Correlation between Electric Field Pulse Induced Long-Lived Permeabilization and Fusogenicity in Cell Membranes

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ABSTRACT Electric field pulses have been reported to induce long-lived permeabilization and fusogenicity on cell membranes. The two membrane property alterations are under the control of the field strength, the pulse duration, and the number of pulses. Experiments on mammalian cells pulsed by square wave form pulses and then brought into contact randomly through centrifugation revealed an even stronger analogy between the two processes. Permeabilization was known to affect well-defined regions of the cell surface. Fusion can be obtained only when permeabilized surfaces on the two partners were brought into contact. Permeabilization was under the control of the pulse duration and of the number of pulses. A similar relationship was observed as far as fusion is concerned. But a critical level of local permeabilization must be present for fusion to take place when contacts are created. The same conclusions are obtained from previous experiments on ghosts subjected to exponentially decaying field pulses and then brought into contact by dielectrophoresis. These observations are in agreement with a model of membrane fusion in which the merging of local random defects occurs when the two membranes are brought into contact. The local defects are considered part of the structural membrane reorganization induced by the external field. Their density is dependent on the pulse duration and number of pulses. They support the long-lived permeabilization. Their number must be very large to support the occurrence of membrane fusion.

INTRODUCTION

The cell plasma membrane protects the cytoplasmic volume from being entered by exogenous molecules and prevents the spontaneous fusion of two different cells. This barrier can be overcome only through dramatic treatments of the cell surface.

It was shown previously that subjecting cells to short strong electric pulses makes the membrane permeable in a spontaneously reversible way (Neumann and Rosenheck, 1972). Later it was observed that such an electropulsing of cells in contact induced their fusion (Senda et al., 1979). More recently, fusion was observed to occur when prepulsed cells were brought into contact (Sowers, 1986, 1987). This was called the pulse first method (PF), as opposed to the contact first approach (CF), which was more commonly used. This has been demonstrated for many systems: ghosts (Sowers, 1986, 1987, 1989a,b; Sowers and Kapoor, 1987; Dimitrov and Sowers, 1990b; Wu et al., 1992), mammalian cells (Teissié and Rols, 1986, 1988; Rols and Teissié, 1989, 1990a), and plant protoplasts (Montané et al., 1990). Heterofusion can be obtained between erythrocyte ghosts and mammalian cells (Rols et al., 1994). This last observation is the definitive evidence that fusion occurs between cells that are not preassociated during the pulse. These methods of permeabilization and fusion are now routinely used in cell biotechnology for obtaining cell hybrids and in gene transfer procedures, but very little is known about the molecular mechanisms involved (Neumann et al., 1989). It is thought that the leading force is a modulation of the membrane potential difference inducing a membrane structural change. This was indeed observed through video monitoring of fluorescence of voltage sensitive dyes in the pulsed cells (Gross et al., 1986; Ehrenberg et al., 1987; Kinosita et al., 1988). As soon as the membrane potential difference was elevated to a critical value of ~200 mV, the membrane becomes permeabilized and fusogenic in that part of the cell surface (Teissié and Rols, 1993). But the precise analysis of this new organization is still missing, except for a ^{31}P NMR study (Lopez et al., 1988). One major question is the extent to which the processes of permeabilization and fusion are related to each other. Supposing that a membrane was a viscoelastic film, Dimitrov (1995) has suggested a correlation between "electroporation" and electrofusion in the case of contact first approach (CF).

In the present study, a strong correlation was observed between the long-lived properties of permeability to small molecules and fusogenicity induced by the field pulse. The two phenomena are controlled in the same way by the physical parameters of the pulse: the field strength and the pulse duration. The data on the molecular changes of the cell membrane associated with electropermeabilization suggest that the reorganization of the membrane that increases its permeability parallels the induction of random defects, which are highly fusogenic, in defined parts of the cell membrane.

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METHODS

CHO culture

The WTT clone of CHO (Chinese hamster ovary) cells was selected because it is not strictly anchorage dependent. It has been adapted for

suspension culture at 37°C under gentle agitation in minimum essential medium supplemented with 8% new born calf serum, antibiotics, and L-glutamine. Cells were maintained in the exponential growth phase. The advantage of this clone was its ability to be replated readily on petri dish, making the observation of the level of polynucleation very convenient.

CHO cell pulsation

Cells were washed in a pulsing buffer (10 mM phosphate, 250 mM sucrose, 1 mM $MgCl_2$, pH 7.2) and resuspended at different concentrations between 10^5 cells/ml and 10^7 cells/ml. A volume (0.1 ml) of the cell suspension was placed between the thin stainless steel parallel electrodes in contact with the bottom of a culture dish (Nunc, Roskilde, Denmark). The two electrodes were connected to a square-wave pulse generator (CNRS Cell Electropulser; Jouan, St. Herblain, France). The voltage pulses applied to the cells were monitored on line, using an oscilloscope incorporated in the cell electropulser (Enertec, St. Etienne, France). The delay between pulses was 1 s. All of these parameters were electronically controlled by the electropulser. As the cell suspension formed a rather stable drop on the bottom of the dish, cells were pulsed in different directions by changing the orientation of the electrodes relative to the dish (Teissié and Rols, 1986). Experiments were conducted at room temperature or on ice.

Cells were washed in a permeabilization buffer containing dye (100 μM propidium iodide). One hundred microliters of the cell suspension (10^6 cells) was poured between two parallel stainless-steel electrodes in contact with the bottom of a culture dish (diameter 35 mm; Nunc). After pulsing, cells were incubated for 10 min at room temperature. Cells were analyzed by flow cytometry (Becton Dickinson FACScan) to determine the percentage of stained cells in the population.

Post-pulse contact between the cells was created in a random way by simply spinning them down at 50 $\times$ *g* (Jouan centrifuge). The cell pellet was then incubated at 37°C for 10 min, and then after gentle deaggregation the cell suspension was poured into a petri dish (Nunc) of fresh culture medium. Viable cells can then spread on the dish surface. After a 2–4-h incubation in an air/CO_2 atmosphere, cells were observed under an inverted microscope (Leica, Wetzlar, Germany) by video monitor (Sony, Aomori, Japan), and the percentage of polynucleated cells was evaluated. The fusion index (FI) was taken as the the number of nuclei in polynucleated cells as a percentage of the total number of nuclei. However, an endogenous background of polynucleation (due to a nucleus division without a cell division) was always present (Teissié and Rols, 1986) and was observed to increase upon aging of the culture. Because of this spontaneous level of polynucleation, the net fusion index was obtained by substracting the background polynucleation value.

Experimental conditions, in which only plated cells were observed, were chosen so as to select viable cells (i.e., those able to grow after pulsation). Indeed, significant dispersion could be observed in the cell survival measurement, because of its modulation by cell culture aging (Gabriel and Teissié, 1995).

Lifetime of the fusogenic state of CHO cells

The lifetime of the fusogenic state was measured by centrifuging the pulsed cells (10 times 100 μs, 1.2 kV/cm) at progressively longer times after the pulse treatment at room temperature. In the experiments for the zero incubation time, it required less than 10 s to move the cells from the pulsing chamber to the centrifuge, and an additional time was needed to create their contact during the centrifugation. The fusion index was evaluated as above.

Experiments on ghosts

All results were taken from the experiments published by A. E. Sowers (1986, 1987, 1989a). Pulses were obtained with a capacitor discharge set-up. Contacts in the case of fusion were obtained by dielectrophoresis.

THEORY

As shown previously (Neumann et al., 1989; Ehrenberg et al., 1987; Hibino et al., 1993), the main effect of an applied field is to modulate the membrane potential difference. This is done in a position-dependent manner:

$$\Delta V = frE \cos\theta(1 - \exp(-t/\tau_c)) \quad (1)$$

where ΔV is the membrane potential difference modulation (Fig. 1), f is a physiological factor that is dependent on the cell (Teissié and Rols, 1993), r is the radius of the cell, E is the strength of the field, θ is the angle between the direction of the bulk applied field and the normal to the cell surface at any given point, t is the duration of the field application, and τ_c is the charging time of the membrane.

As permeabilization is triggered when the membrane potential difference is larger than a critical threshold ΔV_p of ~200–300 mV (Teissié and Rols, 1993), it is easily shown

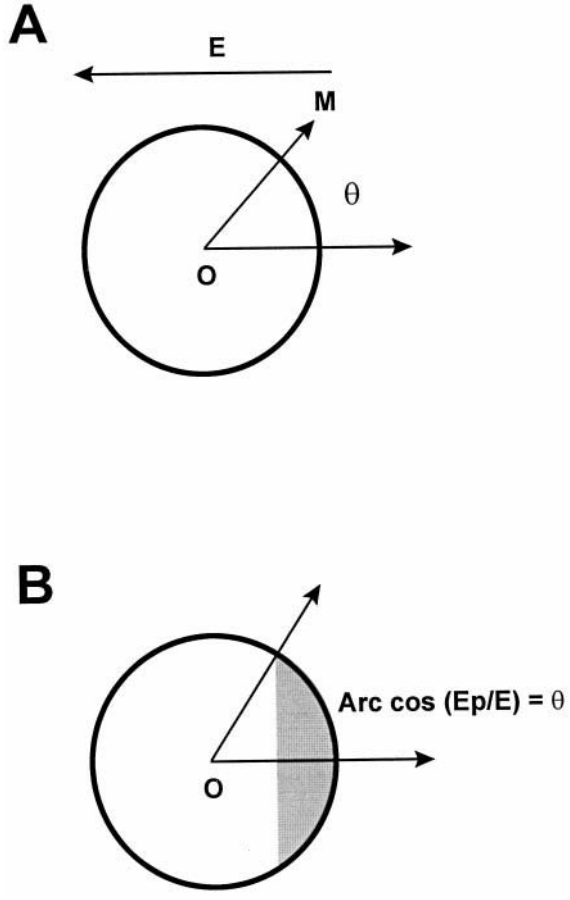

FIGURE 1 Field effects on a spherical cell. (*A*) The cell is exposed to a pulsing uniform field E. θ is the angle between the bulk direction of the field and the normal to the cell surface at position M. The induced potential difference is position dependent on the cell surface. (*B*) When the cell is pulsed by a field with a strength E (larger than E_p, the critical value that triggers membrane electropermeabilization), the surface cap within the angle Arc cos (E_p/E) (i.e., θ_p) is permeabilized (the gray part of the surface on the cartoon) and brought to a fusogenic state.

that only the part of the cell surface facing the electrode, and inside a cone with a half-angle θ_p such that

$$\cos \theta_p = E_p/E \quad (2)$$

is affected by the field.

E_p is given by

$$\Delta V_p = frE_p \quad (3)$$

This is indeed observed experimentally when square-wave pulses are used to induce ATP leakage in CHO membranes (Rols and Teissié, 1990b). With square-wave pulses, the field is a constant (i.e., it is not time dependent). The outflow Φ is related to the field strength, as soon as $E > E_p$, by the definition of the extent of the affected cell surface (Fig. 1):

$$\Phi = K(1 - E_p/E) \quad (4)$$

where K is a parameter that does not depend on the field strength.

The pulse duration has been shown to strongly control the extent of permeabilization. As mentioned in the Methods, two permeabilization steps are observed. One is present during the pulse and allows the exchange of large macromolecules (Sowers, 1988b). The second one is much more long-lived and supports the diffusion of small molecules (up to 1–2 kDa) according to Fick's law, as shown by quantitative analysis (Rols and Teissié, 1990b). The field is active only as long as it is able to induce a membrane potential difference larger than the critical threshold ΔV_p. The flow $\Phi(Z)$ of a given molecule Z across the membrane during the long-lived alteration is given by

$$\Phi(Z) = A\Delta ZF(T, t, n)(1 - E_p/E) \quad (5)$$

where ΔZ is the concentration gradient in Z, A is a constant, T is the pulse duration, n is the number of pulses, t is the delay after the pulse (the resealing process being first order), and F corresponds to the fraction of the actually permeabilized area and describes a progressive transition in the organization of membrane during the pulse. F is the probability of creating permeabilized defects in the part of the cell surface that is prone to permeabilization, i.e., inside the θ_p cone given by Eq. 2 (Fig. 1).

F is therefore not a function of the pulse strength when a square-wave pulse is used and is uniform over the cell surface where permeabilization may take place (Rols and Teissié, 1990a) (Fig. 2). F is a function of T and n (Rols and Teissié, 1990a). F decreases after the pulse with a first-order kinetic:

$$F(t) = F(0)(\exp(-t/\tau_p(T, n))) \quad (6)$$

where t is the time after the pulse and $\tau_{p(T, n)}$ increases with pulse duration T and number n, but does not depend on E (Rols and Teissié, 1990a).

When a capacitor discharge pulse generator is used, as in the case of the experiments with ghosts, the function F will be more complicated, because the duration of the effective part of the pulse is going to be position dependent on the cell surface.

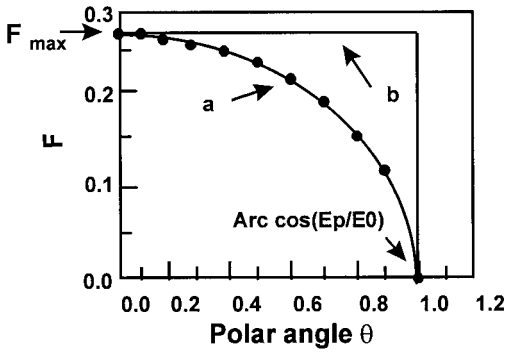

FIGURE 2 Control of F by the polar angle on the cell surface. The cell is assumed to be a sphere. As explained in the text and in Fig. 1, the polar angle θ is between the direction of the field and the normal at the cell surface. If an exponentially decaying field is applied, F decreases with an increase in θ (*curve a*), but drops to zero at the same critical position θ_p, Arc cos(E_p/E_0). If a square wave pulse is applied, F is constant up to Arc cos(E_p/E_0) and then is null (*curve b*). The same intensity E_0 is chosen for the initial field in the decaying field case and for the field in the square wave condition.

The condition for the temporal dependence of the field strength for permeabilization at a position θ, which is drawn from the previous conclusions, is then

$$E(t)\cos \theta > E_p \quad (7)$$

where t starts at the onset of the field, i.e.,

$$E(t) = E_0 \exp(-t/RC) \quad (8)$$

This gives the definition of the effective permeabilizing pulse duration T_θ at position θ on the cell surface, if $E_0 > E_p$:

$$E_0 \exp(-T_\theta/RC)\cos \theta = E_p \quad (9)$$

where E_0 is the initial field intensity, R is the equivalent resistor of the sample, and C is the capacitance of the discharge network. In fact, RC is easily monitored on-line with a storage screen oscilloscope:

$$T_\theta = RC \ln(E_0 \cos \theta/E_p) \quad (10)$$

If we take into account the conclusions obtained with square-wave pulses that under our experimental conditions (short pulse duration) F is a linear function of the number of pulses n and of the permeabilizing pulse duration T_θ, then F is position dependent on the cell surface. We then get the expression of F_θ:

$$F_\theta = BnRC \ln(E_0 \cos \theta/E_p) \quad (11)$$

where B is a proportionality constant.

Furthermore, as described in Eq. 6, F decreases with time after pulsing with a rate constant that decreases with an increase in the effective pulse duration (Rols and Teissié, 1990a).

We conclude that first, with the capacitor discharge system, permeabilization is not uniform on the cell surface (Fig. 2). A very small and short-lived flow will occur when θ is large up to the limit:

$$\cos \theta_p = E_p/E_0 \quad (12)$$

But permeabilization should be large and long-lived at $\theta = 0$.

Second, increasing E_0 will increase the percentage of the cell surface that is permeabilized and, for a given value of θ, the local value of F and its decay time after the pulse. This is due to the increase in T_θ, as shown in Eq. 10.

Third, increasing RC will increase the local value of F and its postpulse decay time for a given position, but not the percentage of the cell surface that is prone to permeabilization.

This complex dependence of F not only on the electrical parameters E_0 and RC, but mainly on the position that is considered on the cell surface (Fig. 2), provides a convenient tool for observing whether F is related to FI.

F_θ can be written as a function of the position by rearranging Eq. 11 as

$$F_\theta = D \ln(\cos \theta) + G \quad (13)$$

where

$$D = BnRC \quad (14)$$

$$G = D \ln(E_0/E_p) \quad (15)$$

F_θ is then related by a rather simple mathematical expression to the experimental parameter θ when cells are pulsed under the same E_0 and RC parameters.

RESULTS

Field strength effects

Permeabilization (i.e. the percentage of stained cells in the population) is under the control of field intensity (Fig. 3 *A*). A threshold field intensity is required to observe the occurrence of permeabilization. When the field strength was less than 400 V/cm, it was impossible to detect stained cells. The number of stained cells then increased with the increase in field strength. Nearly all of the cells were stained when 1.4 kV/cm was used.

The effect of the field strength on PF FI was evaluated for CHO cells (Teissié and Rols, 1986). FI is clearly increased over the background with an increase in the field strength (Fig. 3 *B*). More interestingly, FI is strongly increased for the same field strength just by pulsing the cells in two perpendicular directions (compare conditions 3 and 4 in Fig. 4). Under such experimental conditions, different caps on the cell surfaces will be brought to the permeabilized states. By choosing the field intensities in such a way that there is no overlap between the permeabilized surfaces, the total area will be the sum of the areas of the two caps. FI has been plotted as a function of the affected surface in Fig. 5. A good fit is obtained between FI and the square of the

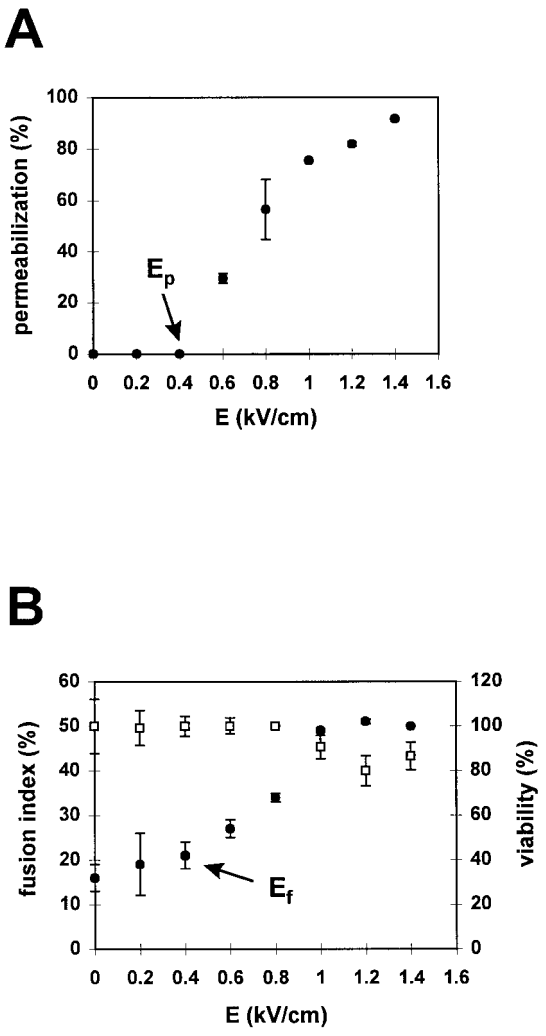

FIGURE 3 Field strength effects. (*A*) Electropermeabilization as a function of field intensities. Cells were pulsed 10 times for 100 μs with fields of increasing strength in the presence of propidium iodide. The level of permeabilization (i.e., the percentage of stained cells in the population) is plotted as a function of the electric field strength. (*B*) Fusion index (●) and viability (□) of pulsed CHO as a function of field intensities. Cells were pulsed 10 times for 100 μs. The level of fusion index (i.e., the percentage of fused cells) is plotted as a function of the electric field strength. The level of viability (i.e., the percentage of viable cells 24 h after the procedure) is plotted as a function of the electric field strength.

permeabilized surface. This observation can be explained by taking into account that the contact between the cells is obtained in a random way along the centrifugation step. We should expect such a correlation if we assume that fusion occurs only when two electropermeabilized areas are brought into contact. The contact between an electropermeabilized surface and a normal one is not prone to fusion. The way to increase the permeabilized surface is not a factor in the definition of FI, because the same fusion yield is obtained either by increasing the field strength or by pulsing in two crossed directions.

Cell viability is not altered by the electric field intensity (Fig. 3 *B*). For intensity ranging from 0 to 1.4 kV/cm, the

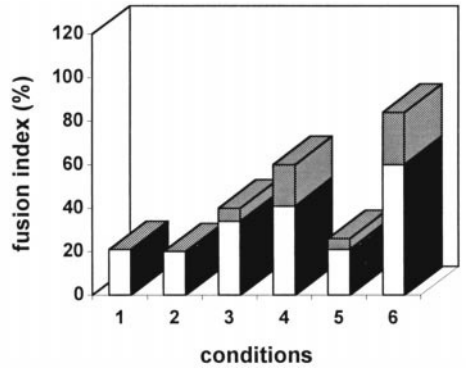

FIGURE 4 Fusion index of CHO cells. Cells were pulsed in suspension under different conditions, except that the square-wave pulse duration was kept at 100 μs. The fusion index (FI) is given by the level of polynucleation. The experimentally observed yield is plotted in white. But as a large polynucleation background level was present in the unpulsed sample (see Methods), the index due to the electric field was obtained after the background was subtracted. This index is indicated by the grey portion of each bar. Note that cells must be pulsed and centrifuged to show the electric field effect. Conditions: 1, no pulse, no centrifugation; 2, no pulse, centrifugation; 3, 1 kV/cm, five pulses, one direction, centrifugation; 4, 1 kV/cm, centrifugation, three pulses in two crossed directions; 5, 1,6 kV/cm, five pulses, no centrifugation; 6, 1.6 kV/cm, five pulses, centrifugation.

viability remained unchanged under our short-pulse, low-number conditions.

Effect of pulse duration on permeabilization and fusion

At a given electric field strength, increasing the duration of pulses induced an increase in the permeabilization efficiency up to a plateau value (~90%) obtained at relatively a low value of the pulse duration (i.e., 100 μs) (Fig. 6 *A*).

The dependence of cell fusion on the pulse duration is shown in Fig. 6 *B*. The polynucleation index increases with the increase in duration of pulses, up to a maximum observed for a pulse duration of 100 μs. Viability is strongly affected by increasing pulse duration. This decrease in viability did not seem to affect the fusion index, which is observed only for viable cells, because of the selective plating step.

The results obtained for permeabilization and fusion show that the pulse duration modulates permeabilization and fusion in a similar way.

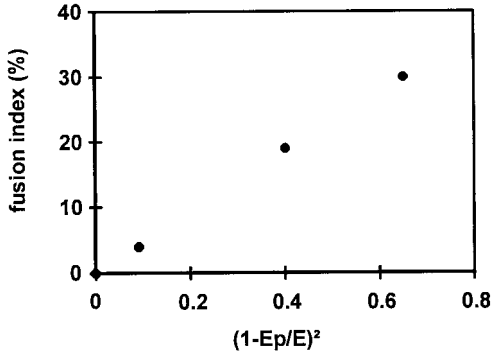

FIGURE 5 Electrofusion index of CHO cells as a function of the permeabilized surface. The permeabilized surface was obtained from the field strength by taking into account the threshold value needed to induce permeabilization. A linear fit is clearly observed when FI (corrected from the background) is plotted versus the square of the area. Conditions: five pulses of 1.6 kV/cm with centrifugation.

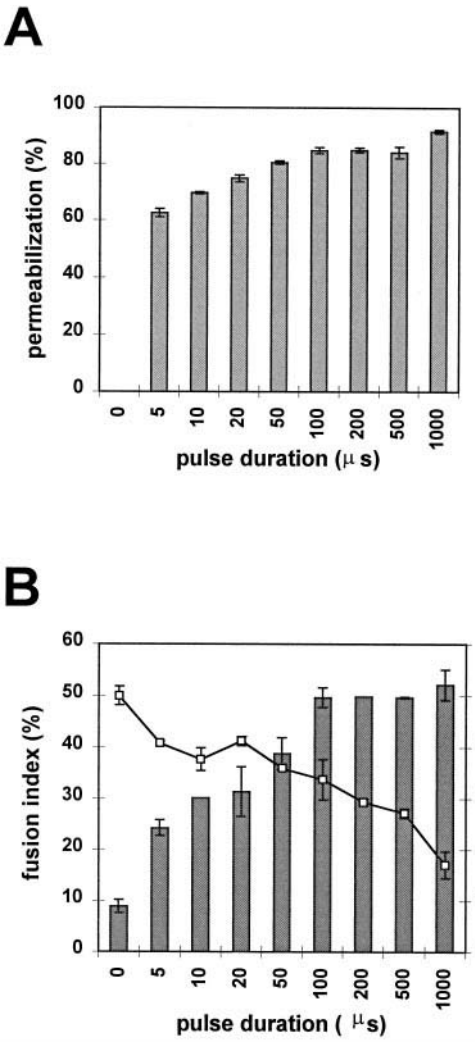

FIGURE 6 Effect of pulse duration on permeabilization and fusion. (*A*) Electropermeabilization as a function of the pulse duration. Cells were pulsed 10 times at 1.2 kV/cm in the presence of propidium iodide in the pulsing buffer. The level of permeabilization (i.e., the percentage of stained cells in the population) is plotted as a function of the duration of pulses. (*B*) Fusion index (▨) and viability (□) of pulsed CHO as a function of the pulse duration. Cells were pulsed 10 times at 1.2 kV/cm. The level of fusion index (i.e., the percentage of fused cell) is plotted as a function of the duration of pulses. The level of viability (i.e., the percentage of viable cells 24 h after the electric treatment) is plotted as a function of the pulse duration.

Effect of number of pulses on permeabilization and fusion

At a given electric field strength (1.2 kV/cm), increasing the number of pulses (duration 100 μs) induced an increase in

the permeabilization efficiency up to a plateau value obtained at a relatively low value of number of pulses (i.e., 10 pulses) (Fig. 7 *A*).

The dependence of cell fusion on the number of pulses is shown in Fig. 7 *B*. Pulses of 1.2 kV/cm lasting 100 μs were applied to the suspension of cells. It should be noted that both parameters (intensity and duration) were chosen to give maximum fusion. After only two pulses, the extent of fusion is already very high; ~80% of the maximum effect is observed. The plateau value was reached after only 10 repeated pulses. But the level of fusion was observed to decrease as the number of pulses increased.

Viability is strongly affected by the increasing number of pulses. The decrease in the index of fusion can be associated with the damaging effect of the pulses, as shown by the associated loss of viability.

This result shows that cell fusion is not dependent only on permeabilization. A cytoplasm reorganization follows the membrane merging.

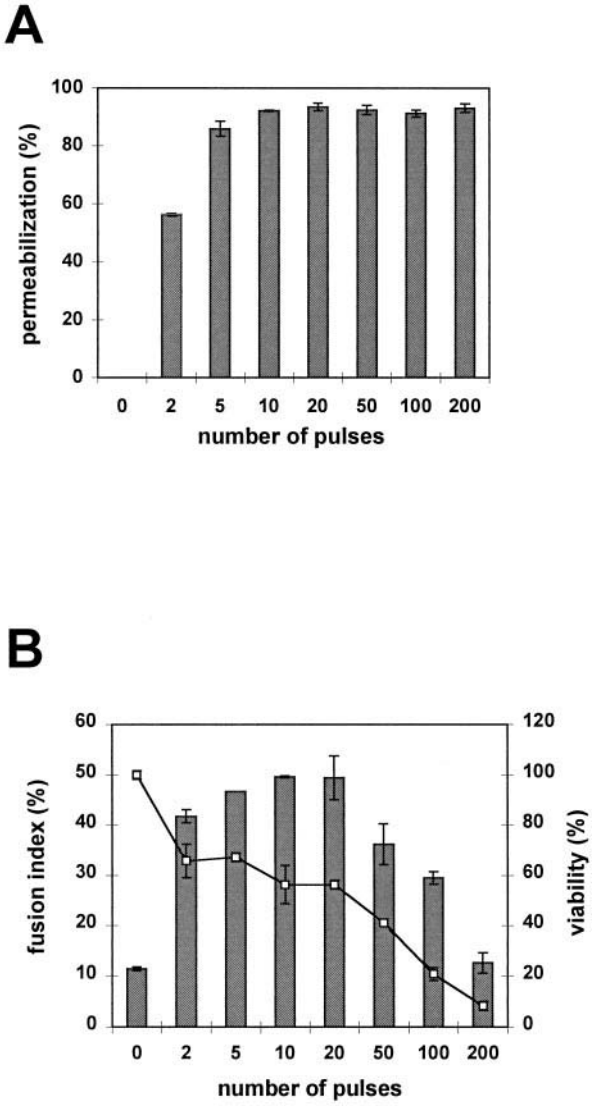

FIGURE 7 Effect of number of pulses on permeabilization and fusion. (*A*) Electropermeabilization as a function of the number of pulses. Cells were pulsed at 1.2 kV/cm for 100 μs in the presence of propidium iodide in the pulsing buffer. The level of permeabilization (i.e., the percentage of stained cells in the population) is plotted as a function of the number of pulses. (*B*) Fusion index (▨) and viability (□) of pulsed CHO as a function of the number of pulses. Cells were pulsed at 1.2 kV/cm for 100 μs. The level of polynucleation index (i.e., the percentage of fused cell) is plotted as a function of the number of pulses. The level of viability (i.e., the percentage of viable cells 24 h after the electric treatment) is plotted as a function of the number of pulses.

Lifetime of the permeable and fusion state

Application of electric field pulses leads to reversible permeabilization of the plasma membrane of cells. The phenomenon is dependent on the electric field intensity, the number of pulses and their duration, and the temperature.

Cells were pulsed by application of 10 pulses of 100 μs duration each. The field intensity was 1.2 kV/cm. Propidium iodide in the pulsing buffer was added at definite times after pulsation, from 0 to 20 min. Fig. 8 *A* shows the kinetics of the recovery of electropermeabilized cell membrane integrity obtained at room temperature. Recovery curves showed that 20 min after electropulsation, all cells regained their initial membrane impermeability to the dye.

The fusion index is strongly dependent on the delay between the electric pulses and centrifugation. Results are shown in Fig. 8 *B*. A high FI is observed if the contact is created as fast as possible, but fusion is almost absent 5 min after pulsing. The value that is then measured is in fact due to the endogenous polynucleation of the control cells. A very fast decrease is indeed present during the first minute.

It is then clear that the lifetime of the fusogenic state is shorter than that of the electropermeabilized state.

As FI is dependent on the F function, FI should decrease as F; i.e., its lifetime should be the one of the electropermeabilized states:

$$\mathrm{FI}(t) = k(F(n, T, t)) \tag{16}$$

$$F(n, T, t) = F(n, T, 0)\exp(-t/\tau_p) \tag{17}$$

$$\mathrm{FI}(t) = \mathrm{FI}(0)\exp(-t/\tau_p) \tag{18}$$

where τ_p is the decay time of the resealing kinetic.

Another model can be proposed from the conclusions obtained from the field effects. FI is dependent on the square of F because permeabilized surfaces must be in contact for fusion to take place. F reflects the density of defects, but is the same on all membrane permeabilized parts, because all cells are pulsed in the same way:

$$\mathrm{FI}(t) = k(F(n, T, t))^2 \tag{19}$$

$$\mathrm{FI}(t) = \mathrm{FI}(0)\exp(-2t/\tau_p) \tag{20}$$

From the results in Fig. 8 and the data on electropermeabilization resealing, even after such a correction, fusogenicity appears to be very short-lived. A critical level of F, F_c must be present for fusion to take place. As soon as F decays

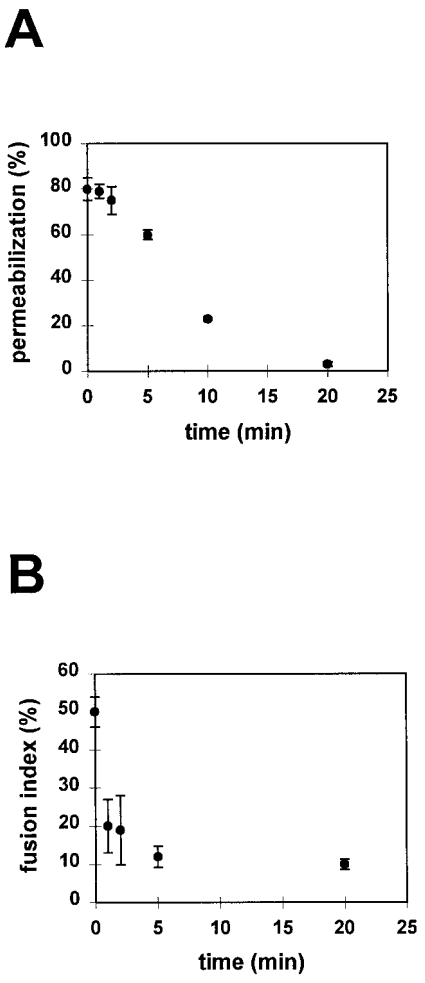

FIGURE 8 Decay of the post-pulse permeability and fusogenicity of CHO cells. (*A*) Electropermeabilization resealing as a function of the time after pulses. Cells were pulsed for 100 μs 10 times at 1.2 kV/cm. The electropermeabilization resealing (i.e., the percentage of cells still incorporating dye at different times after the electric treatment) is plotted as a function of the time after pulses. (*B*) Decay of fusion as a function of the time after pulses. As described in Methods, the contact between the cells was created after increasing delays after pulsing. A background level of polynucleation was present in the control cells (~10 (± 2)%). Conditions: 10 100-μs pulses of 1.2 kV/cm at a frequency of 1 Hz followed by a centrifugation at the indicated delay. Experiments are run at room temperature.

down to this critical level, FI drops to zero. Results in Fig. 8 then indicate that such a critical F_c value is reached less than 1 min after CHO cells are pulsed with square-wave pulses (this value needs to be corrected by the experimental delay described in the Methods).

Effects of the position of the contacts

In the experiments in which the postpulse contact is obtained by dielectrophoresis, it was possible to obtain the cell-cell contact in a specific position, using the direction of the permeabilizing field as a reference (Sowers, 1987). An example is given in Fig. 2.

Using the results relating FI to the direction of the AC field, which was made different from that of the DC pulse (Sowers, 1987), FI can be plotted as a function of ln(cos θ), as shown in Fig. 9. It is clear that as long as θ is small (large values of ln(cos θ)), we obtain a linear relationship showing that for large values of F (i.e., small values of θ), FI is directly dependent on F (i.e., by the structural transition in the membrane organization) (Rols and Teissié, 1990b). When θ is large (i.e., F is small; small values of ln(cos θ)), FI is equal to zero, suggesting either that the lifetime of the permeabilized state is shorter than the delay between pulse and contact or/and that F must be larger than a critical value F_c to induce the fusion when contact is created. This second conclusion gives the following expression for FI:

$$\mathrm{FI} = H(F - F_c) \tag{21}$$

where H and F_c are constant. F_c is $F(\theta = 33°)$ in Fig. 9. F_c is, again, experimental evidence that the structural transition induced by the external field should have reached a critical level for the fusogenicity to be detected.

DISCUSSION

Electric field pulses are known to induce permeabilization and fusion of cell membranes. Previous studies (Sowers, 1986; Teissié and Rols, 1986) showed that the two processes were long-lived. The present work suggests a strong correlation between these two long-lasting properties induced in a cell membrane by an electric pulse treatment. The major parameters triggering these alterations of the specificity of a cell surface, i.e., the field strength, the pulse duration, and the number of pulses, appear to play similar roles in the two processes.

Electropermeabilization is described as a multistep phenomenon. The first two steps, induction and expansion, were first described to explain the conductance changes of

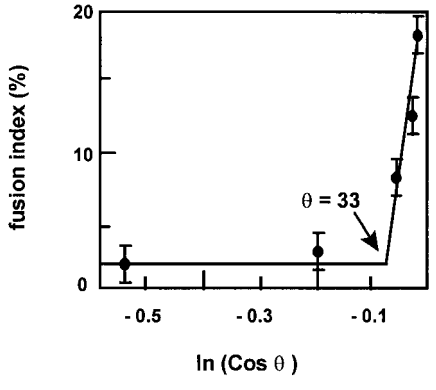

FIGURE 9 The fusion index of ghosts is under the control of F. The plot is obtained from the results in figure 2 in Sowers (1987) and from Eq. 13. The model system was ghosts that were brought into contact under controlled directions relative to the bulk permeabilizing field by dielectrophoresis. F must be larger than a threshold obtained for θ (close to 33°) for fusion to occur. This is indicative of a critical value for F, as shown in Eq. 21. For larger values of F, F_1 is a linear function of F, as shown in Eq. 16.

pulsed red blood cells (Kinosita and Tsong, 1979). A stabilization step was shown by time-resolved video analysis of the pulsed cells (Kinosita et al., 1988; Hibino et al., 1993). The final step, resealing, was known from early studies and is, of course, critical for cell viability (Neumann et al., 1989). Direct video analysis of the leakage of fluorescent molecules from pulsed ghosts showed that macromolecules were able to cross the membrane only during the first three steps (Sowers, 1988b; Dimitrov and Sowers, 1990a). Small molecules were also observed to leak out during the last step (Dimitrov and Sowers, 1990a; Rols and Teissié, 1990b; Marszalek et al., 1990; Tekle et al., 1990, 1991). The last property is the one on which the present study focused.

The present results deal with PF-induced electrofusion and demonstrate that the fusion occurs by a mechanism different from what is observed in CF electrofusion (Abidor and Sowers, 1992a; Dimitrov, 1995). In such a case, the fusion yield is dependent on a first-order reaction, i.e., its logarithm is linearly related to the pulse duration. This suggests that the fusion reaction takes place during the pulse when the membrane organization is highly conductive at yet uncharacterized defects (Hibino et al., 1993). If the rate constant appears to be linearly related to the square of the electropermeabilized part of the membrane (Fig. 5), it is observed to be linearly related to the probability of defect formation ("electropores"), as in the present study. This was explained by the close contact between the two membranes during the pulse, which facilitates double permeabilization and coaxial pore formation, and by a direct action of the field that pushes the edges of the "pores" into contact to form the membrane "tubes" (Abidor and Sowers, 1992a,b). Such an explanation is not valid for postpulse contact, in which close contact is induced randomly between pulsed cells, when the field is no longer applied. But the same observation is obtained if there is a high probability of fusogenic defects on the contact surface between the two partners.

Electron microscopy studies were on two different time scales. Quick freezing techniques suggested the occurrence of short-lived dramatic alterations such as cracks and craters (Stenger and Hui, 1986; Chang and Reese, 1990). The short lifetime of such structures (on the order of seconds) strongly suggests that they are not relevant to long-lived permeability (which lasts several minutes). Other studies showed an eruption of villi and the formation of blebs, which may be due to a weakening of the cell matrix (Escande-Geraud et al., 1988; Gass and Chernomordik, 1990). Hydrophobicity of pulsed protoplast membranes was observed through contact angle measurements to be increased (Hahn-Hagerdal et al., 1986). Indirect conclusions were obtained from the analysis of ATP leakage in pulsed CHO cells (Rols and Teissié, 1990b). The permeabilized surface was well localized on the cell surface and was not spread out along the resealing step. This was in agreement with previous video observations (Mehrle et al., 1985). These observations lead to a model of the electropermeabilized membrane in which lipids cannot be the only target, because of their fast lateral diffusion coefficient. This is in agreement with recent results showing that the resealing step was under the control of the cytoskeleton (Rols and Teissié, 1991).

As far as PF electrofusion is concerned, the most significant fact was that the fusogenic part of the cell surface was on laterally immobile sites (Sowers, 1987). Fusion involves a decrease in the magnitude of the repulsive forces known to exist when two cells are in close contact (Zimmerberg et al., 1993). The activation of the fusion step is entropy driven, and an increase in the surface hydrophobicity is always observed under conditions leading to membrane fusion (Ohki, 1987). The above-reported increase in the membrane hydrophobicity in electropermeabilized cells is in agreement with an increase in their fusogenicity.

The conclusion of the present study is that there is a strong correlation between the two effects of cell electropulsation. Fusion occurs only between parts of the cell surface that have been brought to the permeabilized state. The decrease in the fusion yield that is observed over time in the experiments in which the contact was topologically controlled by dielectrophoresis (Sowers, 1987) can then be a consequence of the slow rotation of the pulsed ghosts due to their Brownian motion. The two partners in the fusion must be electropermeabilized for fusion to occur. The dependence of the FI on the position is clearly related to the level of real permeabilization, which is dependent on the effective pulse duration and the number of pulses (Rols and Teissié, 1990b). This level is associated with the extent of the membrane structural transition that is induced by the field. It was suggested that fusion may occur either through a merging of flaps (a short-lived process) or by a contact between local defects or fluctuations (Stenger, 1988). The long-lived fusogenicity can of course be related only to the second process. Fusion should then be described by a dynamic or local process and be triggered by a strong interaction between small portions of the cell surfaces. A prediction was that FI should increase under high ionic content conditions that are likely to increase the number of local defects (Rols and Teissié, 1989; Sowers, 1989b). It should be then suggested that the number of local defects, in which contact triggers the fusion, and the extent of the structural transition, which controls the level of permeabilization (i.e., the parameter F in the flow equations), are two forms of the same alteration of the membrane organization. This is again supported by the present study, which demonstrates a control of FI by the effective pulse duration and number of pulses shown to control F. This alteration is clearly triggered by a lipid matrix change, but its lifetime and its lack of lateral mobility suggest that membrane proteins and cytoskeleton are involved in the process (Teissié and Rols, 1994). We should mention that cell fusion resulting from membrane fusion is facilitated by osmotic stress (Song et al., 1993; Stoicheva and Hui, 1994; Li and Hui, 1994), but remains controlled by cytoskeleton reorganization (Blangero et al., 1989; Wu et al., 1994). But these are

associated effects of the critical membrane fusion linked to membrane electropermeabilization.

A key conclusion is obtained from the present study. It is assumed in many cases that the long-lived permeability of pulsed cells is due to the creation of a limited number of "pores" with sizable diameters (Kinosita and Tsong, 1979; Sowers and Lieber, 1986; Dimitrov, 1995). This was supported by a electron microscopy study on ghosts under hypoosmolar conditions (Chang and Reese, 1990). A different description was proposed, in which a large number of small defects due to structural mismatches were present (Cruzeiro and Mouritsen, 1989). This is in line with the molecular model for the movement of molecules across lipid membranes proposed by Träuble (1971). This second description is in better agreement with the ^{31}P NMR studies, which showed that most lipids in the permeabilized area were in an out-of-equilibrium state during the electropermeabilized state (Lopez et al., 1988). The "pore" hypothesis cannot explain the present results if it is assumed that fusion occurs by the "coaxial" process. The "pores" are created in a random way at a very low density on the cell surface (Dimitrov, 1995). The probability that they should face each other when cells are brought into contact in a random way is extremely low, even if it is assumed that they diffuse during the contact step, which lasts ~1 min (the lifetime of the fusogenic state). This assumption of a movement of "pores" is furthermore not supported by the observation that fusogenic parts are not mobile (Sowers, 1987). We must then conclude that fusogenicity is due to the creation of a high number of small defects created randomly during the pulse in a well-defined part of the cell surface. This density of defects increases with the pulse duration and the number of pulses, but not with the field strength, as shown by the dependence of F and FI on pulse duration, number of pulses, and resealing. The structure and dynamics of these defects remain unknown but induce a decrease in the intermembraneous repulsive forces that prevent spontaneous fusion. If we make the assumption that the defects are a "stable" nonclassical lipid organization, when FI is 0.4 we get 10^8 defects per cell (or less) (see Appendix). If a description based on dynamics is preferred, these "defects" are described as "fluctuations" in the packing density of the lipid matrix. The probability of such fluctuations is then $5 \times 10^5/\mu m^2$/min (see Appendix). This conclusion supports the mismatch description of the long-lived permeabilization. This probability decreases after the pulse (resealing), but must be larger than a threshold for fusion to take place, as shown by the presence of F_c (Eq. 21).

The final conclusion of the present study is that the PF electrofusion obeys two of the electrofusion mechanisms that were proposed in the case of CF electrofusion (Abidor and Sowers, 1992a):

1. The "trigger" mechanism: The field locally brought the membrane to a long-lived fusogenic state.

2. The "stochastic" mechanism: Fusion takes place through the merging of random defects occurring in the part of the cell surface prone to electropermeabilization. The density of these fusogenic defects is dependent on the pulse duration.

APPENDIX: DENSITY OF FUSOGENIC DEFECTS

CHO cells, when pulsed with a 1.6 kV/cm field (5 pulses of 100 μs), can fuse with a FI of 35% (Fig. 4). As the E_p is 0.6 kV/cm,

$$(1 - E_p/E)^2 = 0.36$$

the optimum density of fusogenic defects is then obtained:

$$FI = (1 - E_p/E)^2$$

As the cell diameter is 12 μm, the fusogenic surface is

$$\pi D^2(1 - E_p/E) = \pi(12)^2(1 - 0.6/1.6) = 260\ \mu m^2$$

Making the assumption that a "defect" is at least four lipids in contact (square array), with a molecular area per lipid of 0.5 nm^2, this gives a density of $5 \times 10^5/\mu m^2$ ($3.3 \times 10^5/\mu m^2$ will be obtained under the assumption of a hexagonal array, six lipids in contact to build the defect) and a total number of defects of 1.3×10^8 (i.e., $5 \times 10^5 \times 260$) (0.8×10^8 defects in the case of an hexagonal area).

As the lifetime of the fusogenic state is ~1 min, the probability of fusogenic fluctuations is $5 \times 10^5/\mu m^2$/min ($3.3 \times 10^5/\mu m^2$/min for six lipids).

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