Electropermeabilization of Mammalian Cells to Macromolecules: Control by Pulse Duration

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ABSTRACT Membrane electropermeabilization to small molecules depends on several physical parameters (pulse intensity, number, and duration). In agreement with a previous study quantifying this phenomenon in terms of flow (Rols and Teissié, *Biophys. J*. 58:1089–1098, 1990), we report here that electric field intensity is the deciding parameter inducing membrane permeabilization and controls the extent of the cell surface where the transfer can take place. An increase in the number of pulses enhances the rate of permeabilization. The pulse duration parameter is shown to be crucial for the penetration of macromolecules into Chinese hamster ovary cells under conditions where cell viability is preserved. Cumulative effects are observed when repeated pulses are applied. At a constant number of pulses/pulse duration product, transfer of molecules is strongly affected by the time between pulses. The resealing process appears to be first-order with a decay time linearly related to the pulse duration. Transfer of macromolecules to the cytoplasm can take place only if they are present during the pulse. No direct transfer is observed with a postpulse addition. The mechanism of transfer of macromolecules into cells by electric field treatment is much more complex than the simple diffusion of small molecules through the electropermeabilized plasma membrane.

INTRODUCTION

Electropermeabilization of cells has many applications in cellular biology and biotechnology for gene transfer, cell hybridization, and loading of cells with extracellular molecules (Neumann et al., 1982; Zimmermann et al., 1976), as in medicine for gene therapy, cancer chemotherapy, and transdermal drug delivery (Titomorov et al., 1991; Mir et al., 1991; Prautsnitz et al., 1993a); but the mechanisms involved in such phenomenon remain unclear (Tsong, 1991). Although it has been suggested that hydrophilic pores are formed in the lipid matrix (Neumann et al., 1989), their existence has never been clearly shown. Molecular processes involved in the mechanism are not fully understood despite many theoretical studies on the formation of "pores" under the influence of an electric field (Crowley, 1973; Chernomordik et al., 1983; Saulis, 1997). However, due to the high complexity of natural membranes, these models cannot explain all the experimental results, even the more recent of them including the effect of cytoskeleton (Sung and Park, 1997).

Electropermeabilization can be described as a three-step process of creation, stabilization, and annihilation of transient structures of permeation (Kinosita and Tsong, 1977; Rols and Teissié, 1990a). Electric field intensity appears to be crucial in this phenomenon. It induces a rapid (microsecond) increase in transmembrane potential, as experimentally observed by submicrosecond imaging studies (Hibino et al., 1993). Number and duration are the two other pulse parameters shown to modulate permeabilization efficiency (Kinosita and Tsong, 1977; Rols and Teissié, 1989, 1990a, b; Tsong, 1991). Clear differences in processes by which molecules of different sizes translocate across the electropermeabilized membrane have therefore been proposed. While small molecules could rather freely cross the permeabilized membrane for a time much longer than the duration of the electric pulse application, macromolecule transfer could involve more complex steps of interaction with the membrane and no free diffusion after pulse application (Morgan and Day, 1995).

Only a few experimental studies have provided determinations of permeabilization versus the different electric field parameters (Schwister and Deuticke, 1985; Liang et al., 1988). In a previous work, performed in the case of mammalian cells, we studied the effect of the different electric field parameters on membrane permeabilization to small molecules. That work led us to suggest a quantitative analysis of the phenomenon where the electric field pulse strength E was observed to be crucial (Rols and Teissié, 1990a). A quantitative study of molecular transport of bovine serum albumin by erythrocyte ghosts has been described in a model where viability, however, could not be evaluated (Prautsnitz et al., 1993b, 1994). In that study, BSA uptake was correlated with the total time integral of field strength ($E_o\tau$). In another study, transfer of DNA has been reported to be proportional to the power of the pulse ($E^2\tau_{1/2}$) according to a polarization mechanism (Kubiniec et al., 1990; Neumann et al., 1996). The respective effects of the electric field pulse parameters on the transfer of mac-

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Abbreviations used: E, pulse field strength; β-Gal, β galactosidase; BSA, bovine serum albumin; CHO cells, Chinese hamster ovary cells; E_o, peak field strength; FD, fluorescein-coupled dextran; LDH, lactate dehydrogenase; N, number of pulses; PI, propidium iodide; T, pulse duration; τ, decay time constant; $\tau_{1/2}$, half decay time.

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romolecules by electric fields therefore remain unclearly defined.

The aim of this study was to determine the control by electric field parameters (in particular the respective effect of electric field pulse strength, duration, number, and frequency) on the exchange of macromolecules across plasma membrane of mammalian cells under optimized conditions compatible with the maintenance of their viability. CHO cells were used because of the wide extent of data we obtained about their electropermeabilization (Rols and Teissié, 1989, 1990a, b). Trypan blue and PI were used to study the relationship between small molecule electropermeabilization efficiency and the electric field parameters. Fluorescent macromolecules (FITC-dextran and BSA-FITC), enzyme (β-Gal), and plasmid DNA were used as probes with high molecular weight. The integrity of the oligomeric enzyme was assayed by observing its activity.

MATERIALS AND METHODS

Cell culture

CHO cells have been used in a large number of somatic cell genetic laboratories [see Gottesman (1985) for a review]. We selected the WTT clone, which is not strictly anchorage-dependent. It grows at 37°C in monolayers on petri dishes (35 mm in diameter, Nunc, Denmark), but has been adapted for suspension culture under gentle agitation (100 rpm) in Eagle's minimum essential medium (MEM0111, Eurobio, France) supplemented with glucose (3.5 g/l), tryptose phosphate (2.95 g/l), sodium bicarbonate (3.5 g/l), vitamins, and 8% new-born calf serum. Antibiotics penicillin (100 U/l) and streptomycin (100 μg/ml), and glutamine (0.585 ng/ml) are added extemporally. Cells are maintained in the exponential growth phase (4×10^5-1.2×10^6 cells/ml) by daily dilution of the suspension. Cells in suspension have a mean radius equal to 6.5 μm (Teissié and Rols, 1993). Cells grown in suspension can be replated readily on petri dishes and kept at 37°C in a 5% CO_2 incubator (Jouan, France). The ability of cells in suspension to replate is evidence of their viability. This property was used to select viable cells after their electric treatment.

Application of electrical pulses to the cells

The cell permeabilization protocol for cells has been described elsewhere (Teissié and Rols, 1988). Briefly, cells in suspension were centrifuged for 5 min at $350 \times g$ (1000 rpm, C500 centrifuge; Jouan, France) and resuspended in pulsing medium (10 mM phosphate, 250 mM sucrose, 1 mM $MgCl_2$, pH 7.2) at a concentration of 10^7 cells/ml; 100 μl of suspension were placed between the flat parallel electrodes on a culture dish. Voltage pulses were then applied (CNRS cell electropulsator, Jouan, France). In the case of high-frequency experiments, the cell electropulsator was coupled to a Hameg HM8035 pulse generator that applied pulses at various frequencies up to several kilohertz (Hameg, Germany). All these experiments were performed under sterile conditions under a laminar flow hood (ESI, France).

Determination of permeabilization

Electropermeabilization of cells was quantified by the penetration of impermeant dyes. Penetration of trypan blue (T0887, 4 mg/ml in the pulsing buffer; Sigma, St. Louis, MO) and PI (P4170, 100 μM in the pulsing buffer, Sigma, St. Louis, MO) were used to monitor permeabilization of cells to small molecules. Cells were pulsed, incubated 5 min at room temperature, and then observed under an inverted light microscope (Leitz, Germany) or analyzed by flow cytometry (Becton Dickinson, FACScan). The percentage of permeabilized cells was 100 times the ratio of the number of blue-stained or fluorescent cells to the total number of cells. Flow cytometry gives access both to the percentage of fluorescent cells and to the amount of fluorescence associated with each of these cells, i.e., the extent of loading in each cell (Rols and Teissié, 1998). Laser excitation was at 488 nm and detection of fluorescence was performed at 650 nm.

Penetration of fluorescein isothiocyanate dextran with an average molecular weight of 71.2 (FD-70) was performed under the same protocol. Concentration of FD-70 in pulsing buffer was equal to 2 mg/ml. After the postpulse incubation time, cells were washed twice with pulsing buffer, put into culture for 24 h to select the viable ones, and observed under a fluorescence microscope or analyzed by flow cytometry.

Penetration of β-Gal (Sigma) was performed under the same protocol with a final concentration in pulsing buffer equal to 0.25 mg/ml. After pulsing, cells were incubated 5 min, washed, and incubated in culture medium at 37°C in an air/CO_2 incubator for 24 h to keep only plated, i.e., viable cells, on the dish. Activity was detected by using X-Gal as the substrate of the enzyme (Rols et al., 1994). Electropermeabilized cells expressing the electrotransferred β-Gal activity appeared stained blue within an hour in the microscope. In contrast, control cells appeared colorless. Percentage of permeabilization was 100 times the ratio of the number of blue-stained cells to the total number of cells. Due to the culture of cells for 24 h after pulsation, this percentage is related to viable cells.

Penetration of plasmid DNA was performed by using a 6.8-kb pBR322 shuttle vector carrying the β-Gal gene under the control of the SV40 promotor. It was prepared by using the Qiagen Maxiprep Dna purification system. For each assay, 6 μg plasmid were added to 100 μl cell suspension, corresponding to 0.8×10^6 cells. After 10 min incubation on ice, electric pulses were applied at room temperature; 10 min later, cells were put into culture medium for 24 h before revealing the β-Gal activity on viable, i.e., plated, cells (Rols et al., 1994).

Determination of lifetime of permeabilization

This was assayed by a postpulse permeabilization assay. Cells were pulsed in the pulsing buffer, which was replaced by the reporter molecule containing buffer after the indicated time span. Cells were incubated 5 min thereafter; penetration was used to monitor the induced permeabilization of the membrane and not to assess the cell viability, as is routinely done with trypan blue or PI.

Determination of electropulsed cell viability

Cells were pulsed in the pulsing buffer in the absence of any added molecules, but under the same conditions as for permeabilization assays. They were kept 5 min at room temperature and grown in petri dishes with 2 ml culture medium. Viability was measured by observing the growth of the cells over 48 h (about two generations) under an inverted phase-contrast light microscope (Leitz, Germany).

Determination of the release of proteins from electropulsed cells

Determination of the amount of proteins released from electropermeabilized cells was performed by using the Biorad microassay procedure derived from the Bradford procedure (Bradford, 1976), which allows us to measure total protein amount. Cells were pulsed in petri dishes on ice in 1 ml pulsing buffer. Ten minutes later, 800 μl buffer were taken off and added to 200 μl Biorad reactive; 15 min later, optical density was measured at 595 nm and allowed to calculate the amount of protein in the sample with a reference curve obtained with known quantities of bovine serum albumin. The lactate dehydrogenase was used to measure a cytosolic enzyme release. Cells were pulsed in petri dishes on ice in 1 ml pulsing buffer; 10 min later, 200 μl buffer were taken off and added to 1 ml reactive medium (50 mM phosphate buffer, pH 7.4; sodium pyruvate, 2.5 mM; NADH, 10 mM). Kinetic absorption was performed at 340 nm.

Statistical treatment of experiments

Due to the physiological state of the cells, all experiments were repeated at least three times at intervals of several days to avoid fluctuations of results within a given set of experiments (Rols et al., 1992).

THEORY

Electroinduced permeabilization only occurs for electric field values above a threshold (E_c) in cell membrane regions where the transmembrane electric potential difference reaches values of 250 mV (Teissié and Tsong, 1981; Marszalek et al., 1990; Teissié and Rols, 1993). At a point M of the cell membrane, the electric field-induced potential difference increases, as described by Neumann et al. (1989):

$$\Delta V_M = fgrE \cos \theta \quad (1)$$

where f is a cell shape factor, g is a membrane conductance factor, r is the cell radius, E is the applied electric field amplitude, and θ is the angle between the electric field vector and the membrane normal vector at the point M. ΔV_M is added to the cell resting potential difference $\Delta\psi$. E_c increases the resting membrane potential difference up to the critical permeabilizing value at the cell pole facing the electrodes. For an applied pulse of intensity E, with $E > E_c$, it is clear that from Eq. 1 the permeabilizing potential condition is reached inside a cone of half-angle θ such as

$$E \cos \theta = E_c \quad (2)$$

The area of permeabilizable cell surface is therefore proportional to (Schwister and Deuticke, 1985)

$$A_{perm} = K(1 - E_c/E) \quad (3)$$

where K is a constant.

The geometrical and kinetic dependence of ΔV_M has been experimentally demonstrated at the single cell level by the use of potential electrochromic dyes (Gross et al., 1986; Kinosita et al., 1988). Furthermore, these electroinduced permeabilized membrane areas have been visualized during a microsecond pulse or in the milliseconds after it by ultra-rapid video systems (Hibino et al., 1991, 1993; Gabriel and Teissié, 1997).

The resulting electroinduced permeabilization of the cell membrane can be quantified in terms of the flow of molecules S (F_s), which cross the plasma membrane. For small molecules, F_s obeys Fick's law but occurs only on the permeabilizable cell surface (Rols and Teissié, 1990a):

$$F_s(t) = P_s y(N, T) A_{t/2}(1 - E_c/E)\Delta S \exp[-k(N, T)t] \quad (4)$$

Where P_s is the permeation coefficient of the solute S, y is a function that depends on the duration and number of pulses, N is the number of pulses, T is the pulse duration, A_t is the cell surface, E is the electric field intensity, ΔS is the concentration difference of S between cytoplasm and external medium, k is the resealing process constant (in the case of small molecules), and t is the time after the pulse.

RESULTS

The use of a square-wave pulse generator made it possible to deliver repeated pulses at 1 Hz frequency of defined intensity and duration and to study the effect of either number of pulses, pulse duration, or intensity on electropermeabilization, the two others being kept constant.

Electric field strength effect with short pulse duration

CHO cells were exposed to electric pulses in the presence of various impermeant molecules having various molecular weights. Under electric field pulse conditions (i.e., 10 pulses, 100 μs duration up to 1.2 kV/cm) where the cell viability was partly preserved, it was not possible to detect penetration of macromolecules such as FITC dextran or β-Gal (Fig. 1 *A*). Penetration of FD-70 was only detected for electric fields >1.5 kV/cm, where all cells were lysed, and was associated with a release of LDH (a cytosolic protein) (data not shown). Penetration of macromolecules was therefore possible, but restricted to damaged cells. In agreement with previous publications, penetration of low molecular weight molecules was detected for electric field values >0.3–0.4 kV/cm. Above this value, increasing electric field strength resulted in an increase in the percentage of permeable cells in a sigmoidal way (Escande-Géraud et al., 1988; Rols and Teissié, 1990a).

Electric field strength effect with long pulse duration

As penetration of macromolecules was not detected when cell viability was preserved when using short pulses, pulse duration was increased to 5 ms [i.e., to the conditions used in cell transfection (Hui, 1996; Rols et al., 1994)]. Fig. 1 *B* shows the permeabilization efficiency as a function of electric field strength. Permeabilization occurs for field intensities >0.3 kV/cm for small as well as large molecules. Increasing the field intensity therefore results in an increase in permeabilization efficiency for small and large molecules. Under those conditions, it is possible to permeabilize 80% of the viable cells to β-Gal while preserving the viability of 80% of the population at a 0.8 kV/cm electric field intensity.

When permeabilization is plotted as a function of the reciprocal of E, a linear fit is observed with linear regression coefficients between 0.94 and 0.99 (Fig. 1 *C*). A similar relation was reported for data of plasmid transfer (Wolf et al., 1994). From Eq. 3, these results support a process where macromolecule transfer takes place only in the part of the cell surface in which electric potential difference is brought to 200–250 mV, i.e., in the part of the cell membrane surface which is known to be permeabilized to small molecules (Hibino et al., 1991, 1993; Gabriel and Teissié, 1997).

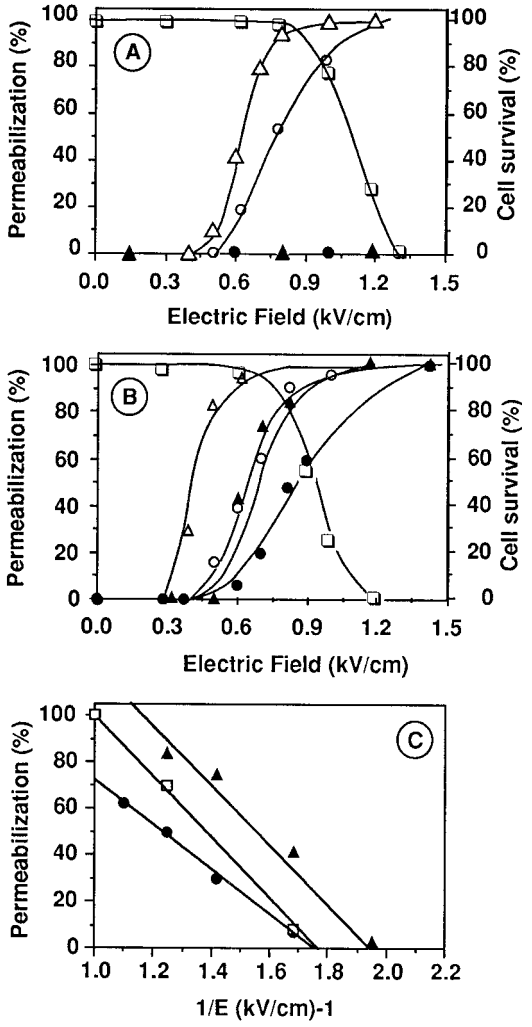

FIGURE 1 Effect of electric field intensity on permeabilization efficiency and cell survival. CHO cells were pulsed 10 times, at 1 Hz, at various electric field strengths in the presence of different molecules. (△), propidium iodide; (▲), β-Gal; (○), trypan blue; (●), FD-70. (*A*) Pulse duration was equal to 100 μs. The percentage of permeable cells and of viable cells (□) are plotted as a function of electric field strength. (*B*) Pulse duration was equal to 5 ms. The percentage of permeable cells and of viable cells (□) are plotted as a function of electric field strength. (*C*) Pulse duration was equal to 5 ms. The percentage of permeable cells is plotted as a function of 1/*E*; □ is related to FD-40. Regression coefficients being respectively equal to 0.980, 0.998, and 0.942 for β-Gal, FD-40, and FD-70.

Electric field pulse duration effect

Cells were submitted to 10 repetitive pulses, at a constant field strength of 1 kV/cm, at a 1 Hz frequency, in pulsing buffer containing various reporter molecules at various pulse durations from 20 μs to 15 ms. Results plotted in Fig. 2 *A* show that the longer the duration of pulses, the higher the efficiency of permeabilization. Only pulse durations equal to or longer than 1 ms are associated with the detection of macromolecules in pulsed but viable cells, while permeabilization of cells to PI was already detected for the microsecond duration. Our methodology takes only plated, i.e., viable, cells into account in the assay of permeabilization for large molecules. The percentage of permeabilized cells plotted corresponds to the percentage of permeable and viable cells.

These results show that pulse duration allows a controlled penetration of macromolecules into mammalian cells without being dramatically associated with a loss in their viability. When permeabilization, performed at various conditions of electric field intensities and pulse duration, was plotted as a function of the reciprocal of *E*, a linear relationship was obtained whatever the pulse duration from the tested range of values (Fig. 2 *B*).

Number of pulses effect

Cells were submitted to pulses at a constant field strength of 1 kV/cm in pulsing buffer containing different reporter molecules ($N = 1–20$). Results plotted in Fig. 2 *C* show that the higher the number of pulses, the higher the efficiency of permeabilization, but pulse durations must be long to detect macromolecules in pulsed but viable cells. For a 5-ms pulse duration, the electroloading efficiency to β-Gal was proportional to the number of pulses (Fig. 2 *C*).

These results show that the number of pulses allows controlled penetration of macromolecules into viable mammalian cells, but for pulse duration in the millisecond time range. When permeabilization was plotted as a function of the reciprocal of *E*, a linear relationship was obtained whatever the number of pulses (Fig. 2 *D*). This supports the conclusion that transfer occurs only in a limited part of the cell surface.

Lifetime of permeabilization

Electropermeabilization of cells can be only transient. In the case of small molecules, the extent of permeabilization decreases as a function of time as a first-order process (Rols and Teissié, 1990a). The lifetime of the permeabilized state to PI has been measured at various pulse durations and numbers of pulses. The time at which half the cell population becomes impermeable again ($t_{1/2}$) is plotted versus pulse duration (Fig. 3 *A*) and number of pulses (Fig. 3 *B*). A linear dependence is observed.

In the case of macromolecules, no direct transfer to the cell cytoplasm can occur when macromolecules are added after application of the electric pulses. This is observed whatever the number and duration of pulses, and is in agreement with previous studies (Wilson et al., 1991; Wolf et al., 1994; Rols et al., 1995).

Number of pulses versus pulse duration

Permeabilization was checked at various numbers of pulses (*N*) and durations (*T*) at a constant cumulative duration (*NT*). Two sets of numbers of pulses/pulse duration (*NT*) products were tested (0.5 and 5 ms) at a 1 Hz frequency, and at 1 kV/cm intensity. The percentage of permeabilized cells

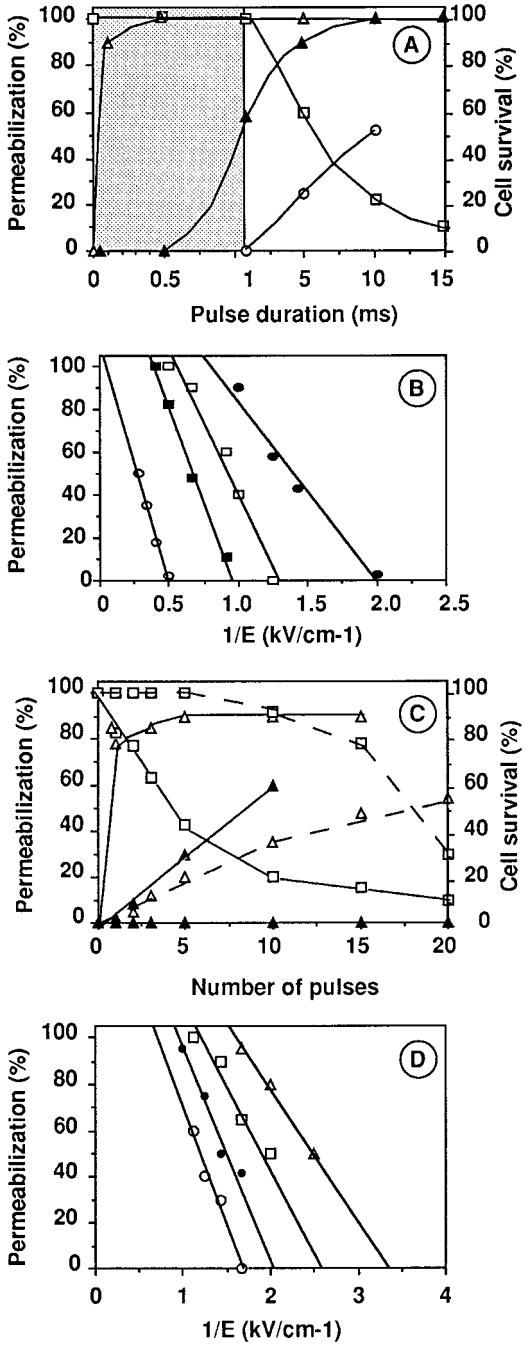

FIGURE 2 Effect of pulse duration and number of pulses on permeabilization efficiency and cell survival. CHO cells were pulsed at a 1 Hz frequency in the presence of different molecules: (△), propidium iodide; (▲), β-Gal; (○), trypan blue; (●), FD-70. (*A*) The percentage of permeabilized and viable cells is plotted as a function of pulse duration for cells pulsed 10 times at 1 kV/cm intensity; □ is related to cell viability. (*B*) The number of pulses was equal to 10. The percentage of permeable cells to trypan blue is plotted as a function of 1/*E* for various pulse duration values [(○), 20 μs; (■), 50 μs; (□), 2.5 ms; (●), 10 ms], regression coefficients being respectively equal to 0.97, 0.99, 0.97, and 0.98. (*C*) The percentage of permeabilized and viable cells is plotted as a function of number of pulses for cells pulsed at 100 μs (*dotted lines*), or 5 ms (*straight line*) at 1 kV/cm intensity; □ is related to cell viability. (*D*) The pulse duration was equal to 100 μs. The percentage of permeable cells to trypan blue is plotted as a function of 1/*E* for various numbers of pulse values [(○), 1; (●), 2; (□), 5; (△), 20], regression coefficients being respectively equal to 0.98, 0.97, 0.99, and 0.98.

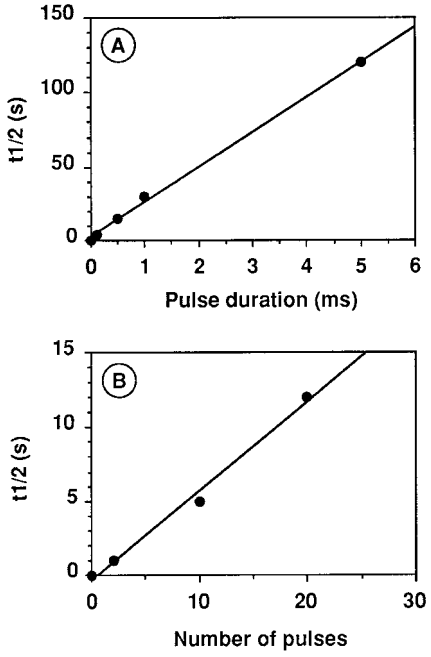

FIGURE 3 Effect of number of pulses and pulse duration on lifetime of permeabilization. CHO cells were pulsed at 1 Hz at 1 kV/cm intensity at various numbers of pulses and duration, and incubated at 21°C. At the indicated time, propidium iodide was added to the cell suspension, which was tested for permeabilization. The time $t_{1/2}$ at which half of the cell population becomes impermeable again is plotted versus pulse duration (*A*) at $N = 10$, and versus number of pulses (*B*) at 1 ms. Regression coefficients are respectively equal to 0.998 and 0.992.

did not change significantly for a given couple of *NT* products, but the associated fluorescence intensity was increased for the highest number of pulses for the two *NT* products (Fig. 4). Resealing experiments were performed under the same conditions. The $t_{1/2}$ was shown to increase

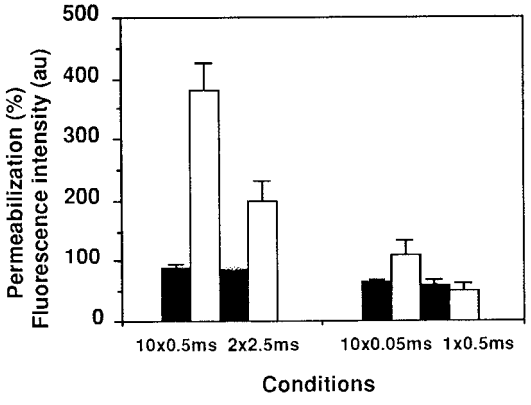

FIGURE 4 Effect of pulse duration and number of pulses at a given electropulsation time. Cells were pulsed at 1 kV/cm intensity at various pulse durations and numbers of pulses whose product was kept constant and equal to 0.5 or 5 ms (i.e., 10 pulses lasting 0.5 ms and 2 pulses lasting 2.5 ms or 10 pulses lasting 50 μs and 1 pulse lasting 0.5 ms) at a 1 Hz frequency. The percentage of permeabilized cells to propidium iodide (*black histogram*) and the associated fluorescence intensity (*white histogram*) are plotted as a function of these different conditions.

1) by increasing the *NT* product value, and 2) at a given *NT* electropulsation time, by increasing the pulse duration (data not shown).

In the above experiments, performed at various numbers of pulses and pulse durations, the frequency of pulse application was kept constant and equal to 1 Hz. In order to go further in the study of the respective effects of number of pulses and pulse duration on cell permeabilization, a constant cumulated electropulsation time (*NT*) was applied, but with a constant time. In the following experiments, various combinations of number of pulses/pulse duration products were applied, with a cumulated constant value of 50 ms. Frequencies *F* at which these pulses were applied were therefore determined to keep the time between the first and the last pulses constant and equal to 10 s to avoid artifacts due to cell rotation. The relation between the number of pulses and the frequency was $N/F = 10$ s. An increase in frequency means an increase in the number of pulses and a decrease in the pulse duration.

Cells were pulsed at a 0.6 kV/cm electric field intensity. Permeabilization was determined by penetration of PI, β-Gal, and plasmid DNA. Efficiency of electropermeabilization to small molecules, quantified by the incorporation of PI, decreases by increasing the pulse frequency. This is shown in Fig. 5 *A* both by the decrease in the percentage of permeabilized cells and by the amount of molecules incorporated in these permeabilized cells. This decrease reaches a factor of 4 between 1 Hz and 500 Hz frequencies. Cell viability remained not significantly affected in all the tested ranges of frequency values (data not shown).

A similar, but more pronounced, dependence of efficiency of permeabilization on frequency of pulse application was observed in the case of macromolecules (Fig. 5 *B*). The percentage of permeabilized cells to β-Gal decreases by a factor of 10 by simply increasing the frequency from 1 to 5 Hz (i.e., by decreasing pulse duration from 5 ms to 1 ms, while increasing pulse number from 10 to 50) and then slightly decreases to reach zero at 100 Hz. The transfection level also strongly decreases, by a factor of 5 between 1 and 10 Hz, and then to almost zero beyond 100 Hz (Fig. 5 *B*).

These results show that, at a constant number of pulses/pulse duration product, pulse duration is the key parameter leading to efficient cell permeabilization to macromolecules.

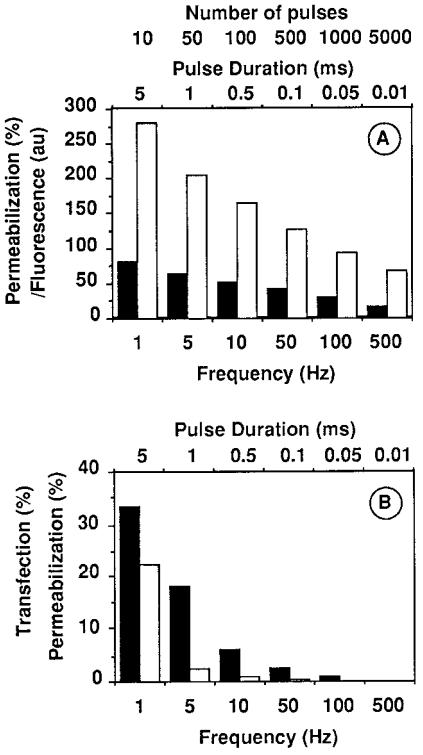

FIGURE 5 Effect of pulse duration and number of pulses at various frequencies. Cells were pulsed at 0.6 kV/cm intensity at various pulse durations and number of pulses whose product was kept constant and equal to 50 ms (i.e., from 10 pulses lasting 5 ms to 5000 pulses lasting 10 μs), i.e., with various frequencies from 1 to 500 Hz. (*A*) The percentage of permeabilized cells to propidium iodide (*black histogram*) and the associated fluorescence intensity (*white histogram*) are plotted as a function of pulse frequency. (*B*) The percentage of transfected cells (*black histogram*) and of permeabilized cells to β-Gal (*white histogram*) are plotted as a function of pulse frequency.

DISCUSSION

The application of electric field pulses to CHO cells results in the permeabilization of their plasma membrane. Increasing molecular weight of the molecule to be transferred results in an apparent shift of curves to high field strengths with a concomitant decrease in the process efficiency (decrease in the slope of the curve at 50% permeabilization, Fig. 1, *A* and *B*). Under electrical conditions maintaining cell viability, no permeabilization can be detected for molecules with molecular mass higher than 10 kDa for pulse durations shorter than 1 ms. Pulse duration is therefore a key parameter for macromolecule electrotransfer. When data of permeabilization for small and large molecules are plotted as a function of $1/E$, linear fits are obtained whatever the values of pulse number and pulse duration (Figs. 1 *C* and 2, *B* and *D*). These results show that macromolecule transfer takes place only in the part of the cell surface in which electric potential difference is brought to 200–250 mV, i.e., in the part of the cell membrane surface known to be permeabilized to small molecules. These conclusions therefore do not agree with previous reports in which transfer of DNA was proportional to $E^2\tau_{1/2}$, i.e., to the polarization energy (Kubiniec et al., 1990; Neumann et al., 1996), and BSA uptake was a function of the time integral of field strength ($E_o\tau$) (Prautsnitz et al., 1994), a relation obtained with a fit of 0.6.

A strong increase in the electric field intensity is not needed to incorporate macromolecules into cells, as previously shown in the case of bacteria (Xie and Tsong, 1992). Optimum conditions for membrane electropermeabilization are obtained at high pulse duration *T* values and moderate values of the electric field intensity *E* to preserve the viability. The electropermeabilized membrane area can there-

fore be defined as follows: E topologically determines at the cell level the membrane area where permeabilization can occur, while T and N determine the density and/or size of permeated structures (Fig. 6). So, the same permeabilization efficiency can be obtained either at low values for N and T but high E or at a low value for E and high values for N and T. The last electric field conditions are less damaging for the cells. This is in agreement with previous works showing the importance of optimizing E and T in transfection experiments (Xie and Tsong, 1992; Hui, 1996). It was suggested that excessive field strength could lead to permeation of a wider area, or forming larger pores that may exceed the resealing limit (Hui, 1996). The same conclusion was also reported by Rosemberg and Korenstein (1990); E controlling the total area of membrane that undergoes permeabilization, and T the diameter of "electropores." Longer pulses corresponding to low field strength were reported to be more advantageous than the short ones in terms of transfection success (Kubiniec et al., 1988). It has been shown that increasing N led to an increase in permeabilization in mammalian cells too (Kwee et al., 1989). This is what we observed too in the case of low molecular weight molecules (Fig. 4) in agreement with previous work (Rols and Teissié, 1993). But, as far as macromolecule transport is concerned, pulse durations in the millisecond time range are required (Figs. 1 *B* and 2 *A*) as previously reported by others (Chu et al., 1987; Potter, 1989), suggesting a contribution of electrophoretic forces (Sukharev et al., 1992; Wolf et al., 1994). However, accumulation of a high number of pulses with shorter duration at a constant accumulated value, allowing efficient permeabilization to small size molecules, does not give access to macromolecules (Fig. 5, *A* and *B*). This appears to be in conflict with electrophoresis-driven transfer.

Several models assumed that transfer across the membrane occurred across pores, which has never been observed. Different calculations of the "electropores" diameter have been proposed, from 0.39 to 5.8 nm, that correspond to 0.01–0.1% of the membrane area (Sowers and Lieber, 1986; Kinosita et al., 1988; Rosemberg and Korenstein, 1990; Hibino et al., 1993; Neumann et al., 1996). If these values are compatible with the size of small molecules ($M_w < 10$), they cannot support the penetration of macromolecules such as plasmid DNA and enzymes. According to the pore percolation model of Sugar et al. (1987), if coalescence and fusion of permeated structures could occur when increasing their number with the number of pulses, they should enhance permeabilization. This is what we experimentally observed in the case of PI (Fig. 4). This model of pore percolation has recently been extended to DNA transport via the formation of transient complexes with the lipids in the pore edges of electropercolated hydrophilic pore zones (Hristova et al., 1997), but pore percolation should not be dependent on the way pulses are applied. This prediction is in conflict with our observations. As we previously suggested (Wolf et al., 1994), electric pulses could lead to the formation of a complex between DNA and the membrane. DNA could then cross the membrane in the seconds after electropulsation. The efficiency of the transfer would therefore depend on the stability of the complex. Our conclusion is that it depends on the pulse duration. The duration of each individual pulse is the critical factor, not the accumulation of several short pulses.

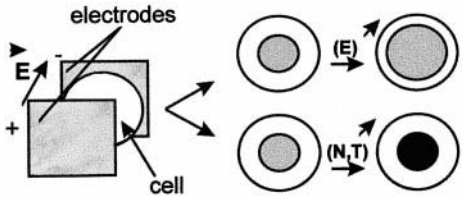

FIGURE 6 Representative scheme for the respective roles of the different electric field parameters on cell membrane permeabilization. A cell between two electrodes is submitted to an electric field. The gray area represents the cell surface that is prone to be permeabilized, i.e., the cell membrane area whose potential is brought to 200–250 mV. This area increases with the electric field intensity. When the cell is submitted to a constant electric field intensity value but to increasing values of pulse numbers and/or pulse duration, the size of the potentially permeabilizable area remains constant, but the density of transient permeated structures in that area, i.e., the permeabilization, increases by increasing these parameters (which is represented by gray hatching).

Furthermore, the major difference between electropermeabilization of cells to small and large molecules is the lifetime of the competent state for transfer. In the case of macromolecules, the molecule must be present during the pulse for a direct transfer. In the case of FITC-dextran and β-Gal, the addition of these macromolecules in the minutes after pulsation was associated with a vesicular distribution due to a macropinocytosis-induced phenomenon (Rols et al., 1995). As far as gene transfer is concerned, it only occurs when DNA is present during pulsation (Sukharev et al., 1992; Wolf et al., 1994). These results agree with other studies showing that recovery of membrane integrity after the pulse treatment assayed from membrane conductance revealed the existence of at least two recovery processes. Conductance decreased strongly in a submillisecond after the pulse, a fast process that alone did not lead to complete recovery (Kinosita et al., 1988; Hibino et al., 1993). The existence of two kinds of permeated structures for small and large molecules can therefore be suggested as being in agreement with previous reports. In 1988 Hui et al. (see Kubiniec et al., 1988) suggested the implication of two populations of pores in which the macromolecules can penetrate only at high field strength or long pulse duration via a population of larger size pores. Free access to cell cytoplasm can occur in the minutes following pulsation for small molecules (Rols and Teissié, 1990a), a process related to both N and T (Fig. 3, *A* and *B*). We previously described a three-state energetic model for the lifetime of electropermeabilization in which membrane and cytoskeletal proteins were involved in the stabilization of the permeated state due to their interaction with lipids (Teissié and Rols, 1994). In that model, electrical alterations first trigger a conformational change in the lipids, as shown by NMR (Lopez et al., 1988). In the permeabilized state, a metastable level can be

found associated with a conformational change of some proteins. This model, described in the Appendix, predicts that the half-time of electropermeabilization increases with an increase in the rate constant k_3, related to the transition between the state where only lipids are altered, and the permeabilized one where both lipids and proteins are affected. As shown on Fig. 3, the half-time of electropermeabilization is linearly related to N and T, leading to the conclusion that increasing N and/or T facilitates the transconformation of proteins either by a more stringent action on lipids or by a direct field effect.

The molecular mechanisms underlying the electropermeabilization phenomenon at the membrane level therefore remain to be elucidated, but from the results reported in this work, it appears that

1. The transfer of macromolecules is correlated with the reciprocal of E. It occurs in membrane areas topologically controlled by electric field intensity E;
2. Increasing E may increase transport of macromolecules, but as it may induce a loss in cell viability, it is associated with a decrease in plasmid expression;
3. The effects of N and T are not the same on small and large molecule transfer. Transfer of macromolecules depends on T in a more complex way than for small molecules;
4. At a constant NT product, T plays a major role in the transport of macromolecules, no significant effect being observed in the case of small molecules;
5. To obtain transfer of macromolecules under electrical conditions preserving cell viability, it is better to apply long pulse durations at low electric field strength than to apply short pulses at high electric field strengths to really affect a small area of cell membrane rather than to slightly alter a large surface area.

APPENDIX

Mathematical prediction of the three-state model for the lifetime of electropermeabilization (Teissié and Rols, 1994)

The long-lived electropermeabilized state implies that proteins are involved in cell membrane electropermeabilization. This is supported by experiments (Teissié and Tsong, 1980; Rosemberg et al., 1994). The field effect was modeled by a three-state scheme: the intact membrane (state A) is brought to a highly unstable state B by the pulse; B is spontaneously converted to the metastable state C:

$$\begin{array}{ccccc} & \mathrm{E} & & \mathrm{E(?)} & \\ & \downarrow & & \downarrow & \\ & k_1 & & k_3 & \\ \mathrm{A} & \rightleftarrows & \mathrm{B} & \rightleftarrows & \mathrm{C} \\ & k_2 & & k_4 & \end{array}$$

C has a long lifetime because the system must follow the reverse set of events to recover its initial, and stable, configuration.

Transitions between the different states take place with different rate constants k_i. As membranes can be permeabilized only when an electric field is present with an intensity larger than a threshold E_c,

$$k_1 = 0 \quad \text{if} \quad E = 0, \tag{5}$$

which is the case in the postpulse incubation.

The permeabilized state is therefore described by the population of both B and C. Just at the end of the electric pulse, only state B is populated. At $t = 0$,

$$B = B_o \tag{6}$$

$$C = 0. \tag{7}$$

The following equations describe the different reactions that took place during resealing:

$$-\mathrm{d}B/\mathrm{d}t = k_2B + k_3B - k_4C \tag{8}$$

$$-\mathrm{d}C/\mathrm{d}t = k_4C - k_3B. \tag{9}$$

The resealing process is described by the decay of the populations B and C. This can be obtained by a numerical simulation of the set of Eqs. 8 and 9, which show that resealing obeys a first-order kinetic. Its half-lifetime is linearly related to k_3 (Teissié and Rols, 1994).

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