquad (6)$$

Extrapolation for infinite values of T allowed to determine the critical threshold value *Es* equal to 0.3 kV/cm. In the case of CHO cells in suspension, we observed that *Es* is equal to 0.7 kV/cm (data not shown). As a transmembrane potential difference induced by the electric field is directly proportional to the cell radii (Eq. n°1), this difference can be explained by the cell size and shape difference between cells in suspension and in monolayers.

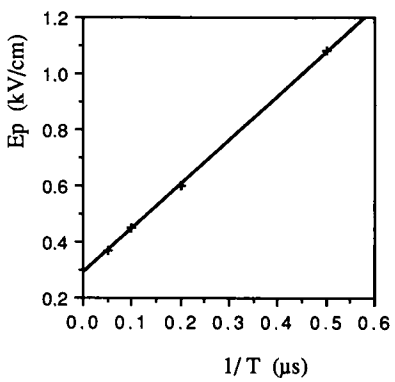

FIGURE 5 Effect of pulse duration T on the apparent threshold value *Ep* for permeabilization. Cells were pulsed 10 times. Threshold values *Ep* determined for different value of T, $E_{p,T}$, are reported as a function of $1/T$. The correlation coefficient of the straight line is equal to 0.999.

Effect of pulse intensity below the threshold of permeabilization *Es*

Pulses of 1-ms duration were applied to the CHO cells in suspension. Field intensity was either 1.2 or 0.4 kV/cm, i.e., above or below *Es*. The pulse number was equal to 1 or 10. As shown in Table 1, no permeabilization was detected for field intensity equal to 0.4 kV/cm. Application of nine successive pulses with an intensity smaller than *Es* (400 V/cm) after a permeabilizating pulse (1.2 kV/cm) had no further effect on the level of the amount of ATP released, i.e., on the permeabilization extent (Table 1). Similar results were obtained by using trypan blue as an indicator for cell permeabilization (data not shown). Both experiments show that application of electric pulses with lower intensity than *Es*, after a pre-permeabilizating pulse, has no effect on the extent of permeabilization. It is only when it is larger than *Es* that the electric field alters the state of the membrane.

Effect of the electric field vectoriality on permeabilization

The use of flat and parallel electrodes leads to the induction of a homogeneous electric field, where field lines are parallel. Electrodes being in a fixed position, effect of the electric field direction on permeabilization could be performed by rotation of the petri dish containing the cells. Cells in suspension were used due to their regular spherical shape as opposed to the random pattern found with plated cells. The rotation rate of a cell is given according to the Einstein relation:

$$Drot = k \cdot T/6 \cdot Vh \cdot \eta, \quad (7)$$

where *Drot* is the rotational diffusion coefficient, T the temperature, k the Boltzman constant, *Vh* the hydrated volume of the cell, and η the viscosity of the solution. The corresponding rotational coefficient time *Tc* is then given by:

$$Tc = Drot/6. \quad (8)$$

In the case of CHO cells, having an average radius of 6 μm, and for η equal 0.013 poise, *Tc* is equal to 200 s. As the time needed to run the experiment is much shorter (3 s), cells can be considered to be immobile during the rotation of the dish, the drag effect being negligible.

In this experiment, permeabilization was detected by trypan blue penetration into pulsed cells. Electric pulses were applied either in one direction or in two perpendicular directions. Results are reported in Table 2. At constant pulse number, the percentage of trypan blue permeated cells was higher when pulses were applied in the same direction than when pulses were applied in two perpendicular directions. In the latter case, the number of trypan blue colored cells was not changed compared with the number obtained when half of the pulses were applied in only one direction. At a given field intensity E, the cell membrane area which is permeable is inside the cone with a pole angle θ_s given by:

$$Es = E \cdot \cos \theta_s, \quad (9)$$

where θ_s is the polar angle in reference to the direction of the applied field vector (Schwister and Deuticke, 1985; see Appendix). For *Es* and E, respectively, equal to 0.7 and 0.8 kV/cm, θ_s is equal to 30°. There was no overlap between the two perpendicular permeabilized areas A and B. These results showed a nonadditivity of the effect of pulses when there is no overlap between permeabilized area and are confirmative of the vectorial character of the electric field effect (Teissié and Blangero, 1984; Mehrle et al., 1985; Dimitrov and Sowers, 1990).

Effect of the electric field parameters on the resealing process

Electropermeabilization of CHO cells in suspension under controlled pulsing conditions (i.e., 10 pulses, 100 μs

TABLE 1 Effect of pulses, 1 ms duration, with intensity below the threshold for permeabilization, on the permeabilization efficiency of CHO cells in suspension

Pulse number	Field intensity	Amount of ATP released
	kV/cm	*nmol*
10	0.4	0
10	1.2	400 (±80)
1	1.2	120 (±35)
1/9*	1.2/0.4*	120 (±65)

*Pulses were applied in the following sequence: 1 pulse 1.2 kV/cm intensity and then 9 pulses 0.4 kV/cm intensity. Pulses were applied with a frequency of 0.1 Hz.

TABLE 2 Effect of electric field direction on permeabilization efficiency

Pulse number			Percentage of permeabilized CHO cells
Total	In direction A	In direction B	
0	0	0	2 (±1)
5	5	0	8 (±3)
10	10	0	34 (±3)
10*	5	5	9 (±2)

*Cells were pulsed according to two perpendicular directions A and B. Pulse duration was equal to 0.1 ms. Permeabilization was determined by trypan blue uptake.

duration, field up to 2 kV/cm) does not affect their viability. The electric field-induced permeability disappeared spontaneously with time at room temperature (Teissié and Rols, 1986).

Cells were pulsed by application of 10 pulses, 100 μs duration. Field intensity was either 0.9, 1.2, or 1.5 kV/cm. Calcein in pulsing buffer was added at definite times T after pulsation, from 30 s to 4 min. Quantification of the amount of incorporated calcein in a single pulsed cell, C, was performed by using digital image processing. In the three different electric field intensity experiments, resealing curves were observed to fit a first order kinetic well:

$$C = Co\, e^{-kT}. \tag{10}$$

As observed above, the amount of incorporated calcein into pulsed cells at time zero, Co, depends on the electric field intensity (it increased by increasing electric field intensity) but the rate constant of the reaction, k, was found constant and equal to 0.35 +/− 0.04 min^{-1} with a correlation coefficient of 0.98 whatever the field strength at given pulse duration and number (Table 3). This data showed that the annealing process of induced permeabilization does not depend on the electric field intensity. Similar experiments where the number of pulses and their duration were changed, showed that the resealing process was in this case dependent on pulse number and duration, with the higher the value of these parameters the smaller the value of the constant k (the longer the permeabilized state of the membrane for calcein incorporation was maintained [Table 3]).

ANALYSIS OF RESULTS

It has been experimentally shown, in agreement with theory, that membrane area given to diffusion after electropulsing depends on the electric field intensity (Schwister and Deuticke, 1985). Previous studies showed that permeabilized zones of the membrane were facing the electrodes (Rossignol et al., 1983; Mehrle et al., 1985; Dimitrov and Sowers, 1990). The permeability coefficient of the membrane, P, is therefore equal to zero for electrically unperturbed parts of membrane (i.e., for regions where θ is such that the relation $E \cdot \cos\theta < Es$ is obeyed).

TABLE 3 Effect of electric field parameters on the resealing rate constant

Electric field intensity	Pulse number	Pulse duration	Rate constant
kV/cm		*ms*	*min^{-1}*
1.2	10	0.1	0.34 (±0.04)
0.9	10	0.1	0.33 (±0.03)
1.5	10	0.1	0.35 (±0.02)
1.2	10	1	0.19 (±0.02)
1.2	20	0.1	0.23 (±0.03)

Furthermore, the fact that at constant field intensity (i.e., at constant membrane area able to be permeabilized), the long lived permeabilization efficiency depends on both pulse number and duration (Figs. 3 and 4), lead us to propose that in the well-defined area A of the potentially electropermeabilizable cell membrane (θ being such that $E \cdot \cos\theta > Es$), only a fraction x is really permeabilized. P is equal to Po (close to zero) in nonperturbed zones and to: $Pp\, x + Po\,(1 - x)$ in the perturbed ones. Po is the permeated coefficient for unpermeant areas and Pp the permeated coefficient for the totally permeated ones. In fact, Po is so small that it may be considered equal to 0.The function $x(E, N, T, \theta)$, which corresponds to the fraction of the really permeabilized area, is describing a progressive transition between two membrane states with respective permeability equal to zero ($P = 0$) and different from zero ($P = Pp$). This transition depends on pulsing conditions such as: field intensity, E, pulses number and duration, N and T, and the localization of the considered point M on the membrane surface. Initial flow expression is then given by:

$$dF = Pp \cdot S \cdot x(E,\ N, T, \theta) \cdot dA. \tag{11}$$

Calculation of the surface integral is explained in detail in the annexes. When pulsing experiments were run at N and T constant, x is only dependent on E then relation between F and E is through the dependence of x on E. Several models can be proposed:

(a) x is not dependent on the electric field E, except the obvious condition: $E > Es$

$$x(E) = y, \quad y \text{ being a constant}$$
$$F = Pp \cdot y \cdot At/2 \cdot (1 - Es/E) \cdot S. \tag{12}$$

(b) x is proportional to E:

$$x(E) = yE$$
$$F = Pp \cdot y \cdot At/2 \cdot (E - Es) \cdot S. \tag{13}$$

(c) x is proportional to the electric field-induced transmembraneous potential difference. For $E > Es$, the effect depends on the localization of M on the cell surface

$$x(E) = y \cdot E \cdot \cos\theta, \quad E\cos\theta > Es$$
$$F = Pp \cdot y \cdot At/2 \cdot (E - Es^2/E) \cdot S. \tag{14}$$

(d) x is proportional to the square potential (Abidor et al., 1979). For $E > Es$, this effect is a function of the position of M on the membrane surface.

$$x(E, \theta) = y \cdot E^2 \cdot \cos^2 \theta, \quad E \cos \theta > Es$$

$$F = Pp \cdot y \cdot At/2 \cdot (E^2 - Es^3/E) \cdot S. \qquad (15)$$

In all these equations, the flow is equal to zero when the electric field intensity is below Es, i.e., when the area membrane A given to the flow is nill. At represents the total cell surface.

Initial flows of released ATP from CHO cells in suspension depend on the electric field intensity (see Fig. 2). Fitting the results to the four preceeding hypothesis showed an excellent correlation between the released ATP flow from pulsed cells and the applied field only according to the first hypothesis (Fig. 6). Adjustments obtained for the three other conditions were not statistically acceptable (correlation coefficients between 0.82 and 0.94, with a very poor extrapolation at the origin).

This mathematical approach shows that the efficiency of electropermeabilization, i.e., the fraction x of the membrane brought into a permeable state is independent of the electric field intensity E; N and T being kept constant. The role of E is only to determine the regions of cell membrane potentially permeabilizable (according to the relation $E \cdot \cos \theta > Es$). The role of T and N is then to control the permeabilization extent of this potentially electropermeabilizable membrane surface defined by E.

As reported above, at low values of T, permeabilization is only detected for field intensities larger than a threshold Ep (larger than Es) which depends on the duration T of the pulses. The experimentally observed linear dependence between Ep and the inverse of T (Fig. 5) can be explained as follows. At the level of the detection threshold Ep, the flow of diffusing molecules F is very low: $F = Fmin$. $Fmin$ being the threshold for detection, an experimental constant. Flow equation is then given by:

$$Fmin = Pp \cdot y \cdot At/2(1 - Es/E_{p,T})S. \qquad (16)$$

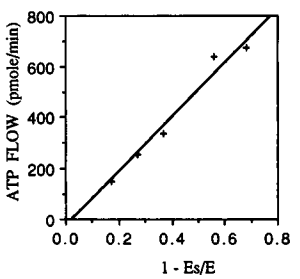

FIGURE 6 Effect of membrane area extent submitted to electric pulses on initial ATP flow from pulsed CHO cells in suspension.
Cells were pulsed 10 times with pulses of 100 μs duration. ATP flows were determined according to the initial slope of the curves of released ATP against electric field intensity. They are reported in this figure as a function of $1 - Es/E$, with Es equal to 0.7 kV/cm. Correlation coefficient for the straight line was found to be 0.99.

As x is a linear function of T and N ($x = c \cdot N \cdot T$), it comes:

$$Fmin = Y \cdot T(1 - Es/E_{p,T}),$$

with $Y = Pp \cdot c \cdot N \cdot At/2 \cdot S$, which gives:

$$1/T = Y'(1 - Es/E_{p,T}),$$

with $Y' = Y/F$ which has a high value

then:

$$1 - 1/TY' = Es/E_{p,T} \text{ and } E_{pT}/Es = 1/(1 - 1/TY')$$

but $1/TY'$ is very small and

then:

$$E_{p,T}/Es = 1 + 1/Y'T \qquad (17)$$

which shows a linear dependence between Ep and the inverse of T, as experimentally observed (Fig. 5).

DISCUSSION

Electropermeabilization of CHO cells in suspension, underlined by penetration of unpermeant molecules such as trypan blue and calcein, or by release of intracellular compounds such as ATP, is only detected for electric field intensities stronger than 0.7 kV/cm (Fig. 2). Permeabilization extent depends on both pulse number and duration (Figs. 3 and 4), but increasing the value of these parameters does not result in a decrease of the permeabilization threshold Ep below the threshold Es equal to 0.7 kV/cm. Moreover, pulse accumulation in case of applied electric field strength lower than Es does not lead to a more efficient permeabilization of prepermeabilized cells (Table 1).

These results show that the electric field intensity is the specific trigger of the electropermeabilization process only when larger than the characteristic threshold Es. As soon as electric field intensity becomes higher than the threshold Es, membrane can locally change from a native unpermeable state to a permeable one. The role of the field intensity is to make it possible for the membrane to become permeable as soon as the electric field intensity creates a local transmembrane potential difference at least equal to the permeabilizing one, i.e. ~200–300 mV (Teissié and Tsong, 1981). Increasing the electric field intensity only results in an increase of the membrane area able to be permeabilized (according to the θ angle, such as $Es = E \cos \theta$) but not of the permeability efficiency, x, which depends on both pulse number and duration.

Such a result can be explained by taking theory into account. As soon as the transmembrane potential difference reaches the threshold value required for permeabilization, the electro-induced potential difference increase is

limited by the concomittant decrease of the cell membrane permeability factor f (see Eq. 1; Kinosita et al., 1988). There is a counterbalancing effect of the permeabilization on its trigger. The resultant transmembrane potential difference then reaches a maximum value above which it can no longer increase.

Several theoretical models have been proposed to explain the phenomenon of electropermeabilization. One of them is based on the existence of natural membrane organization defects in lipid bilayers. It assumes that these defects can grow in size under the application of a transmembraneous electric field up to a critical value where hydrophilic pores are formed (Abidor et al., 1979). The process is described as depending on the square of the transmembrane potential difference (i.e., to the square of the applied electric field). Our results describing the dependence of the ATP flow on the applied field intensity (see hypothesis d in Analysis of Results) show that the model is not consistent with experimental results. Moreover, no explanation concerning the effect of pulse duration and number are given in theoretical models. Only experimental results on red blood cells pointed to the implication of N and T in the phenomenon (Kinosita and Tsong, 1979; Schwister and Deuticke, 1985). We show here that these two parameters play a very important role in the process. The field intensity, when higher than the threshold Es, leads the membrane to be potentially permeable. Field intensity determines fraction of the cell membrane where permeabilization can occur. In this localized region, only a part is in fact permeabilized to an extent determined by pulse numbers and durations. The probability of any shift from the initial unpermeant state to the permeated one increases with time, x tends then to 1. As previously described by others (Kinosita and Tsong, 1979) and recently confirmed by us (Rols and Teissié, 1989), electropermeabilization of cells is a two step process consisting in (a) a step of induction of transient permeated structures for field intensities greater than a threshold and (b) a step of expansion of these structures depending on pulse number and duration. Electropermeabilization can then be described as follows:

$$I \xrightarrow{a} I^* \xrightarrow{b} P,$$

where I is the initial, unpermeant state of the membrane and P the electro-induced permeated one. The induction step (a) occurs in regions for which the relation $E \cos \theta > Es$ is verified. It is very rapid (<1 μs). The expansion step (b) depends on both pulse number and duration. The probability for the membrane to become permeable i.e., the expansion step, depends on pulse number and duration according to the function x which increases linearly for small values of pulse number and duration.

Permeability is long lived. Life time of the permeated state was experimentally observed as depending on several parameters such as incubation temperature, field intensity, number, and duration of the pulses (Kinosita and Tsong, 1979; Rols and Teissié, 1989). It appears that stability of the phenomenon is largely affected by the nature of the membrane. Pure phospholipid vesicles present very short lived permeability (Teissié and Tsong, 1981; Benz and Zimmermann, 1981), whereas natural cell membranes can be maintained permeable for longer periods (i.e., from seconds to hours; Kinosita and Tsong, 1977; Lopez et al., 1988). We show here that, although stability of permeability is apparently enhanced with electric field intensity (Rols and Teissié, 1989), the rate constant of the resealing process in the case of mammalian cells is not dependent on field intensity but is affected by pulse number and duration. These results indicate that, as for the permeabilization efficiency, the resealing rate of the process depends on both pulse number and duration but does not depend on electric field intensity of the creating pulse.

In the case of exponential electric field pulses, the resealing rate of the electro-induced fusogenic state of electropermeabilization has, however, been indirectly reported to be dependent on the localization of the considered point at the electropermeabilizated membrane surface (Sowers, 1987). Such an apparently conflicting result can, however, be explained by taking into account our results by the fact that in the case of exponential decay, the only active part of the pulse for permeabilization is when electric field intensity $E(E = Eo\ e^{-t/\tau})$ is greater than the critical threshold E_S. At a given initial electric field intensity Eo, the angle θ in fact depends on the pulse duration t in the case of exponential decay. In this case, the resealing process is then nonhomogeneous on all the cell surface because of the dependence of θ on t.

Taking the theory into account, electro-induced permeable area should be strictly localized on the cell membrane, in regions where the transmembrane potential difference is brought to a threshold value. This was experimentally demonstrated in terms of the fusogenic property of the electropermeabilized membrane (Sowers, 1987; Teissié and Blangero, 1984): transient permeated structures do not spread with time on the surface and are strictly localized in regions facing the electrodes which extend in relation to the electric field intensity. We here report direct evidence that this phenomenon is still observed as far as permeabilization is concerned (Table 2). Permeabilization efficiency depends on the direction of the field; permeabilized regions do not spread at least during the time of the experiment.

To sum up, electropermeabilization of cells can be described in terms of membrane organization transition

as suggested in by Sugar, 1983 and Robello and Gliozi, 1989. This appears when transmembrane potential difference reaches a critical value. These structural membrane reorganization are not homogeneously localized on cell surface in the case of an electric field-induced modulation according to the theory: their positions depend on electric field strength. The extent of permeated structures appears to be strictly dependent on both pulse number and duration by a mechanism which is still to be determined. As pure phospholipid vesicles can be electropermeabilized (Teissié and Tsong, 1981), phospholipids appear to be the principal target of the electric field. Moreover, stability of the electro-induced permeabilization in the case of cells and the fact that the transient permeated structures do not spread with time suggest that membrane compounds other than lipids, i.e., proteins due to their interaction with the cytoskeleton, must be involved in the process (Sowers, 1987; Lopez et al., 1988; Escande-Géraud et al., 1988). The effects of electric field on cell membrane organization have then still to be elucidated.

APPENDIX

Calculation of the flow equations

The transmembrane potential difference, induced by the electric field, is given by:

$$\Delta\psi = frE \cos\theta.$$

When the electric field intensity is equal to the threshold Es, the transmembrane potential difference needed to permeabilize cell membranes, $\Delta\Psi c$, is reached. Permeabilization appears first in the region facing the anode $\Delta\Psi c = frEs$. Increasing electric field intensity results in an increase in the permeabilized region according to the angle θ as follows:

$$\Delta\psi c = frE \cos\theta.$$

A relation between E and θ is then given by:

$$Es = E \cos\theta.$$

Membrane area A able to be permeabilized is then a function of the angle θ, and is calculated according to the differential element of the angle $d\alpha$:

$$dA = rd\alpha 2\pi r \sin\alpha$$

then:

$$A = 2\pi r^2 \int_0^\theta \sin\alpha \, d\alpha.$$

The flow equation of a solute S through the membrane A is then given by:

$$F = PS \int_0^\theta x \, 2\pi r^2 \sin\alpha \, d\alpha.$$

Thanks are due to J. Zalta for her help in cell culture and to Dr. Robb for reading the manuscript. M. P. Rols was a recipient of a post-doctoral fellowship of the Association pour la Recherche sur le Cancer (ARC).

Received for publication 16 May 1990 and in final form 22 June 1990.

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