Control by Pulse Parameters of Electric Field-Mediated Gene Transfer in Mammalian Cells

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ABSTRACT Electric field-mediated gene transfer in mammalian cells (electrotransformation) depends on the pulsing conditions (field intensity, pulse duration, number of pulses). The effect of these parameters was systematically investigated using the transient expression of the chloramphenycol acetyltransferase and the β-galactosidase activities in Chinese hamster ovary cells. Pulsing conditions inducing reversible permeabilization of the cell plasma membrane are not sufficient to induce gene transfer. 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. Pulse duration plays a key role in the magnitude of the transfer. The field induces a complex reaction between the membrane and the plasmid that is accumulated at the cell interface by electrophoretic forces. This leads to an insertion of the plasmid, which can then cross the membrane.

GLOSSARY

E	electric field
I	impermeant state of the membrane
P	permeabilized state of the membrane
Tr	transformation yield
T	pulse duration
N	pulse number
u	electrophoretic mobility
L	electrophoretic displacement

INTRODUCTION

Transfer of foreign information in the genome of cells is a key problem in cell biology and biotechnology. This is crucial in the study of eucaryotic cells where there is no spontaneous transfer such as exists in bacteria. Transfer is obtained by chemical or viral approaches, although with many limitations (Malissen, 1990). A physical approach using the effect of electric field pulses on cells was pioneered in the early 1980s (Neumann et al., 1982). Electrotransformation is now routinely used in molecular biology (Potter, 1992) because of the broader range of cells that are sensitive to the electric technique as compared to other techniques. Large volumes of cells can be routinely treated (Rols et al., 1992). But very few studies have focused on the electric field-mediated mechanism of gene transfer, except in the case of bacterias (Xie and Tsong, 1990, 1992; Xie et al., 1990, 1992; Eynard et al., 1992). It is proposed that in the case of mammalian cells the plasmid crosses the membrane during the pulse due to the induction of an electropermeabilized state and under the effects of electrophoretic forces associated with the external field (Klenchin et al., 1991; Sukharev et al., 1992). But this conflicts with the results on *Escherichia coli* where no role for electrophoretic forces was observed. Indeed very few experimental results have been gathered on the reversible organization of the permeabilized membrane. Different theoretical descriptions have nevertheless been proposed: breakdown (Crowley, 1973), pores (see for reviews Neumann et al., 1989; Chang et al., 1992) or mismatches (Cruzeiro-Hanssen and Mouritsen, 1989). ^{31}P NMR studies have shown that the structural organization of the membrane phospholipids is affected inducing a fusogenic character in the electropermeabilized cell membrane (Sowers, 1986; Teissié and Rols, 1986; Lopez et al., 1988). Reorganization of the membrane/solution interface was proposed to be a key step in the induction of permeabilization (Rols and Teissié, 1990a). Electron microscopy investigation under isoosmolar conditions only revealed very short lived electrocracks (Stenger and Hui, 1986) and longer lived eruptions of villi (Escande et al., 1988; Gass and Chernomordik, 1990). "Volcano"-shaped pores were detected in red blood cells under hypoosmolar conditions (Chang and Reeves, 1990), but were correlated with the hemoglobin induced osmotic swelling (Chernomordik, 1992). The time dependence of the eruption of these pores was very different from that of the conductance changes (Kinosita and Tsong, 1979; Hibino et al., 1993). A kinetic model of the induction of electropermeabilization was proposed where a multistep process comprising induction steps (nucleation) followed by fast collective coalescence of the defects in ramified cracks was present (Sugar et al., 1987). Qualitative investigations of the process demonstrated that the magnitude of the field controlled the geometrical definition of the permeabilized part of the cell surface (Schwister and Deuticke, 1985) but that the cumulated pulse duration determined the local magnitude of the alteration (Rols and Teissié, 1990b).

In the present study, the effect of the different parameters controlling both transient electrotransformation and electropermeabilization of Chinese hamster ovary cells

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was investigated. The effect of electric field pulses is mediated by polarization effects in which time dependence is more easily monitored by the use of square wave pulses (Neumann, 1989). This technology was chosen as being more suitable than the more primitive capacitor discharge type (Neumann et al., 1982; Chu et al., 1987; Winterbourne et al., 1988). From these results, a new description of the molecular events responsible for electric field mediated gene transfer is proposed taking into account the decisive role of the pulse duration we observed.

MATERIALS AND METHODS

Chemicals

Trypan blue and propidium iodide were obtained from Sigma (USA). Salts were of analytical grade. Solutions were prepared in Milli Q water (Millipore, (USA)).

Cells

Chinese hamster ovary (CHO) cells are used in many somatic cell genetics laboratories (see Gottesmann, 1985). The WTT clone, which was given to us by Prof. Zalta (this Institute), was selected for the present study due to its ability to grow in suspension under gentle stirring. Cells were grown in Eagle's minimum medium (MEM 0111; Eurobio, France) supplemented with 6% newborn calf serum (Boehringer, Germany), antibiotics and Glutamine. They were maintained in exponentional growth phase by daily dilution.

Plasmids

PSV2CAT was obtained from Dr. Stevens (CRBGC-CNRS, Toulouse). PUT 531 was obtained from Pr. Tiraby (UPS, Toulouse). A pBR322 shuttle vector carried the SH-Gal fusion gene under the control of the SV40 promotor. Plasmids were prepared by standard procedures (Birnboim and Doly, 1979).

Electropermeabilization

The procedure was previously described (Teissié and Rols, 1988). Cells were washed in a permeabilization buffer containing the indicating dye (1% Trypan blue or 0.1 mM propidium iodide). 200 μL of the cell suspension (i.e., 10^6 cells) were poured between two flat parallel stainless steel electrodes in contact with the bottom of a culture dish (Nunc, diam. 35 mm, Denmark). The electric field was generated by a square wave high voltage of electronically selectable duration applied between the electrodes (CNRS Cell electropulser, Jouan, France). The pulse was monitored on line with an oscilloscope (Enertec, France). All experiments were run at 21°C. After pulsing, cells were incubated for 5 min. at room temperature and the percentage of stained cells was determined in the population.

Reversibility was assayed by pulsing in a dye free buffer and by adding trypan blue at selected periods following the pulses, cells being kept at 21°C.

Counting of stained cells was obtained by observing the cells under an inverted microscope (Leitz, Germany) by videomonitoring (JVC, Japan) and tape recording on a VCR (Philips, the Netherlands), 2 times 500 cells (pulsed and control) were observed routinely in order to obtain statistically significant results. Cells pulsed in the presence of propidium iodide were analysed by flow cytometry (Becton Dickinson FACScan).

Cell viability was assayed by following growth 24 h after the pulses which were operated under a laminar flow hood (ESI, France) to obtain sterile conditions. 2 parameters were indicative of viability : plating efficiency and growth rate. Viability was expressed by the amount of proteins in the cell extract.

The pulsing buffer (PB) composition used was: $MgCl_2$ 1 mM, Sucrose 250 mM, Phosphate 10 mM pH 7.4 (low salt buffer).

Electrotransformation

Cells were washed in PB and brought to a concentration of 5 10^6 cells per mL. Plasmids were in Tris-EDTA buffer at a concentration of 0.1 mg/ml for PSV2CAT and of 1 mg/ml from the nontransforming salmon sperm DNA (Carrier DNA). Carrier DNA was used because it is well known from classical transfection, transformation and microinjection procedures that the addition of "carrier DNA" (i.e., nonspecific high-molecular-weight DNA) leads to an increase in the level of gene expression of the specific plasmid transfered (Krens et al., 1982). This is linked to the activity of intracellular nucleases. In the presence of excess carrier DNA, a sufficient amount of the specific coding foreign plasmid DNA is preserved from cleavage. Up to 40 μL of the plasmid solution and 10 μL of the carrier DNA were added to 150 μL of the cell suspension. This mixture was kept for 3 min. at room temperature before pouring it between the two electrodes as described in the electropermeabilization procedure.

After pulsing at room temperature, the electrodes were removed and the suspension was incubated during 10 min. at room temperature. Then 3 mL of the culture medium were added. Cells were incubated during 48 h in an Air-CO_2 95–5 incubator (Jouan, France). Culture medium was changed after 24 h.

Transformation assay

Chloramphenycol acetyl transferase activity was assayed by 2 procedures. The chromatographic method (Gorman et al., 1982) was used in preliminary experiments but a new method (Sleigh, 1986) was prefered, in which the labeled product was continously phase extracted and measured. A direct quantitation of CAT activities from cell extracts was obtained.

All results are expressed following this last procedure and related to 1 mg of protein of the cell extract. Our observations describe the expression of the activity coded by plasmids in viable cells. Transfection efficiency was expressed as the number of dpm/min/mg protein.

β-galactosidase activity was detected by the hydrolysis of X-Gal (Rols et al., 1992) and was expressed as the percentage of blue stained cells (i.e. the percentage of cells expressing the β-galactosidase activity).

All experiments were repeated at least 3 times at 2-day or 3-day intervals in order to avoid possible fluctuations due to different physiological states of cells, mainly due to the age of the culture. Reproducible relative results were obtained in all cases.

RESULTS

Electropermeabilization

As described in the Methods part, permeabilization was observed through the staining of the pulsed cells by an unpermeant dye (Trypan blue or propidium iodide). Results confirm many previous studies performed with cells either plated or in suspension (Rols and Teissié, 1989).

Briefly, permeabilization, i.e. the percentage of stained cells in the population, is under the control of the field intensity, the pulse duration and the number of pulses. The longer the pulse, the lower the field intensity needed to obtain a given permeabilization. But a threshold field intensity is required to observe the occurence of permeabilization. When the field strength was less than 580 V/cm, it was impossible to detect stained cells even if long pulses (up to 24 ms) were applied to the sample. Permeabilization plots appear to be steep staircase-like curves. All cells were stained as soon as the field strength was larger than the threshold (Fig. 1). At a given electric field strength, increasing the number of

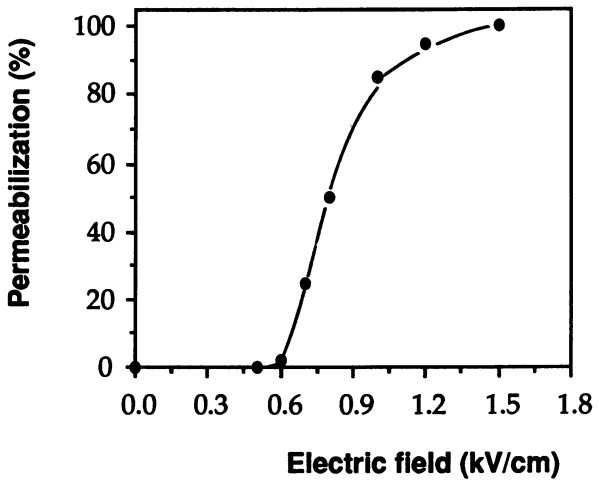

FIGURE 1 Electropermeabilization as a function of field intensities. Cells were pulsed five times at 1-ms duration with fields of increasing strength in the presence of trypan blue (1% w/v). The level of permeabilization (i.e., the percentage of stained cells) is plotted as a function of the electric field strength.

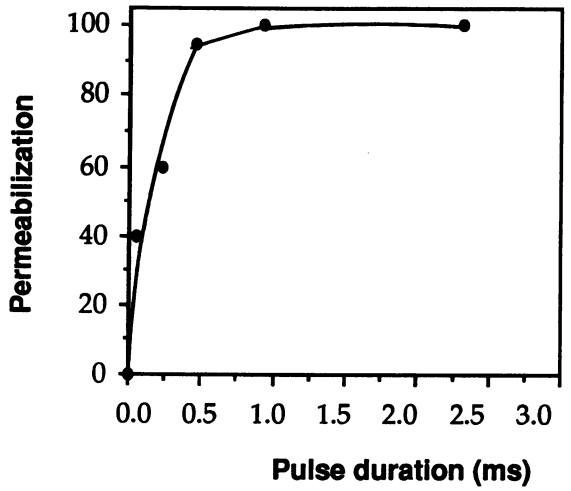

FIGURE 3 Electropermeabilization as a function of the pulse duration. Cells were pulsed at 0.9 kV/cm five times in the presence of propidium iodide in the pulsing buffer. The level of permeabilization (i.e., the quantity of dye accumulated into the cells) is plotted as a function of the duration of pulses.

pulses or their duration induced an increase in the permeabilization efficiency up to a plateau value obtained at relatively low values of the pulse duration and number (i.e. 5 pulses, 1 ms duration) (Figs. 2 and 3).

The electric field induced permeabilization of CHO cells was long lived. This was assayed by adding the dye after the pulses. Trypan Blue was still able to penetrate into the cytoplasm when added several minutes after pulsing. A long period of time was needed to recover membrane impermeability when using the long pulse conditions prone to providing efficient gene transfer. This period was lengthened by increasing both the pulse duration and/or the electric field strength. This confirmed our previous study which showed that electric field parameters control the lifetime of the permeabilized state (Rols and Teissié, 1990b).

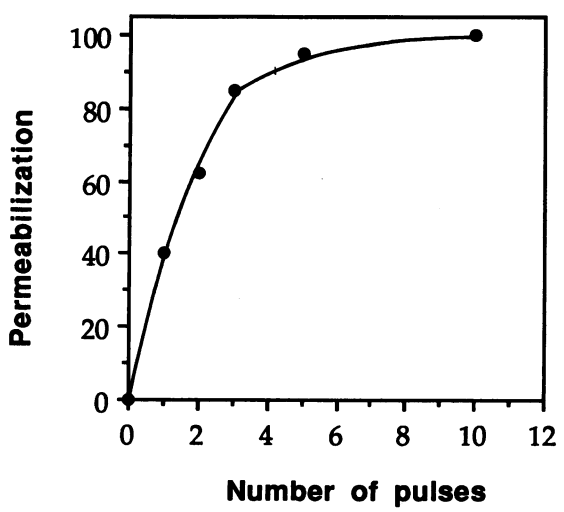

FIGURE 2 Electropermeabilization as a function of the number of pulses. Cells were pulsed at 0.9 kV/cm at 1-ms duration in the presence of propidium iodide in the pulsing buffer. The level of permeabilization (i.e., the quantity of dye accumulated into the cells) is plotted as a function of the number of pulses.

Electrotransformation

Detection of foreign electrotransfered activity was observed only when the plasmid-cell mixture was pulsed under suitable conditions. The level of transient transformation was controlled by the 3 electric parameters: intensity, duration and number of pulses.

Effect of field strength

No gene transfer was mediated when low fields were used. A threshold value of the field strength of 0.58 kV/cm was detected which is the same as the one needed to induce the permeabilization to Trypan blue we first described. The extent of transformation increases sharply when higher field intensities were used. Nevertheless when the field was too strong, the level of foreign activity which was detected per viable cell was low, but the electrical conditions affected strongly the viability of most of the cells. It was indeed observed that whenever cell viability was decreased under 5–10%, this was associated with a decrease in transfection efficiency. Taking into account that the main effect of electropulsing is to make the membrane locally permeabilized when its potential difference is larger than a characteristic threshold (Kinosita et al., 1988), the definition of the permeabilized area Ap has been calculated as being equal to

$$A_p = At\,(1 - Es/E), \quad (1)$$

in which At is the cell surface area, E the electric field intensity, and Es the threshold field above which permeabilization occurs (Schwister and Deuticke, 1985).

As shown on Fig. 4, the level of transformation obtained for increasing field intensities is linearly related to the reciprocal of the field strength, i.e. to the extent of permeabilized cell surface Ap on each cell of the population (Eq. 1) as long as the viability of the cell population is not strongly affected.

Effect of pulse duration

At a given field intensity, the detection of CAT activity depended strongly on pulse duration. When field strength was larger than 0.6 kV/cm, the detected activity was observed to increase steeply at first with pulse duration and then to decrease strongly, which was correlated with a decrease in cell viability. Using the mathematical approach we just described, one can calculate that this biphasic pulse duration effect was detected when more than 30% of cell surface was permeabilized (Es/E smaller than 0.7). The initial increase in transferred activity Tr can be mathematically approximated to the following dependence on the duration T (Fig. 5):

$$\mathrm{Tr} = C(E)T^{2.3} \qquad (2)$$

$C(E)$ describes the effect of the field intensity as reported above.

Effect of number of pulses

Cells can be pulsed several times. Successive pulses were applied at a frequency of 1 Hz. As shown in Fig. 6, the level of detected activity was observed to rise linearly with the number of pulses. This was observed under experimental conditions which did not alter the viability of the pulsed population too greatly.

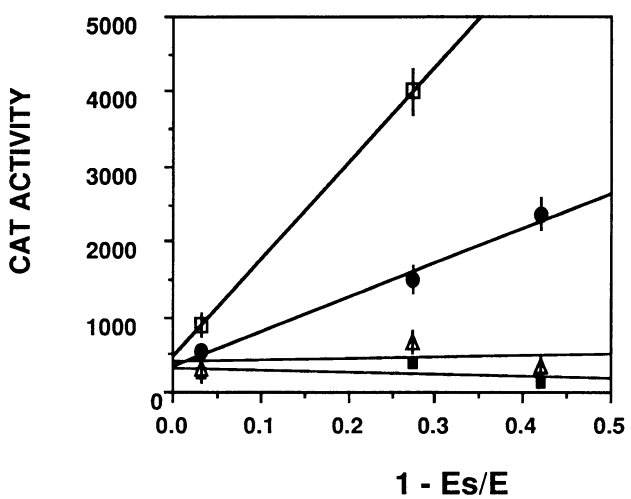

FIGURE 4 Electrotransformation as a function of field intensities. The DNA-cell mixture was pulsed five times with fields of increasing strength. Pulse duration was 0.1 ms (■), 1 ms (△), 2.5 ms (●), and 4 ms (□). The level of activity (counts/mg protein) in the region where the cell viability is not affected is plotted as a function of the fraction of the cell surface whose potential has been brought to a value larger than the permeabilizing threshold.

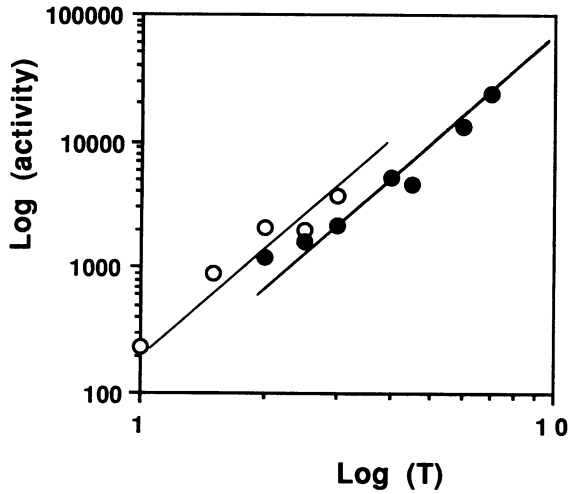

FIGURE 5 Electrotransformation as a function of the pulse duration. The DNA-cell mixture was pulsed five times with different pulse durations. The field intensity was 0.8 (●) or 1.0 (○) kV/cm. The logarithm of the CAT activity is plotted as a function of the logarithm of the pulse duration.

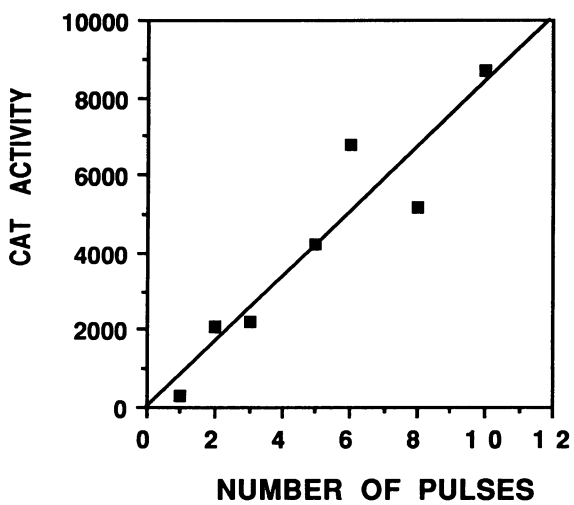

FIGURE 6 Electrotransformation as a function of the number of pulses. The DNA-cell mixture was pulsed with fields of 1.125 kV/cm lasting 1 ms at a frequency of 1 Hz. The level of CAT activity (counts/mg protein) is plotted as a function of applied pulses.

Effect of the period of time between pulses

Cells were pulsed 10 times with a duration of 5 ms under an electric field intensity of 0.8 kV/cm at variable frequency. As shown in Fig. 7, the level of transfection was observed to decrease as the period between pulses diminished.

Comparison with calcium phosphate transformation approach

The chemical method is routinely used in many laboratories (Malissen, 1990) and it was applied as described in (Sambrock et al., 1989). Its efficiency was compared with the electrical ones. 0.9 million cells were mixed with 5.5 μg of PSV2CAT and 10 μg of salmon DNA in a final volume of

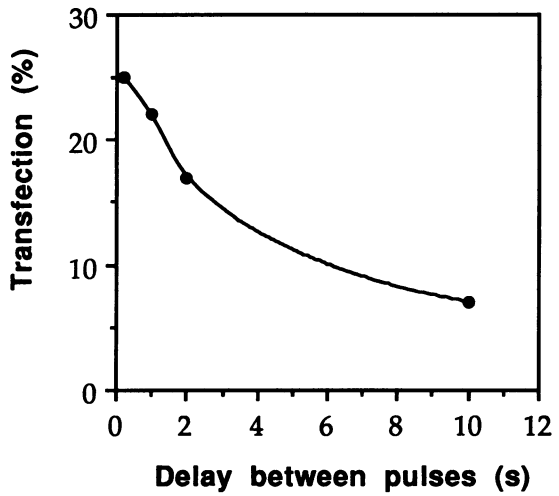

FIGURE 7 Electrotransformation as a function of the period between pulses. The DNA-cell mixture was pulsed ten times at different frequencies with fields of 0.8 kV/cm lasting 5 ms. The level of β-galactosidase activity (the percentage of blue-stained cells) is plotted as a function of the delay between the pulses.

200 μl. In the electrotransfection experiments, 5 pulses lasting 1 ms were applied at a frequency of 1 Hz and at 0.9 kV/cm intensity. A 60-fold increase in gene transfer was obtained with the electric approach with a 40% associated loss of viability.

Control of DNA transfer by the plasmid

The level of transfered activity was related to the concentration of added plasmid as shown in Fig. 8. The amount of added plasmid was changed by diluting the stock DNA with Tris-EDTA buffer. The viability of pulsed cells was affected by the concentration of DNA (Fig. 8). For concentrations ranging from 1 to 13 μg/ml, the viability remained unchanged but decreased for higher concentrations. This suggests a synergic effect of DNA and electric pulses on cell viability. This was confirmed by electropulsing cells in a 1 mg/ml Salmon sperm DNA. All pulsed cells were lysed 24 h after pulsing.

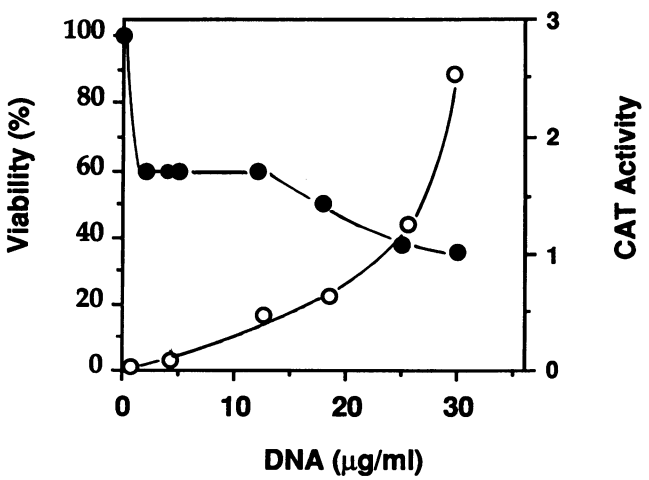

FIGURE 8 Electrotransformation and viability of pulsed CHO cells as a function of the amount of PSV2CAT plasmids. The DNA-cell mixture was pulsed five times with fields of 1.125 kV/cm lasting 1 ms. The level of CAT activity (counts/mg protein) is plotted as a function of the concentration of coding plasmids, no carrier DNA being present.

Different pre- and post-pulse incubation times were checked. Results are in Table 1. The conclusions are that both pre and post incubations improve the level of transformation. But the main fact is that DNA must be present during the pulse. By adding the plasmid only 2 s after pulsing, the shortest period we can operate, we observed that the cell suspension did not undergo any transformantion, in agreement with previous observations (Winterbourne et al., 1988; Klentchin et al., 1991).

DISCUSSION

The purpose of this work was to investigate the mechanism of electrotransformation. Up to now, most experiments dealing with this approach to gene transfer have assumed that the plasmid crossed the membrane due to electrically induced permeability under the effect of electrophoretic forces mediated by the external field (Klenchin et al., 1991; Sukharev et al., 1992). It was implicitly assumed that electropermeabilization and electrotransformation occured through similar processes. The mechanisms of such processes are still to be elucidated although recent models based on experimental data have been proposed (Dimitrov and Sowers, 1990).

Our results indeed indicate that electric field pulses inducing permeabilization are needed to mediate the gene transfer. The intensity of the field must be larger than the characteristic threshold required to permeabilize the cell membrane locally. If one describes electropermeabilization as a local reaction bringing the membrane from the native impermeant state I to a permeabilized one P as observed by videomicrosopy (Kinosita et al., 1988; Hibino et al., 1993)

$$I \underset{E}{\rightarrow} P \qquad (3)$$

in which E is the electric pulse, then electrotransformation can only occur in the part of the cell surface which can be

TABLE 1 Electrotransformation of CHO cells: effect of the timing of the DNA-cell mixing

Conditions	Counts/ mg protein
No pulse	13
Pulse (standard conditions)	1715 (±5)
DNA added 2 S after pulsing	17 (±2)
DNA added 30 S after pulsing	33 (±7)
DNA added 1 min after pulsing	50
DNA added 2 min after pulsing	23
No carrier DNA	2000
Cells were pulsed just after adding DNA	900
The DNA-cell mixture was diluted just after pulsing	740

0.9×10^6 cells were mixed with 5.5 μg of PSV2CAT and 10 μg of salmon sperm DNA in a final volume of 200 μl of PBA. 5 pulses lasting 1 ms were applied at a frequency of 1 Hz with a 0.9 kV/cm intensity.

brought to the P state. This is clearly shown here in the dependence of Tr on field intensity (Fig. 4). No other major direct effect of the field on the gene transfer process can be detected such as a contribution from electrophoretic forces. Tr simply depends on the reciprocal of E and is not a complex function of field intensity.

Other parameters controlling both electropermeabilization and electrotransformation play a role in gene transfer. The pulse duration induces an increase in permeabilization up to a saturating level (see Fig. 3 and Rols and Teissié, 1990b), whereas the transformation increases in a pseudo-exponential way even when permeabilization levels off. No saturation of transformation with the number of successive pulses is observed as opposed to the observation on permeabilization (see Fig. 2 and Rols and Teissié, 1990b). Optimal conditions for permeabilization are different from what is observed for transformation. Cell viability plays a decisive role in transformation; this, of course, is not needed to detect permeabilization. This dependence of transformation per viable cell on the percentage of viable cells in the population strongly suggests that gene transfer and expression of coded activity are not only controlled by the electrical parameters.

Another decisive difference between the two processes of permeabilization and transformation is the time dependence of the transfer on the exogeneous molecule. In the case of small dyes, the permeant state is long lived. This is not the case for transformation. The plasmid must be present during the pulse (Table 1; Winterbourne et al., 1988). This indicates that membrane structural changes which are a key step in gene transfer are either present during the pulse or are very short lived (less than 2 s from our data). In any case, they are different from those supporting the permeability for small molecules which are long lived.This observation has to be correlated with a recent study showing that the increase in membrane conductance induced by the permeabilizing field pulse disappeared very quickly (μs time scale) (Hibino et al., 1993).

The electric pulse affects the DNA molecules by both the field strength and the pulse duration. It is well known that due to the counterion flow polarization and to the associated induced dipole the electric field applies a torque on the elongated DNA molecules and induces their rotation (Porschke et al., 1984; Neumann et al., 1993). This process is very fast (in the microsecond time range). Due to the field, all molecules are oriented with their long axis parallel to the field lines. Furthermore, due to their electric charge, electrophoretic forces move the molecules with an electrophoretic mobility u of 1.5 10–4 cm^2/Vs. As this mobility is dependent on the size and on the electric charge, it is much larger for the plasmid than for the cell. A local interfacial accumulation of DNA is then induced by the pulse. During the pulse, the dispacement L of the plasmid is

$$L = uET = 15\ \mu\text{m} \tag{4}$$

if $E = 1$ kV/cm and $T = 10$ ms, which are conditions routinely used in our electrotransformation experiments. We can then assume that all the plasmid molecules which are in the cylinder coaxial to the cell, its axis parallel to the field lines and with a 15 μm height, are brought into contact with the cell surface during the pulse. This gives a 5 fold increase in the DNA surface concentration (Annexe). This electrophoretic accumulation interpretation of the pulse duration effect is supported by the negative contribution on transformation by an increase in viscosity (Klenchin et al., 1991).This accumulation factor is, under these very simple assumptions, linearly related to N, T, and E. As we did not observe any effect of E associated with such electrophoretic accumulation, we may suggest that under our field conditions, due to the friction forces a limiting value of plasmid velocity is obtained. Such an assumption is in agreement with the viscosity effect we quoted above.

It may be suggested that the transformation process is due to a complex cascade of events where the interfacial DNA is first inserted into the permeabilized cell membrane to form a complex which then crosses the membrane by a still unknown process before releasing the free DNA in the cytosol where the activity it codes for is expressed (transient expression):

$$\begin{array}{l} \mathrm{DNA_{bulk}} \underset{4}{\overset{1}{\rightleftarrows}} \mathrm{DNA_{interface}} \\ \qquad\qquad\qquad\qquad \underset{6}{\overset{3}{\rightleftarrows}} \mathrm{DNA_{inserted}} \rightarrow \text{Translocation} \\ \quad I \underset{5}{\overset{2}{\rightleftarrows}} P \end{array} \tag{5}$$

in which $\mathrm{DNA_{bulk}}$ is the plasmid freely dispersed in the solution, $\mathrm{DNA_{interface}}$ is the interfacial plasmid, $\mathrm{DNA_{inserted}}$ is the plasmid which has partly penetrated the membrane during the pulse, I is the intact cell membrane, and P is the permeabilized cell membrane during the pulse.

Steps 1 and 2 depend on field strength E and pulse duration T and pulse number N as described above. P of course depends on N, T, and E (Rols and Teissié, 1990b). It is the state of the membrane during the pulse (or very shortly after it) because transfer is detected only if the plasmid is present during the pulse (Table 1) (Winterbourne et al., 1988; Sukharev et al., 1992).

Step 3 as well as the translocation can be dependent on the pulse but we have no experimental evidence. Step 3 and translocation are two different events as recently shown in *E. coli* (Eynard et al., 1992) and Cos cells (Sukharev et al., 1992). Step 3 occurs during the pulse in the part of the cell surface which is permeabilized (from Fig. 4).

Steps 4 to 6 are not dependent on the pulse. They may occur after the pulse through brownian motions. It is then clear that any process which prevents step 6 would facilitate the translocation by increasing the lifetime of the inserted form of the plasmid. This is what occurs if the plasmid remains overconcentrated close to the membrane as through the electrophoretic accumulation in the present experiments and would explain why non permeabilizing pulses, which drive nevertheless a plasmid accumulation, following a strong permeabilizing one, increase the transformation yield

(Andreason and Evans, 1989; Sukharev et al., 1992). They maintain an electrophoretic mediated plasmid interfacial accumulation. This could explain why both an increase in the number of pulses (Fig. 3) or a decrease in the time between them (Fig. 7) induces an increase in transfection efficiency by facilitating accumulation of plasmids at the interface. This could also explain why diluting the cell-DNA mixture immediately after electropulsation induces a decrease in transfection efficiency (Table 1). One dramatic consequence of electropermeabilization, cell lysis, is not taken into account in this scheme. It is critically dependent on step 2 where the cell structure is brought from *I* to *P* but is clearly increased by step 3. We recently showed that the percentage of lysed cells was higher when cells were pulsed with increasing amounts of plasmids (Rols et al., 1992), maybe as a consequence of the permeabilization enhancing effect induced by carrier DNA described by others (Sukharev et al., 1992).

The hypothesis, that step 3 is not enough to give the expression, is brought by the observations that i) the translocation step was slow for *E. coli* and occurs after the pulse (Eynard et al., 1992) and ii) that a train of low intensity pulses after the permeabilizing one improved the transformation efficiency in some cases (Andreason and Evans, 1989; Sukharev et al., 1992).

The field strength affects the transformation yield almost only by controlling the part of the cell surface which is prone to being permeabilized as shown in Fig. 4. Its contribution to the plasmid interfacial accumulation (step 1) and to the plasmid insertion in the membrane (step 3) can be considered as a constant in the rather narrow range of intensities prone to effective transformation. This geometrical function is in agreement with the positive contribution of bipolar pulses where a larger part of the cell surface is brought to a "competent" state (Tekle et al., 1991).

Pulse duration plays a decisive role in almost all steps in agreement with the results in Fig. 5. If we make the simplified assumption that steps 1 and 2 are linearly dependent on *T*, then the transformation will be related in a non linear way to the pulse duration. Increasing *T* would accumulate the plasmid at the cell interface (a linear dependence may be assumed), would facilitate its insertion by increasing the *P* form of the membrane organization or maybe through a direct effect on the plasmid. Assuming that plasmid transfer is only due to electrophoretic forces (Sukharev et al., 1992) predicts at the most a linear dependence on *T* if no saturation at the interface is taken into account. This conclusion is the theoretical support to the observation by many groups that efficient transformation is obtained when using long pulses (Chu and Berg, 1987; Kubiniec et al., 1990; Potter, 1989, 1992). It is only in the case of RF pulses (Chang et al., 1990; 1991; Teckle et al., 1991) that trains of uni or bipolar pulses with short duration are shown to be efficient for gene transfer. But it was proposed that RF fields acted on a membrane in a mechanical way by inducing a local sonication which enhanced permeabilization by a mechanical fatigue (Chang, 1989a,b).

APPENDIX

The bulk DNA concentration is 20 μg/ml, i.e., 2×10^{-11} $\mu g/\mu m^3$. If we assume for a pBR plasmid a molecular weight of 2×10^6, this means that the number of plasmid copies per μm^3 is

$$6 \times 10^{23} \times 2.10^{-11}/2 \times 10^{12}, \qquad \text{i.e., } 6$$

10^6 CHO cells with a 13-μm diameter gives an exclusion volume of

$$10^6\ 4/3\ \pi\ (13/2)^3, \qquad \text{i.e., } 1.15\ \mu l$$

if we assume that all DNA copies which are inside the excluded volume are bound on the cell surface, $7\ 10^3$ DNA molecules are bound per cell.

The cylinder whose contents are electrophoretically accumulated on the electropermeabilized cell surface has a volume of

$$15\pi(5)^2, \qquad i.e., 1125\ \mu m^3$$

(the 5 μm radius is computed from the 1 kV/cm applied field and 0.6 kV/cm threshold needed to induce permeabilization and transformation).

Its contents are then

$$1125 \times 6, \qquad \text{i.e., } 6.8 \times 10^3 \text{ DNA copies}$$

which are accumulated on a surface *A*

$$A = At/2\ (1 - 0.6/1) = 0.2\,At,$$

i.e., in which only $7 \times 10^3 \times 0.2$ (i.e., 1.4×10^3) copies were present before the pulse.

The accumulation factor is then

$$6.8/1.4 = 5$$

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