An Experimental Evaluation of the Critical Potential Difference Inducing Cell Membrane Electropermeabilization

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ABSTRACT When applied on intact cell suspension, electric field pulses are known to induce membrane permeabilization (electropermeabilization) and fusion (electrofusion). These effects are triggered through a modulation of the membrane potential difference. Due to the vectorial character of the electric field effects, this modulation, which is superimposed on the resting membrane potential difference, is position-dependent on the cell surface. This explains the difference between the experimentally observed critical field strengths requested to trigger the processes of permeabilization and fusion. The critical membrane potential difference which induces membrane permeabilization can be calculated from these experimental observations. It is observed that its value is always about 200 mV for many different cell systems as we previously reported in the case of pure lipid vesicles. This is much less than assumed in most previous studies.

INTRODUCTION

The organization and the functionality of cell membranes are known to be strongly affected when external electric field pulses are applied to cell suspension. ATP synthesis can be triggered (1) as well as ion pumping (2). Dramatic alterations can be mediated leading to the occurrence of reversible membrane permeabilization (3) and/or membrane fusion (4). These effects are thought to be due to a modulation of the transmembrane potential difference induced by the external field and associated with the dielectric properties of the membrane. This membrane potential difference ΔU is taken as the potential outside of the cell minus the cytoplasmic potential ($U_{ext} - U_{in}$). Theory predicts that this modulation should obey (5)

$$\Delta U(E,M,t) = -fgr\,E\cos\theta(M)\,(1 - e^{-t/\tau}) \qquad (1)$$

in which M is the point on the cell surface we are considering, E is the intensity of the electric field, f is a factor reflecting the shape of the cell, g is controlled by the electric permeability of the membrane, r is the size of the pulsed cell (assumed to be a sphere), $\theta(M)$ is the angle between the direction of the field and the normal to the cell surface at M pointing out of the cell, t is the time after the field is turned on, and τ is a characteristic time constant (in the microsecond time range).

The geometrical and kinetic dependence of this membrane potential difference modulation was experimentally checked by use of fluorescence video microscopy (6–8).

A recent investigation showed that the electric field-mediated effects on the membrane structure, such as permeabilization, were position-dependent on the cell surface (8). Previous studies indicated that electropermeabilization occurred preferentially on one side of the pulsed cell (9, 10). This vectoriality of the effects was explained by the contribution of the membrane resting potential difference ΔV (8). In most cases, the value of this physiological parameter is positive (mammalian cells) or null (liposomes, ghosts). The applied field contribution is added to it, giving a position-dependent resulting membrane potential difference (Fig. 1). The total imposed membrane potential difference is then not symmetrical when the resting value is different from zero. Its consequences have the same characteristic. In simpler words, electropermeabilization would occur for lower field intensities on one side of the cell rather than on the other (8). The critical membrane potential difference giving rise to electropermeabilization was not experimentally accessed in these works. Its definition was obtained by use of Eq. 1 under the following assumptions: (*a*) the cell is presumed to be a sphere, f is then equal to 3/2; (*b*) a membrane is considered as a pure dielectric, g takes a value of 1; (*c*) the charging time τ is much smaller than the pulse duration T.

Equation 1 can then be written as follows.

$$\Delta U(M) = -(3/2)\,r\,E\cos\theta(M) \qquad (2)$$

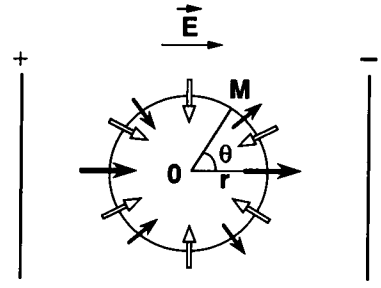

FIGURE 1 The external electric field induces a position-dependent modulation of the membrane potential difference which is superimposed on the resting potential difference. The arrows are the vectorial representation of the electrical potential gradient direction. Open arrows, resting potential; closed arrows, electric field induced potential. Their length is indicative of the magnitude of the potential difference.

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As permeabilization is first observed for $\theta = \pi$ (8), the critical threshold in potential difference is then:

$$\Delta U_p = (3/2)\, r\, E_p \qquad (3)$$

ΔU_p is then obtained experimentally by measuring the size of the cells and by determining the lowest field intensity which triggers permeabilization. However, two facts are not taken into account in this approach. First, as mentioned above, the potential difference is the sum of the resting value and of the electric field contribution. Second, due to its complex nature, a membrane is far from a pure dielectric. It was shown experimentally that surface charges may play a role and that g was controlled by the intrinsic membrane conductance (11, 12) as follows.

$$g = \frac{2\lambda_o\lambda_i}{(2\lambda_o + \lambda_m)(2\lambda_m + \lambda_i) + (r/d)\lambda_m(2\lambda_o + \lambda_i)} \qquad (4)$$

λ_o, λ_i, and λ_m are the electric conductivities of the buffer, of the cytoplasm, and of the membrane, respectively, and d is the thickness of the membrane. Using a value of 3/2 for fg, i.e., making the assumption that a biological membrane is not leaky ($\lambda_o = \lambda_i >> \lambda_m$), is then an overestimation leading to artifacts in the computation of ΔU_p. A new approach to its determination is proposed in the present communication in which associated effects of electropermeabilization are taken into account.

Cell electrofusion was shown to be a long-lived effect of cell electropermeabilization (13–15). The permeabilization of the two membranes in contact was experimentally observed during the ghost electrofusion process (16). This observation leads to the technological development of the use of radiofrequency pulses (17). Bringing electropermeabilized cells into contact induces their fusion. But on the other hand, critical field values which induce fusion of cells in contact during the pulse are larger than intensities which trigger permeabilization (Fig. 2 *A*). This is illustrated by our experiments with plated Chinese hamster ovary cells in which permeabilization was observed by pulsing directly on the dish through the penetration of Trypan blue (18) and fusion by the formation of polykaryons (19). Rather than using cytoplasmic content mixing or membrane coalescence as reporter (20, 21), membrane fusion was assayed by its physiological consequence: cell fusion. By such a mean, we are sure to observe events where the cell viability is preserved. Technically, for permeabilization as for fusion, it is necessary to apply long cumulated pulse duration to increase the sensibility of the method (22). But, in order to reduce the power of long pulse and the associated increase in temperature leading to cell damage, repetitive short pulses (10 × 100 μs) were applied to obtain a long cumulated pulse duration and a facilated detection of the phenomena (18, 19). If the direction of the applied field is inverted between the successive pulses, as in Ref. 8, the permeabilization profile is not affected but the fusion profile becomes similar (Fig. 2 *B*). As experiments are run on plated cells, no movement of the cells occurs between the pulses. This shift in the profiles shows that the contribution of the resting potential difference must be taken into account in the process of electrofusion. Fusogenic electropermeabilization occurs asymmetrically when the field is applied in only one direction.

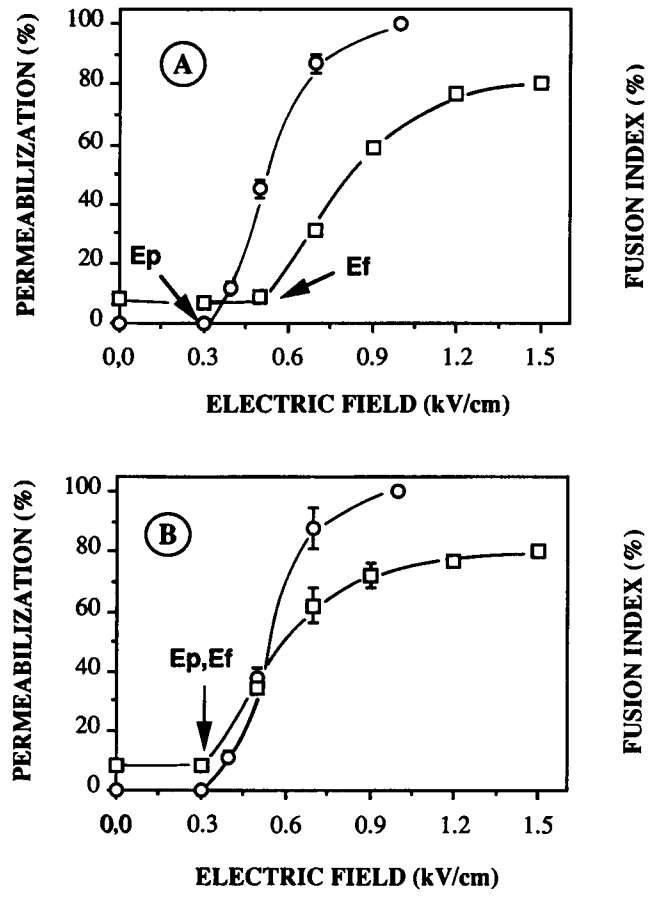

FIGURE 2 Electropermeabilization and electrofusion of plated Chinese hamster ovary cells in relation to the external field strength. *A*, the direction of the field remains the same between the different pulses. Permeabilization (○) was assayed by the penetration of Trypan blue (18) and fusion (□) by the percentage of polykaryonic cells (19). Ten pulses with a duration of 100 μs were applied to facilitate the detection of the events. *B*, the direction of the field was inverted between the pulses. Cells were grown in suspension in Eagle's minimal essential medium supplemented with 8% new born calf serum, antibiotics and L-glutamine (0.584 mg/ml). They were replated readily on Petri dishes (35-mm diameter, Nunc, Denmark) and pulsed according to previous works (18, 19). Briefly, they were pulsed by using a square wave field generator (Jouan, France) connected to two thin stainless-steel parallel electrodes. The electrodes were seated on the bottom of the culture dish what form the experiment chamber. The pulsing buffer was 10 mM phosphate buffer, pH 7.2, 1 mM $MgCl_2$, and 250 mM sucrose. Trypan blue (0.4% m/v) was added for the permeabilization assay. Permeabilization was assessed by the ratio between cells stained in blue to the total number of cells. At least 200 cells were counted by use of a videomicroscopy detection (Leitz Diavert inverted microscope, Wetzlar, Germany; JVC video system, Tokyo, Japan).

As permeabilization is detected as soon as one side of the cell has been affected, the resting potential difference is

added to the field contribution. Then:

$$\Delta U_c(\theta = \pi) = \Delta V + fgr\,E_p \quad (5)$$

in which ΔV is the resting potential difference (which is positive for mammalian cells), and E_p is the critical field intensity above which permeabilization is triggered as shown on Fig. 2. Fusion is observed only when the opposite side ($\theta = 0$) is affected. Then:

$$\Delta U_c(\theta = 0) = |\Delta V - fgr\,E_F| = fgr\,E_F - \Delta V \quad (6)$$

in which E_F is the critical field intensity above which fusion is triggered as shown in Fig. 2

Permeabilization being triggered as soon as a critical potential difference is present which is not position-dependent:

$$|\Delta U_c(\theta = \pi)| = |\Delta U_c(\theta = 0)|. \quad (7)$$

From Eqs. 5–7 one gets:

$$\Delta U_c = \Delta V(E_F + E_p)/(E_F - E_p) \quad (8)$$

and

$$frg = (\Delta V + \Delta U_c)/E_F. \quad (9)$$

From such equations, it can be concluded that, in the case of membranes having a resting potential equal to zero, the thresholds E_p and E_F are the same. This is what was supposed in the coaxial-pore mechanism of cell membrane fusion (23) and experimentally observed in the case of pea protoplasts (24) and lipid bilayers (25). But, when ΔV is not equal to zero, the thresholds E_p and E_F should not be the same as empirically suggested by Zimmermann (26) and experimentally reported by us.

One of the experimental problems in this approach is the determination of the critical field intensities. As described in several approaches (22, 27), electropermeabilization occurs through a multistep process: induction, expansion, and stabilization. Expansion, which is controlled by the pulse duration and number, is the key parameter in the definition of the flow of exogeneous molecules. This flow is crucial for the experimental visualization of the occurrence of permeabilization. As the flow is controlled by the intensity of the field (27), when using too short a pulse and too low a number of pulses, permeabilization would be detected for a larger field than the critical threshold. Then:

$$E_F < E_F^{exp} \quad (10)$$

$$E_p < E_p^{exp} \quad (11)$$

the index *exp* being indicative of the experimental values. If x and y are the overestimations of the critical fields:

$$E_F + x = E_F^{exp} \quad (12)$$

$$E_p + y = E_p^{exp} \quad (13)$$

with $x > 0$ and $y > 0$. Then:

$$\Delta U_c^{exp} = \frac{\Delta V(E_F + E_p + x + y)}{(E_F - E_p - y + x)} \quad (14)$$

$$\Delta U_c^{exp} > \Delta U_c \quad (15)$$

as long as x and y are close to each other. The experimental value is then an overestimation of the real one.

Inverting the polarity of the applied field was reported previously in the "radio frequency" method (17). In those experiments, the frequency of the AC field was very high (10 kHz) as compared to our conditions (1 Hz). As reported by the author, the positive effect of the AC field was due to the occurrence of a mechanical disturbance on the membrane. Two stresses were then present: this mechanical one which is similar to a local "sonication" of the membrane and the electrical one associated to the modulation of the membrane potential difference. In our conditions, it is only the electrical component which is acting on the membrane. By use of the results in Fig. 2, in which the same pulse duration and number were used for the two assays, the permeabilizing membrane potential difference for Chinese hamster ovary cells can be calculated as less than 250 mV. This is much smaller than the estimation by use of the approximation described in Eq. 3 which gives a value of 450 mV. Such a lower value agrees with the estimation on pure lipid vesicles in which a value of 200 mV was calculated (28) and with plasma membrane of murine myeloma cells in which a value of 330 mV was determined and was probably overestimated due to the membrane charging time which was not taken into account for calculation (26). We have checked the validity of this approach on other systems by using results from other laboratories or from our group (Table 1). In all cases, values close

TABLE 1 Estimation of permeabilizing membrane potential difference

Cells	ΔV	E_p	E_F	ΔU_c	r	fg	References
	mV	kV/cm	kV/cm	mV	μm		
Chinese hamster ovary	60	0.3	0.5	240	6	0.8	This work
Yeast protoplasts	60	3	7	150	2	0.15	(31)
Bacterial protoplasts	120	2	6	240	1	0.5	(32)
Plant protoplasts	20	0.35	0.5	114	12	0.3	(33, 34)

ΔU_c and fg were computed from Eqs. 8 and 9, respectively.

to 200 mV were found. An interesting comment was published some years ago in which it was observed that, from a compilation of experimental data, E_F was always in the order of 1.5–2 times E_p in the case of mammalian cells (29). This gives values of ΔU_c ranging between 180 and 300 mV, close to the results in Table 1. The other conclusion is that the fg parameter of Eq. 1 is much smaller than 3/2. Values as low as 0.15 are found in the case of protoplasts in which the proteolytic treatment used to remove the wall have played a damaging effect. But even with untreated mammalian cells, the value is only 0.8 showing that the mammalian cell membrane cannot be considered as a pure dielectric, and the field-induced potential difference is much smaller than previously described. As a general conclusion, a membrane in a cell is permeabilized when its potential difference is brought to 200–250 mV by the external field. This is a much smaller value than previously published when a value of 1 V was reported.

Note added in proof

In a recent theoretical paper (Grosse, C, and H. P. Schwan. 1992. *Biophys. J.* 63:1632–1642), it was suggested that the induced membrane potential difference was affected by the surface conductance and the membrane conductance. These corrections are proned to decrease the magnitude of the phenomena as we experimentally observe in the present work.

APPENDIX

It has been suggested that when one side of the cell is permeabilized, the field effect becomes two times larger on the intact one (30). Under such an assumption Eq. 6 should be written as

$$\Delta U_c^*(\theta = 0) = 2fgr E_F - \Delta V \tag{16}$$

and Eq. 8 as

$$\Delta U_c^* = \Delta V(2E_F + E_p)/(2E_F - E_p). \tag{17}$$

Then

$$\Delta U_c^* < \Delta U_c. \tag{18}$$

Under such an assumption the critical potential difference is even smaller than calculated above.

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