Control by Osmotic Pressure of Voltage-Induced Permeabilization and Gene Transfer in Mammalian Cells

Muriel Golzio, Marie-Pierre Mora, Catherine Raynaud, Christine Delteil, Justin Teissié, and Marie-Pierre Rols

Institut de Pharmacologie et de Biologie Structurale du CNRS UPR 9062, 31062 Toulouse Cedex, France

ABSTRACT   Cells can be transiently permeabilized by a membrane potential difference increase induced by the application of high electric pulses. This was shown to be under the control of the pulsing buffer osmotic pressure, when short pulses were applied. In this paper, the effects of buffer osmotic pressure during electric treatment and during the following 10 min were investigated in Chinese hamster ovary cells subjected to long (ms) square wave pulses, a condition needed to mediate gene transfer. No effect on cell permeabilization for a small molecule such as propidium iodide was observed. The use of a hypoosmolar buffer during pulsation allows more efficient loading of cells with β-galactosidase, a tetrameric protein, but no effect of the postpulse buffer osmolarity was observed. The resulting expression of plasmid coding for β-galactosidase was strongly controlled by buffer osmolarity during as well as after the pulse. The results, tentatively explained in terms of the effect of osmotic pressure on cell swelling, membrane organization, and interaction between molecules and membrane, support the existence of key steps in plasmid-membrane interaction in the mechanism of cell electrically mediated gene transfer.

INTRODUCTION

Transfer of exogenous material into the cytoplasm of cells is a puzzling problem in cell biology and biotechnology, because of the highly selective barrier character of the plasma membrane. Transfer can be achieved by chemical, viral, or physical approaches, although there are many limitations (Malissen, 1990). A physical method, based on the application of electric field pulses to cells, was developed in the early 1980s (Neumann et al., 1982). This method of gene transfer, called electrotransfection or electrotransformation, is now routinely used, mainly because of the broad range of cells that are sensitive to it (Potter, 1992). But the mechanism that mediates gene transfer remains to be elucidated, because of conflicting results reported in the literature. Several models have been proposed in the case of mammalian cells, where the plasmid crosses the membrane 1) due to the existence of long-lived "electropores" (Neumann et al., 1982), 2) eventually after a binding step at the cell surface and then diffusion to the electropores (Xie and Tsong, 1993) (in such models plasmid DNA could enter the cell when added after application of electric pulses), or 3) only during application of the electric pulses due to electrophoretic forces associated with the external field (Klenchin et al., 1991; Sukharev et al., 1992). However, we obtained experimental results leading to the conclusion that plasmids have to be present during electropulsation but can cross the electropulsed membrane after pulse application, in agreement with results on bacteria and yeast (Eynard et al.,

1992; Ganeva et al., 1995; Neumann et al., 1996). We indeed showed that the plasmid must be present during the electric pulse if it is to be transferred across the membrane into the cytoplasm. Only the localized part of the cell membrane brought to the permeabilized state by the external field is competent. The field should induce a complex reaction between the membrane and the plasmid that can be accumulated at the cell interface by electrophoretic forces. During the pulse, this could lead to insertion of the plasmid, which can then cross the membrane in the minute after pulsation (Wolf et al., 1994). This postpulse step is controlled by the cell.

There are only a few experimental observations of the events affecting the membrane during the electropermeabilization process. Various theoretical descriptions such as breakdown (Crowley, 1973), pores (see, for review, Chang et al., 1992), or mismatches (Cruzeiro-Hansson and Mouritsen, 1989) have nevertheless been proposed. ^{31}P NMR studies point out alteration of phospholipid organization during the permeated state of the membrane caused by reorientation of the polar heads (Lopez et al., 1988). Reorganization of the membrane/solution interface was then proposed as a key step in the induction of electropermeabilization (Rols and Teissié, 1990a). It was proposed that this was associated with a decrease in the membrane surface organizing forces, part of the energy required to permeabilize cells being used to modify structural organization inducing the order of the interfacial water molecules (Gruen et al., 1981). The thermodynamic implications of these conclusions have been checked by altering the membrane order (Rols et al., 1990) and the osmotic pressure of the pulsing buffer (Rols and Teissié, 1990a). Decreasing membrane order was shown to hinder permeabilization. Decreasing osmotic pressure, observed to decrease membrane surface undulations, was shown to facilitate it.

As gene transfer is strongly associated with electropermeabilization, in this work, we studied the effect of buffer osmotic pressure on Chinese hamster ovary cells during electropulsation. Pulses were applied in the millisecond range to permeabilize cells to macromolecules (enzyme: β-galactosidase and plasmid coding for the β-galactosidase activity) (Wolf et al., 1994; Rols et al., 1995). Propidium iodide was used as a small-size permeabilization dye to assay permeabilization. We analyzed the effect of osmotic pressure both during pulsation and during the minutes after it to deconvolute the various steps in the transfer.

MATERIALS AND METHODS

Chemicals

Propidium iodide was obtained from Sigma (St. Louis, MO), and β-galactosidase was from Gibco-BRL. Salts were of analytical grade. Solutions were prepared in MilliQ water (Millipore).

Cells

Chinese hamster ovary (CHO) cells were used. The WTT clone was selected for its ability to grow in suspension and to be plated on petri dishes. They were grown as previously described (Rols et al., 1995). Their ability to grow on a support after being maintained in suspension is good evidence of their viability.

Plasmids

A 6.8-kb pBR322 shuttle vector carrying the β-Gal gene controlled by the SV40 promoter was used. It was prepared by using the Qiagen Maxiprep DNA purification system (Ehlert et al., 1993).

Electropermeabilization and electroloading

The procedure was as previously described (Teissié and Rols, 1988). Electropulsation was operated by use of a CNRS cell electropulsator (Jouan). It gave square-wave electric pulses. Two thin stainless-steel parallel electrodes, connected to the voltage generator, gave a uniform electric field. Cells in suspension were centrifuged for 5 min at $120 \times g$ and resuspended in the pulsation buffer, which was a low ionic strength pulsing buffer (10 mM phosphate, 1 mM $MgCl_2$, 250 mM sucrose, pH 7.4). Its osmolarity could be changed and was controlled by its sucrose content: iso-, hypo-, or hyperosmolarities were obtained with 250, 125, and 400 mM sucrose. Osmolarity values were checked with an osmometer (OSMOMAT 030; Gonotec, Berlin, Germany).

Penetration of propidium iodide (0.1 mM in pulsing buffer) was used to monitor permeabilization. Just before application of the pulses, the culture medium was replaced by the dye containing buffer. After 10 min of incubation at room temperature, 100 μl of the cell suspension (i.e., 10^6 cells) was poured between the electrodes in contact with the bottom of a petri dish for each assay. After pulsation, cells were left at 30°C for 10 min. Cells were analyzed by flow cytometry (Beckton Dickinson FACScan) to determine both the percentage of cells permeable to propidium iodide and the associated fluorescence per cell. For protein electroloading experiments, cells were washed by centrifugation and then resuspended in pulsing buffer in the presence of 0.2 mg/ml β-galactosidase. After electropulsation, cells were put in culture medium for 24 h at 37°C and then tested for β-galactosidase activity (Rols et al., 1995). Only viable cells, which were able to plate on the dish, were taken into account in the assay. Cells expressing the β-galactosidase activity were stained blue green within 1 h.

Electrotransfection

Cells in suspension were washed and resuspended in plasmid DNA containing pulsation buffer. For each assay, 100 μl of cell suspension was used, corresponding to 0.7×10^6 cells and 6 μg plasmid. This preparation was incubated for 10 min at 0°C before pulsation, pulsed, and then incubated for 10 min at 30°C after pulsation (Rols et al., 1994). It was then put into culture in a petri dish with 2 ml of culture medium for 24 h at 37°C in a 5% CO_2 incubator before testing for β-galactosidase activity (Rols et al., 1995). Only plated (i.e., viable) cells were taken into account in the assay.

Cell viability

Viability was measured by quantifying the growth of plated cells over more than one generation ($\sim$24 h) by crystal violet staining (Gabriel and Teissié, 1995). Briefly, the cell monolayer was washed with phosphate-buffered saline and incubated with isotonic pulsing buffer containing crystal violet (0.1% w/v), under gentle agitation. The monolayer was washed three times with phosphate-buffered saline, and cells were lysed with 500 μl of 10% acetic acid solution. The absorption of the cell extract at 595 nm was measured. Nonpulsed cells were 100% viable.

Cell diameter determination

Cell diameter was obtained by directly measuring the size of cells. An acquisition card (DC20; MIRO) captured frames from a video camera (SONY) connected to an inverted microscope with a 63$\times$ objective (Leica DM IRB). More than 30 cells were assayed per condition.

To study the effect of postpulse osmotic pressure on cell diameter, glass slides were pretreated with 50 μl of L-polylysine 0.1 ml/ml (P5899; Sigma) to maintain cell adhesion while the buffer was changed after pulsation.

All experiments were repeated at least three times at intervals of several days to avoid fluctuations of results within a given set of experiments due to the physiological state of cells.

RESULTS

Effects of electric pulses and osmotic pressure on CHO cell size

CHO cells in suspension have a mean diameter of 12.2 μm. This diameter can be affected by buffer osmotic pressure changes: it increases with a decrease in osmolarity and decreases with an increase in osmolarity. In fact, these alterations in CHO cell size only occur during the few seconds after osmotic stress. Regulation volume decrease (RVD) and increase (RVI) are carried out within the 10 following minutes. Measurements of cell diameter after 10 min of incubation in hypo-, iso-, or hypertonic buffers did not reveal any significant difference (Table 1A and Fig. 1 *A*).

Cells incubated for 10 min in various osmotic pressure pulsing buffers, were pulsed (10 pulses lasting 5 ms, 0.8 kV/cm field intensity at a 1-Hz frequency) under the microscope, and their diameters were measured after each impulse. Whatever the osmolarity of the pulsing buffer, each pulse induced an increase in cell size. Similar effects were obtained under hypo- and isoosmotic conditions, with an increase of 30% in cell diameter. In hypertonic buffer, the observed increase was lower, reaching 15% (Table 1A).

TABLE 1 Effect of electropulsation and osmotic pressure variations on CHO cell diameter (μm)

	Hypotonic	Isotonic	Hypertonic
A. Effect of the pulsing buffer osmotic pressure			
Diameter of control cells*	12.5 ($\pm$1.5)	12.2 ($\pm$1.5)	11.5 ($\pm$1.5)
Diameter of pulsed cell[#]	16.5 ($\pm$3.9)	15.9 ($\pm$1.9)	13.6 ($\pm$5.7)
B. Effect of the postpulsing buffer osmotic pressure[§]			
Cell diameter 2 s after postpulsing buffer change[¶]	18.1 ($\pm$2)	15.9 ($\pm$3)	12.7 ($\pm$2)
Cell diameter 10 min after postpulsing buffer change[‖]	15.6 ($\pm$2)	15.6 ($\pm$3)	13.8 ($\pm$2)

*Cell diameter was measured 10 min after osmotic pressure change.
[#]These values represent the maximum volume variation after 10 impulses lasting 5 ms at the frequency of 1 Hz.
[§]Cells were pulsed in isotonic medium by application of 10 impulsions lasting 5 ms at the frequency of 1 Hz.
[¶]These values represent the maximum volume variation after osmotic pressure change in pulsated cells.
[‖]These values represent the volume variation 10 min after osmotic pressure change in pulsated cells.

The effect of postpulse osmotic pressure was studied by pulsing the cells in isotonic buffer and by changing the osmolarity of the buffer 1 s later. Cell diameter increased during electropulsation. It then increased in postpulse hypotonic buffer, and it decreased in hypertonic buffer, whereas it did not significantly change in isotonic buffer. The swelling and the shrinking reached their maximum values 2 s after postpulse osmotic pressure change. Then RVD or RVI took place in the following minutes. Under hypotonic conditions, RVD was achieved in less than 2 min, whereas under hypertonic conditions, RVI lasted for 5–10 min (Fig. 1 *B* and Table 1B). The same observations were made when cells were pulsed in hypotonic buffer. A postpulse isotonic buffer led to a decrease in the cell diameter. A greater decrease was observed in postpulse hypertonic buffer. A size increase was observed when cells were pulsed in a hypertonic buffer and then put in iso- and hypotonic buffers (data not shown). Pictures of cells in isotonic buffer are shown Fig. 1 *C*.

Effect of buffer osmotic pressure on the electropermeabilization of CHO cells

Permeabilization of cells was performed by application of long electric pulses, which are required to transfect cells and to load β-galactosidase into cells (Hui, 1995). This was quantified by monitoring the penetration of propidium iodide into cells. By decreasing the electric field intensity, using a long pulse duration makes it possible to preserve cell viability (Wolf et al., 1994; Gabriel and Teissié, 1995).

As described in our previous work (Teissié and Rols, 1988), permeabilization of CHO cells appeared only for electric field values higher than a given threshold. The threshold value was ~400 V/cm and did not depend on pulsing buffer osmolarity. Increasing the field intensity above this resulted in an increase in the percentage of permeabilized cells, whatever the pulsing buffer (Fig. 2 *A*). The fluorescence intensity associated with this permeabilization was an indicator of the number of internalized molecules of PI in each permeabilized cell. Its changes as a function of the electric field intensity were as follows: no increase for field intensity lower than 400 V/cm, continuous increase above that value, no plateau obtained. No strongly significant difference in behavior was observed in iso- or hypoosmotic buffers, whereas under hyperosmotic buffer conditions, the individual fluorescence intensity was lower (Fig. 2 *B*). This electroinduced permeability is associated with a loss in cell viability, as shown in Fig. 2 *C*. At 800 V/cm, the level of permeabilization reaches 75%, whereas the viability decreased to 60%, whatever the osmolarity (Fig. 2, *A* and *C*).

As PI inflow occurred mostly after the pulse (Sixou and Teissié, 1993), the effect of the osmotic pressure during the 10-min incubation after the pulses was investigated by changing the buffer immediately after pulsing. The percentage of permeabilized cells and the associated fluorescence intensity were not affected by this treatment. The effect of osmolarity only existed during the application of the pulses and not during postpulse incubation, when the inflow of molecules of PI took place (Fig. 2 *D*). Resealing experiments were performed. Resealing curves were observed to fit a first-order kinetic well. The rate constant, k, was found to be constant and equal to $0.18 \pm 0.02 \text{ min}^{-1}$, whatever the osmolarity.

Effect of buffer osmotic pressure on β-galactosidase electroloading

As shown in Fig. 3, and in agreement with previous work (Rols et al., 1995), a viable cell population could be electroloaded with β-galactosidase up to 80% in an isoosmolar buffer. Pulsing the cells under hypoosmotic conditions did not dramatically change electroloading efficiency, whereas pulsing the cells under hyperosmotic conditions decreased it by 50%. As for electropermeabilization, the effect of a change in osmotic pressure during the 10-min incubation after the pulses was investigated. No evidence for any difference was found (Fig. 3).

No electroloading was obtained by changing the osmolarity of the medium in the absence of electric field. No influence of the postpulse temperature on the electroloading efficiency was observed when cells pulsed at room temperature were incubated at 4°C or 37°C during the following 10 min (data not shown). Moreover, the addition of trypsin just after the pulsation did not affect the electroloading efficiency (data not shown).

A

B

C
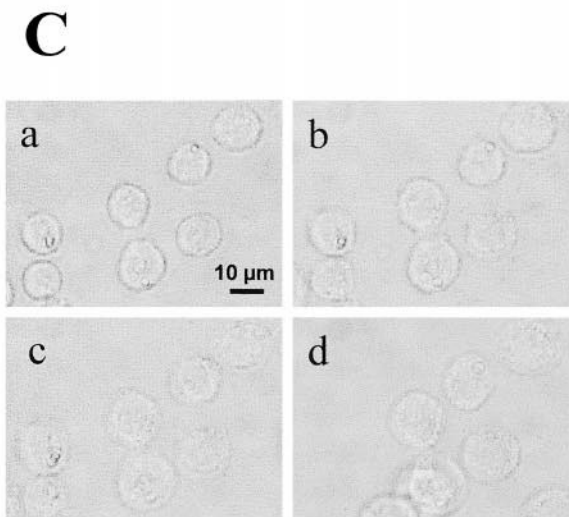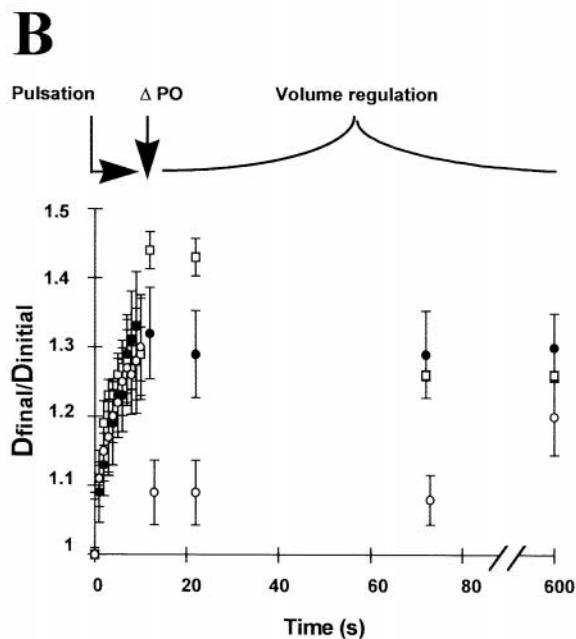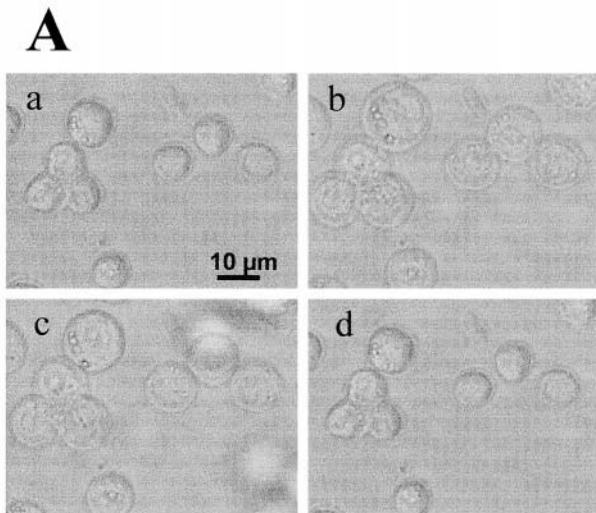
Effect of osmotic pressure on cell electrotransfection

When cells mixed with plasmids were pulsed and incubated in an isoosmolar pulsing buffer (i.e., with 250 mM sucrose buffer), the percentage of viable cells expressing β-galactosidase activity reached values up to 20–25%, as routinely observed (Rols et al., 1994; Rols and Teissié, 1992). This value was increased up to twofold when cells were pulsed under hypotonic conditions. It decreased down to 2.5-fold when cells were pulsed under hyperosmolar conditions, as shown in Fig. 4. Under these conditions, the osmolarity was acting on the three steps of plasmid transfer: prepulse incubation, pulse, postpulse incubation. We checked its effect on the last step by changing the osmolarity of the buffer 2 s after the pulse as described in Methods. A decrease in the osmolarity gave an increase in transfection efficiency, whatever the osmolarity of the pulsing buffer, whereas the opposite effect was observed by increasing the osmolarity (Fig. 4 *A*). The survival of cells was tested under these nine different conditions. Results reported in Fig. 4 *B* show that osmotic pressure had no significant influence on cell viability. No transfection was obtained by changing the osmolarity of the medium in the absence of electric field. Cell viability is affected both by the lytic effect of the electric field and by the toxic effect of the DNA (Rols et al., 1992; Wolf et al., 1994).

To test if 10 min of osmotic stress before the electric treatment could affect the transfection level by acting on the expression of the gene, cells were incubated for 10 min in a hypoosmotic buffer and then put in an isoosmotic buffer before the pulse. No change in transfection level was observed (data not shown). The effect of osmotic pressure on gene expression was performed on cells pulsed in isoosmotic buffer and then incubated for 1 h before the 10 min of osmotic stress. No change in transfection level was observed (data not shown).

DISCUSSION

Electropermeabilization has been described as a multistep process of 1) creation, 2) expansion, and 3) resealing of transient permeated structures. The first two steps occur

FIGURE 1 CHO cell diameter variations due to osmotic pressure change (*A*) and electric field treatment (*B* and *C*). (*A*) Pictures of cells: (*a*) CHO cells in isoosmolar buffer; (*b*) CHO cells in hypoosmolar buffer 5 s after osmotic change; (*c*) CHO cells in hypoosmolar buffer 15 s after osmotic change; (*d*) CHO cells in hypoosmolar buffer 5 min after osmotic change. (*B*) Relative changes in cell diameter: 10 pulses of 5 ms duration at a 800 V/cm electric field intensity were applied to cells suspended in isoosmolar buffer (10 mM phosphate, 1 mM $MgCl_2$, 250 mM sucrose, pH 7.2). After pulsation the osmotic buffer is changed to 125 mM sucrose (hypoosmolar buffer) (□), 125 mM sucrose (isoosmolar buffer) (●), and 400 mM sucrose (hyperosmolar buffer) (○) for 600 s, and the cell diameters are measured. (*C*) Pictures of pulsed cells: (*a*) before pulsation; (*b*) at the fifth impulse (5 ms duration); (*c*) at the tenth impulse; (*d*) 10 min after pulse.
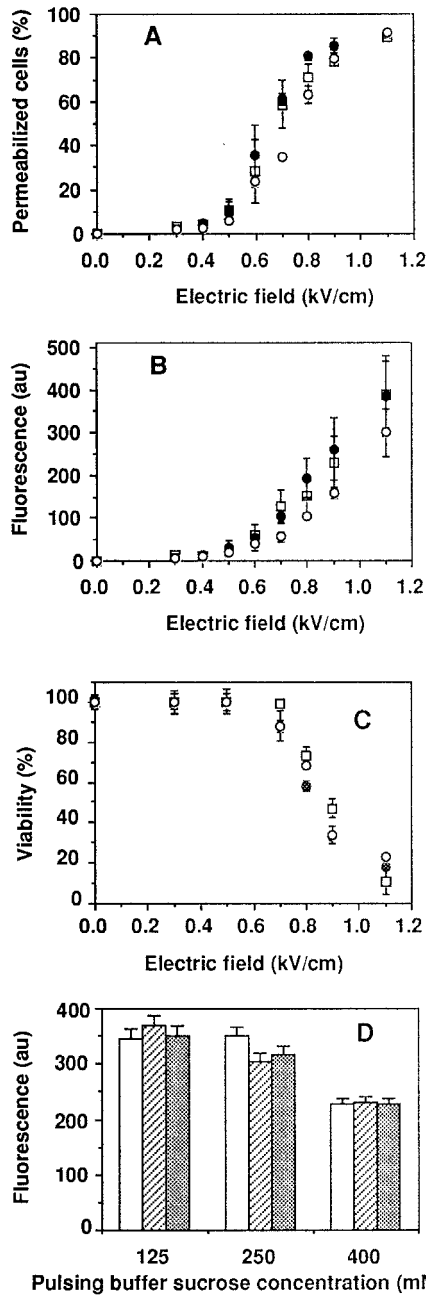
FIGURE 3 Effect of osmolarity of pulsing and postpulsing buffers on the electroloading of the β-galactosidase enzyme into CHO cells. Ten pulses of 5 ms duration at a 900 V/cm electric field intensity were applied to cells suspended in pulsing buffers of varying sucrose concentrations. One second after the pulses, the osmotic pressure of buffers was adjusted to 400 mM (▩), 250 mM (▨), and 125 mM (□). β-Galactosidase activity was detected 24 h later.

modulates the membrane potential difference ΔV (Neumann et al., 1982). It is a complex function of the specific conductivity of the membrane (which is normally low in an intact cell), the specific conductivities of the pulsing buffer

FIGURE 2 Effect of electric field intensity and osmolarity on CHO cell permeabilization. (A) Percentage of permeabilized cells. (B) Fluorescence intensity associated with the permeabilization. (C) Viability (%). Ten pulses of 5 ms duration were applied to cells suspended in different pulsing buffers (10 mM phosphate, 1 mM MgCl$_2$, pH 7.2) with different sucrose concentrations: 125 mM sucrose (hypoosmolar buffer) (□); 250 mM sucrose (isoosmolar buffer) (●); and 400 mM sucrose (hyperosmolar buffer) (○). Permeabilization was quantified by propidium iodide penetration. (D) Effect of osmolarity of pulsing and postpulsing buffers on permeabilization. Ten pulses of 5 ms duration at a 900 V/cm electric field intensity were applied to cells suspended in pulsing buffers of varying sucrose concentrations. One second after the pulses, the osmotic pressure of the buffers was adjusted to 400 mM (▩), 250 mM (▨), and 125 mM (□).

FIGURE 4 Effect of osmotic pressure on the electrotransfection of CHO cells. (A) Effect of postpulse osmolarity on electrotransfection of CHO cells as a function of the pulsing buffer osmolarity. (B) Effect on the survival rate. Ten pulses of 5 ms duration at a 900 V/cm electric field intensity were applied to cells mixed with plasmids and suspended in pulsing buffers of various sucrose concentrations. One second after the pulses, the osmotic pressure of buffers was adjusted to 400 mM (▩), 250 mM (▨), and 125 mM (□). β-Galactosidase activity was detected 24 h later. The absolute value of the maximum transfection depended on the cell culture we used. Relative values of four independent experiments are reported here where the 100% is related to the maximum transfection (obtained for the 125 mM pulsing and postpulsing buffers).

during pulse application, and the third one takes place in the minutes after it (Kinosita and Tsong, 1977; Rols and Teissié, 1990b). It has been demonstrated that electric field
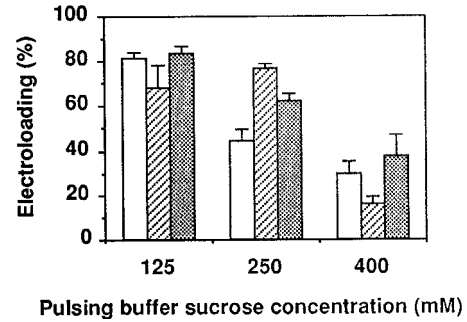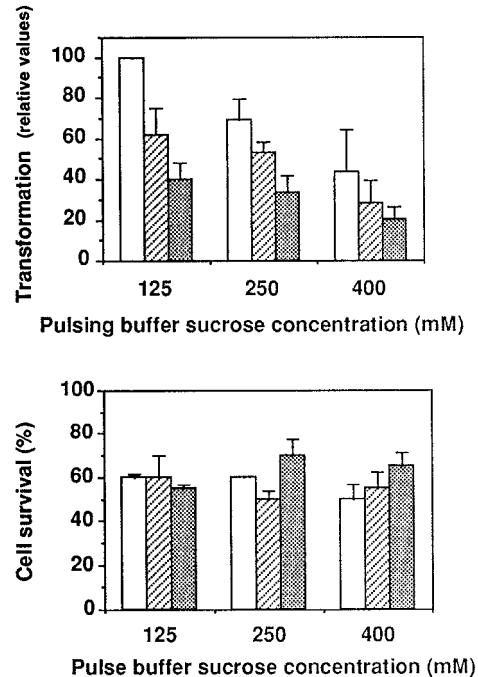
and the cytoplasm, the membrane thickness, and the cell size. Thus,

$$\Delta V_M = f, g(\lambda) r E \cos\theta$$

in which λ designates the various conductivities, θ is the angle between the direction of the normal to the membrane at the considered point M on the cell surface and the field direction, E is field intensity, r is the radius of the cell, and f is a shape factor. ΔV is not uniform on the cell surface. It is maximum at the positions of the cell facing the electrodes. When the resulting transmembrane potential difference (i.e., the sum between the resting value of cell membrane and the electroinduced value) reaches threshold values close to 250 mV, membranes become permeable (Teissié and Tsong, 1981; Teissié and Rols, 1993). As shown by the above equation, ΔV is a function of cell physiology (cell shape and cell size). This could be affected by buffer osmotic pressure and was checked in this study.

Most mammalian cells are able to maintain some control over their intrinsic volume after exposure to an anisosmotic buffer. Hypotonic shock causes water to accumulate in cells by osmosis, because of the high osmotic permeability of cell membranes. This leads first to an increase in the cell size, and then regulated volume decrease (RVD) takes place. It is caused by extrusion of osmolytes, including ions, amino acids, and sugars, through a variety of transport systems (McCarty and O'Neil, 1992). In the present case of CHO cells, we observed this phenomenon: in less than 10 min after osmotic change, the size of the CHO cells was back to its initial value in the range of osmotic pressure used (Table 1B and Fig. 1 *A*).

The permeabilization threshold of 400 V/cm was the same whatever the pulsing buffer osmolarity (Fig. 2 *A*). These observations led us to the conclusion that there is no effect of osmotic pressure on the permeabilization creation step under our conditions. Indeed, we observed that the efficiency of permeabilization (i.e., the expansion step) is significantly less in hyperosmolar buffer (Fig. 2, *B* and *C*). These observations agree with those previously obtained on plated CHO cells under short pulse conditions (Rols and Teissié, 1990a). Increasing osmotic pressure hindered the expansion step of permeabilization. In this work, the cumulated duration of the electric pulses was 50 ms (10 pulses of 5 ms duration) instead of 1 ms (10 pulses of 100 μs duration) (Rols and Teissié, 1990a). This could explain why the slopes of the permeabilization versus field strength curves, which were related to the expansion step of the permeabilization process in Fig. 2 *A*, were less dependent on osmolarity changes than in our previous work. The duration of electric application was long enough to reach maximum permeabilization. As shown in Fig. 2 *D*, the level of fluorescence did not change, whatever the osmolarity of the postpulse buffer. The resealing step also depends on osmolarity, as in previous work. But the two main observations are that in the minutes after the pulse, cells are still permeable whatever the osmotic pressure, and that the osmotic

pressure of postpulse incubation buffer does not alter the resealing kinetic. One should also notice that during electropulsation, the cell diameter increased (Fig. 1, *B* and *C*, and Table 1A). This increase in cell size, which was the same for hypo- and isotonic buffer, was less important in hypertonic buffer. Such an osmotic swelling was already described (Kinosita and Tsong, 1977a, b). Human erythrocytes pulsed in an isotonic NaCl solution kept swelling because of continuous penetration of solutes toward equilibrium. Moreover, this effect could be explained by alteration of the cell cytoskeleton, due to ATP and GTP leakage during electropulsation (Rols and Teissié, 1992). Such alteration has been described as being responsible for cell swelling (Downey et al., 1995).

Previous studies reported high changes in murine L-cell volume produced by alteration of the osmolarity of the cell buffer (Däumler and Zimmermann, 1989). They showed that the transfection rate of murine L-cells and macrophages increased in hypoosmotic buffer. Their result was only discussed in terms of higher permeability of the membrane due to the swollen forces. We observed a dynamic response of the cell size along electropulsation (Table 1A). Cell swelling creates a hydrodynamic influx of water, which could carry the plasmid DNA or the protein into the cytoplasm when the cell is electropulsed. This must be taken into account to explain the differences observed between iso- and hyperosmotic buffers in terms of permeabilization efficiency (Fig. 2 *B*), electroloading (Fig. 3), and electrotransfection (Fig. 4 *A*). But the differences observed between hypo- and isotonic buffers in the case of transfection and electroloading cannot be explained by the existence of this hydrodynamic influx, inasmuch as the swelling rates are the same under these two conditions (Table 1A).

β-Galactosidase was used because the detection of its activity when it is transferred into cell cytoplasm was the proof that the tetrameric macromolecule was intact after being electrotransferred. Its electroloading is clearly controlled by pulsing buffer condition osmolarity (Fig. 3). If the effect of hyperosmolarity can be explained by a smaller cell swelling and associated water influx, this cannot explain the hypoosmolarity effect. The swelling is the same in hypo- and isoosmotic conditions, whereas the loading is different. We may conclude that repulsive forces preventing protein transfer across the membrane are weaker in hypoosmolar conditions. It is known that surface forces, leading to undulation forces when membranes are in close contact, are decreased under hypoosmolar conditions (Rand and Parsegian, 1989). As a hydration shell is present around the protein, we may suggest that hydration repulsion, which was described in homointeraction (Rau et al., 1984), is present in heterointeractions between hydrated bodies (here the membrane and the protein). This controls the transfer when the membrane is brought to a permeabilized state by the external field. Decreasing the surface forces by hypoosmolarity decreases the repulsion and facilitates the transfer, as we observed. As no postpulse effects were detected (Fig. 3) by changing 1) the osmolarity or 2) the temperature, or 3)

by the addition of trypsin, this bears out our previous conclusions that direct transfer of β-galactosidase took place during the pulse. We indeed showed that when added after the application of the electric field, the enzyme has no free access to the cytoplasm, but enters the cell via a macropinocytis-like process (Rols et al., 1995).

The effect of the variation of osmotic pressure on gene transfer to increase the transfection efficiency was investigated by others. Although the transfection rate of macrophages was higher under hypoosmolar conditions (Däumler and Zimmermann, 1989), Van den Hoff et al. (1990) concluded that the use of hypoosmolar buffer had to be avoided in electrotransfection of 5123, FT 02B, and National Institutes of Health 3T3 cells. But they did not take into account the fact they were using a capacitor discharge electropulsator. As they diluted the pulsing medium, in which the ionic content was high, by adding water, they were altering its conductivity and then were acting on the decay time of the pulse (i.e., on the pulse duration). Their observations could not provide definitive conclusions on the osmolarity control of the transfer.

The present study shows a positive control of gene transfer by hypoosmolarity. We show that DNA transfer is controlled by both the pulsing buffer and the postpulsing buffer osmotic pressure. This postpulse dependence leads to the following conclusions on the mechanism of plasmid transfer. First, plasmid penetration into mammalian cells goes on after electric field application and not only during the pulses, as we just showed to be the case for the β-galactosidase enzyme. As when cells and plasmids are pulsed in an isoosmolar buffer, the level of transfection is positively controlled by the cell swelling, we may suggest that the water influx facilitates the plasmid penetration after the pulse. But a more interesting conclusion is obtained from the observations of the osmolarity control during the pulse. As pulsing the cells in the millisecond time range in iso- and hypoosmotic pressure buffers affects neither the permeabilization for small molecules (PI) (Fig. 2, *A*, *B*, and *D*) nor the cell swelling process (Table 1A), we can argue that the osmotic pressure dependence observed in the gene transfer cannot be limited by a more efficient permeabilization or a hydrodynamic efflux. Another factor should be taken into account when considering the control of the transmembrane DNA transfer. It is well known that as DNA is a hydrated body, repulsive forces are present when two such macromolecules are brought into close contact (Rand and Parsegian, 1989). They are due to the local interfacial water organization. A similar water structure is present at the cell surface (McIntosh et al., 1995), producing repulsive interactions when membranes are in contact. In this case, these repulsions are increased by the undulations of the membranes (Rand and Parsegian, 1989). As we described in the case of the β-galactosidase/CHO cell membrane interactions, we suggest that the interfacial water-associated repulsive forces are present when DNA is in contact with cell membranes. Their magnitudes are decreased under hypoosmolar conditions in which the undulations are prevented.
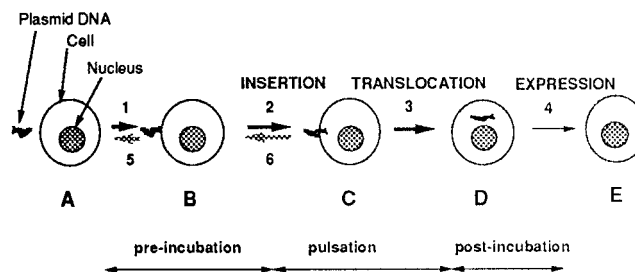

FIGURE 5   Model of mammalian cell electrotransfection. During the application of the pulses, the free DNA (state A) bonds reversibly to the membrane (state B) and begins its reversible insertion into the electropermeabilized membrane (state C). The plasmid is translocated into the cell (state D) not only during pulsation, but also in the second after it (Wolf et al., 1994). Thus the gene activity can be expressed (state E). Steps 1, 2, and 3 are positively controlled by a decrease in osmolarity (*large black arrows*). Step 4 is not controlled. On the contrary, steps 5 and 6 are enhanced by an increase in osmolarity (*gray arrows*).

This decrease in repulsion facilitates DNA insertion during the pulse, a key step in gene transfer (Wolf et al., 1994).

This work supports the model developed by our group and is supported by the results on yeast (Neumann et al., 1996), in which the transfer of plasmid into a cell is not restricted to an electrophoretic process (Ganeva et al., 1995). It is a more complex process, in which an anchoring step connecting the plasmid to the permeabilized membrane takes place during the pulse controlled by water-associated repulsive forces, followed by a postpulse transfer in which a hydrodynamic contribution must be taken into account (Fig. 5).

REFERENCES

Chang, D. G., B. M. Chassy, J. A. Saunders, and A. E. Sowers. 1992. Guide for Electroporation and Electrofusion. Academic Press, San Diego.

Crowley, J. M. 1973. Electrical breakdown of biomolecular lipid membranes as electromechanical instability. *Biophys. J.* 13:711–724.

Cruzeiro-Hansson, L., and O. G. Mouritsen. 1989. Passive ion permeability of lipid membranes modelled via lipid-domain interfacial area. *Biochim. Biophys. Acta.* 944:63–72.

Däumler, R., and U. Zimmermann. 1989. High yield of stable transformants by hypo-osmolar plasmid electroinjection. *J. Immunol. Methods.* 122:203–210.

Downey, G. P., S. Grinstein, A. Sue-A-Quan, B. Czaban, and C. K. Chan. 1995. Volume regulation in leucocytes: requirement for an intact cytoskeleton. *J. Cell Physiol.* 163:96–104.

Ehlert, F., P. Bierbaum, and J. Shorr. 1993. Importance of DNA quality for transfection efficiency. *Biotechniques.* 14:546.

Eynard, N., S. Sixou, N. Duran, and J. Teissié. 1992. Fast kinetics studies of *Escherichia coli* electrotransformation. *Eur. J. Biochem.* 209: 431–436.

Gabriel, B., and J. Teissié. 1995. Control by electrical parameters of short- and long-term cell death resulting from electropermeabilization of Chinese hamster ovary cells. *Biochim. Biophys. Acta.* 266:171–178.

Ganeva, V., B. Galutzov, and J. Teissié. 1995. Fast kinetic studies of plasmid DNA transfer in intact yeast cells mediated by electropulsation. *Biochem. Biophys. Res. Commun.* 214:825–832.

Gruen, D. W. R., S. Marcelja, and B. A. Pailthrope. 1981. Theory of polarization profiles and the hydration forces. *Chem. Phys. Lett.* 82: 315–320.

Hui, S. K. 1995. Effects of pulse length and strength on electroporation efficiency. *Methods Mol. Biol.* 48:29–40.

Kinosita, K., Jr., and T. Y. Tsong. 1977a. Hemolysis of human erythrocytes by transient electric field. *Proc. Natl. Acad. Sci. USA.* 74:1923–1941.

Kinosita, K., Jr., and T. Y. Tsong. 1977b. Formation and resealing of pores of controlled sizes in human erythrocyte membrane. *Nature.* 268: 438–441.

Klenchin, V. A., S. I. Sukharev, S. M. Serov, L. V. Chernomordik, and Y. A. Chidmadzhev. 1991. Electrically induced DNA uptake by cells is a fast process involving DNA electrophoresis. *Biophys. J.* 60:804–811.

Lopez, A., M. P. Rols, and J. Teissié. 1988. ^{31}P NMR analysis of membrane phospholipid organisation in viable, reversibly electropermeabilized Chinese hamster ovary cells. *Biochemistry.* 27:1222–1228.

Malissen, B. 1990. Transfer et expression de gene dans les cellules de mammifères. INSERM, Paris.

McCarty, N. A., and R. G. O'Neil. 1992. Calcium signaling in cell volume regulation. *Physiol. Rev.* 72:1037–1061.

McIntosh, T. J., S. Advani, R. E. Burton, D. V. Zhelev, D. Needham, and S. A. Simon. 1995. Experimental test for protrusion and undulation pressures in phospholipid bilayers. *Biochemistry.* 34:8520–8532.

Neumann, E., S. Kakorin, I. Tsoneva, B., Nikolova, and T. Tomov. 1996. Calcium-mediated DNA adsorption to yeast cells and kinetics of cell transformation by electroporation. *Biophys. J.* 71:868–877.

Neumann, E., M. Schaefer, Y. Wang, and P. M. Holschneider. 1982. Gene transfer into mouse lyoma cells by electroporation in high electric field. *EMBO J.* 1:841–847.

Potter, H. 1992. Protocols for using electroporation to stably or transiently transfect mammalian cells. *In* Guide to Electroporation and Electrofusion. D. C. Chang, B. M. Chassy, J. A. Saunders, and A. E. Sowers, editors. Academic Press, San Diego. 457–464.

Rand, R. P., and V. A. Parsegian. 1989. Hydration forces between phospholid bilayers. *Biochim. Biophys. Acta.* 988:351–376.

Rau, D. C., B. Lee, and V. A. Parsegian. 1984. Measurement of the repulsive force between polyelectrolyte molecules in ionic solution: hydration forces between parallel DNA double helices. *Proc. Natl. Acad. Sci. USA.* 81:2621–2625.

Rols, M. P., D. Coulet, and J. Teissié. 1992. Highly efficient transfection of mammalian cells by electric field pulses. Application to large volumes of cell culture by using a flow system. *Eur. J. Biochem.* 206:115–121.

Rols, M. P., F. Dahhou, K. P. Mishra, and J. Teissié. 1990. Control of electric field induced cell membrane permeabilization by membrane order. *Biochemistry.* 29:2961–2966.

Rols, M. P., C. Delteil, G. Serin, and J. Teissié. 1994. Temperature effects on electrotransfection of mammalian cells. *Nucleic Acids Res.* 22:540.

Rols, M. P., P. Femenia, and J. Teissié. 1995. Long lived macropinocytosis takes place in electropermeabilized mammalian cells. *Biochem. Biophys. Res. Commun.* 208:26–35.

Rols, M. P., and J. Teissié. 1990a. Modulation of electrically induced permeabilization and fusion of Chinese hamster ovary cells by osmotic pressure. *Biochemistry.* 29:4561–4567.

Rols, M. P., and J. Teissié. 1990b. Electropermeabilization of mammalian cells. *Biophys. J.* 58:1089–1098.

Sixou, S., and J. Teissié. 1993. Exogenous uptake and release of molecules by electroloaded cells: a digitized videomicroscopy study. *Bioelectrochem. Bioenerg.* 31:237–257.

Sukharev, S. I., V. A. Klenchin, S. M. Serov, L. V. Chernomordik, and Y. A. Chidmadzhev. 1992. Electroporation and electrophoretic DNA transfer into cells. The effect of DNA interaction with electropores. *Biophys. J.* 63:1320–1327.

Teissié, J., and M. P. Rols. 1988. Electropermeabilization and electrofusion of cells. *In* Dynamic of Membrane Proteins and Cellular Energetics. N. Latruffe, Y. Gaudemer, P. Vignais, and A. Azzi, editors. Springer Verlag, Berlin, Heidelberg, New York, London, Paris, Tokyo. 249–268.

Teissié, J., and M. P. Rols. 1993. An experimental evaluation of the critical potential difference inducing cell membrane electropermeabilization. *Biophys. J.* 68:409–413.

Teissié, J., and T. Y. Tsong. 1981. Electric field induced transient pores in phospholipid bilayer vesicles. *Biochemistry.* 20:1548–1554.

Van Den Hoff, M. J. B., W. T. Labruyere, A. F. M. Moorman, and W. H. Lamers. 1990. The osmolarity of the electroporation medium affects the transient expression of genes. *Nucleic Acids Res.* 18:6464.

Wolf, H., M. P. Rols, E. Boldt, E. Neumann, and J. Teissié. 1994. Control by pulse parameters of electric field-mediated gene transfer in mammalian cells. *Biophys. J.* 66:524–531.

Xie, T. D., and T. Y. Tsong. 1993. Study of mechanisms of electric field-induced DNA transfection. V. Effects of DNA topology on surface binding, cell uptake, expression, and integration into host chromosomes of DNA in the mammalian cells. *Biophys. J.* 65:1684–1689.