Study of mechanisms of electric field–induced DNA transfection II

Transfection by low-amplitude, low-frequency alternating electric fields

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ABSTRACT Electroporation for DNA transfection generally uses short intense electric pulses (direct current of kilovolts per centimeter, microseconds to milliseconds), or intense dc shifted radio-frequency oscillating fields. These methods, while remarkably effective, often cause death of certain cell populations. Previously it was shown that a completely reversible, high ionic permeation state of membranes could be induced by a low-frequency alternating electric field (ac) with a strength one-tenth, or less, of the critical breakdown voltage of the cell membrane (Teissie, J., and T. Y. Tsong. 1981. *J. Physiol. (Paris).* 77:1043–1053). We report the transfection of *E. coli* (JM105) by plasmid PUC[18] DNA, which carries an ampicillin-resistance gene, using low-amplitude, low-frequency ac fields. *E. coli* transformants confer the ampicillin resistance and the efficiency of the transfection can be conveniently assayed by counting colonies in a selection medium containing ampicillin. For the range of ac fields employed (peak-to-peak amplitude 50–200 V/cm, frequency 0.1 Hz–1 MHz, duration 1–100 s), 100% of the *E. coli* survived the electric field treatment. Transfection efficiencies varied with field strength and frequency, and as high as $1 \times 10^5/\mu g$ DNA was obtained with a 200 V/cm square wave, 1 Hz ac field, 30 s exposure time, when the DNA/cell ratio was 50–75. Control samples gave a background transfection of much less than $10/\mu g$ DNA. With a square wave ac field, the transfection efficiency showed a frequency window: the optimal frequency was 1 Hz with a 200 V/cm field, and was ~0.1 Hz with a 50 V/cm field. Transfection efficiency varied with the waveform: square wave > sine wave > triangle wave. If the DNA was added after the ac field was turned off, transfection efficiency was reduced to the background level within 1 min. The field intensity used in this study was low and insufficient to cause electric breakdown of cell membranes. Thus, DNA transfection was not caused by electroporation of the cell membranes. Other possible mechanisms will be considered.

INTRODUCTION

Cells in suspensions, when exposed to short (microseconds to milliseconds) and intense (kilovolts per centimeter) electric pulses, tend to lose cytoplasmic ions and molecules (1, 2). This phenomenon has been shown to be the result of implantation of limited numbers of aqueous pores of diameters ranging from a few nanometers to 100 nm in the cell membranes (3–11). Wong and Neumann (12) and Neumann et al. (13), in 1982, reported a successful introduction of the thymidine kinase (tk) gene into the tk-deficient cultured mouse L-cells and demonstrated the expression of the tk-gene in the transfected cells. Electroporation has now been applied to the transfection of mammalian cells (14–16), plant cells (17, 18), yeast spheroplasts (19), and different strains of bacteria (20–26). In these experiments, electric pulses used were either square dc pulses, exponentially decaying pulses, or dc shifted oscillating electric fields (27). High-intensity electric pulses are effective, and the efficiency for electrotransfection has been shown to reach as high as 80% of the surviving cells in one case (28). However, in most experiments, electric field–treated cells would take a long time to recover from the electric shock. Cells treated with a chemical transfection agent, such as $CaCl_2$, generally recover in a short period. In addition, certain populations of cells are often irreversibly destroyed in an electrotransfection experiment. Attempts to improve the electrotransfection method would require careful studies of mechanisms of the electric field interaction with cell membranes and of physical events leading to the passage of DNA through a cell membrane (26, 29). Previously, Teissie and Tsong (30) and Serpersu and Tsong (31) have shown that reversible activation of certain membrane channels or transport systems may be achieved using low-amplitude, low-frequency ac fields. Electric fields as small as 16 V/cm have been shown to induce membrane conductance, and these effects were completely reversible. Human erythrocytes treated with such ac fields for hours were shown to have normal shape, volume, and permeabilities to K^+ and Na^+ (30, 31). The present study was designed to test whether such low amplitude ac fields

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might also be able to cause DNA transfection of cells, using plasmid PUC[18] DNA and *E. coli* JM105 as model systems.

MATERIALS AND METHODS

Instruments

A model 148A waveform generator (Wavetek, San Diego, CA) was used. This instrument can generate an ac signal with various waveforms (symmetrical square, sine, and triangle waves) between 0.01 Hz and 20 MHz and of strength up to 30 V (peak-to-peak). The chamber for the cell suspension was made of two hollow cylindrical stainless steel blocks, which were tightened into a Plexiglass enclosure as previously described (32). Two platinum sheets in tight contact with the stainless steel blocks served as the electrodes. The distance between the two electrodes was 0.15 cm and the volume of the cell suspension was 50 μl. The temperature of the sample was maintained at 4°C by circulating water through the hollow steel blocks. During or after the electric treatment, the temperature of the sample was carefully monitored by a microthermister probe, which was placed directly inside the sample chamber. Suspension media of low ion concentrations (2–4 mM) were used in these experiments, and temperature rises due to the applied ac fields were <1°C in all cases. The voltage drop between the electrodes was measured directly and displayed on a model 5103 oscilloscope (Tektronix Inc., Beaverton, OR).

Cell and plasmid DNA

E. coli JM105 was grown in Luria Bertani (LB) medium which contained 10 g Bacto-tryptone, 5 g Bacto-yeast extract, and 5 g NaCl per liter, in a shaker at 37°C. The culture grown overnight to the stationary phase was diluted 100× into LB medium and grown, again, to mid–log phase (specific optical density OD_{600nm} of ~0.8). Cells were harvested at this phase by centrifuging at 4,000 *g* for 10 min at 4°C, and they were washed twice with the electroporation medium, which contained 1 mM $MgCl_2$, 30 mM sucrose, and 1 mM Tris-HCl buffer at pH 7.4 (or to different pH values in specified experiments). Cells were resuspended to 1–1.5 × 10^{10}/ml in the electroporation medium and were kept on ice before use.

Plasmid PUC[18] DNA containing the ampicillin-resistant gene was isolated from *E. coli* HB101 by the alkaline lysate procedure, purified with a Sephacryl S-1000 column followed by CsCl/ethidium bromide centrifugation, and diluted to 500 μg/ml in 10 mM Tris-HCl buffer pH 7.5 containing 1 mM EDTA.

Electrotransfection

8 μl of DNA stock solution was mixed with 1 ml of cell suspension (DNA concentration 4 μg/ml, cell concentration 1–1.5 × 10^{10}/ml) and vortexed. The mixture was incubated on ice for 5 min. 50 μl per sample were transferred to the electroporation chamber and treated with an ac field of specified waveform, amplitude (25–200 V/cm), and frequency (0.1 Hz to 1 MHz) for a specified time (1–100 s). 30 s later, the sample was transferred to a 1.5-ml test tube, which contained 500 μl of LB medium, and incubated for 30 min at 37°C. Appropriate aliquots (usually 100 μl each) were subsequently plated on a solid LB medium (containing 15 g agar per liter) with or without 50 mg ampicillin and 25 mg streptomycin per liter. After overnight incubation at 37°C, the number of transformants was counted from plates containing antibiotics, and percent cell survival was obtained from plates containing no antibiotics. Controls were also done with cells, which were not treated with electric fields.

RESULTS

Frequency window for transfection and effects of waveforms

Low amplitude ac fields were found to stimulate the transfection of *E. coli* by plasmid PUC[18] DNA. For example, with a square waveform, 200 V/cm ac field of 1 Hz and an exposure time of 30 s, a transfection efficiency of 4.4 × 10^4 to 5.1 × 10^5/μg DNA was obtained (Figs. 1 and 3). This was four to five orders of magnitude higher than that of controls. In control samples, transfection colony counts were fewer than 10/μg DNA. When the frequency of the ac field was varied, a window for the transfection was observed. In this case, the optimal frequency was found to be 1 Hz. Frequencies exceeding 1 kHz gave diminishing efficiencies, and no transfection was detected using a 1 MHz field. Lowering the frequency to 0.1 Hz also reduced the efficiency of transfection by more than one order of magnitude.

In Fig. 1 transfection efficiency with ac fields of different waveforms are also compared. Transfection efficiency was reduced by one order of magnitude with a sine wave ac field. A triangle wave ac field was even less

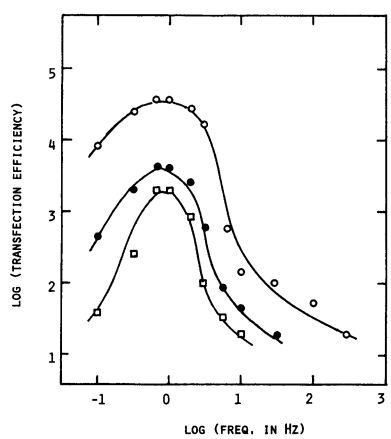

FIGURE 1 Frequency window for DNA transfection by low-amplitude, low-frequency ac electric fields. Experimental details are described in Materials and Methods. The peak-to-peak amplitude of ac fields was 200 V/cm and exposure time for each sample was 30 s. Control samples not treated with ac fields or treated with a dc field of the same strength for 30 s showed transfection colony counts ≪10. Logarithm of the transfection efficiency (expressed in colonies per microgram DNA) is plotted against the frequency of the applied fields for square waveform (○), sine waveform (●) and triangle waveform (□). The initial temperature of electroporation was 4°C, and the temperature rise due to the applied field was <2°C.

effective for electrotransfection. The optimal frequencies in all three cases appeared to be identical at 1 Hz.

Survival of cells treated with low-amplitude ac fields

In a high-intensity electroporation experiment, cells are usually exposed to an electric field for only microseconds to milliseconds. In our low-intensity ac electroporation experiments, cells were exposed to an ac field of 1 to 100 s. To evaluate whether long exposure of *E. coli* to a low-intensity ac field might cause cell death we chose two frequencies, 1 and 5 Hz, to perform experiments. The results are shown in Fig. 2. DNA-cell mixtures were exposed to square wave ac fields of varied amplitudes for a fixed period of time, 30 s. Transfection efficiencies and percent cell survival were plotted vs. electric field strength. The efficiency continued to increase with field strength. In all cases, cell survival rates were indistinguishable from the untreated control samples, i.e., 100%. The ac treated cells grew at the same rate as the control cells with no apparent changes in shape or morphology.

Dependence on field strength and exposure time

In Fig. 3 transfection efficiencies were plotted vs. field strength of square wave ac fields in the ranges 50 to 200 V/cm and 0.1 to 100 Hz. The exposure time was 30 s in all experiments. The logarithm of the transfection efficiencies were nearly linearly dependent on the applied field strength. For each field strength used, there appeared to be a frequency window for transfection. The optimal frequency decreased slightly from 1 Hz as the field strength was reduced. At the field strength of 50 V/cm, the optimal frequency for transfection was ~0.1 Hz, although its value could not be determined with precision because of the low efficiency of transfection.

In Fig. 4, transfection efficiency was plotted vs. exposure time for square wave ac fields of 200 and 150 V/cm, at 1 Hz. With the 200 V/cm field, the transfection

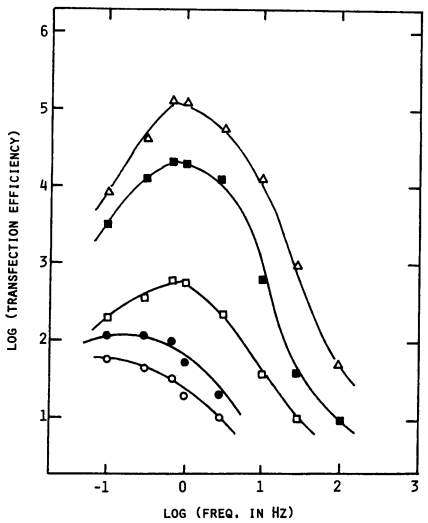

FIGURE 3 Depedence of transfection efficiency on the frequency and the strength of the applied ac field. Square waveform ac fields of 50 to 200 V/cm, 0.1 to 100 Hz was used. The exposure time was 30 s in all cases. Data for 50 (○), 75 (●), 100 (□), 150 (■), and 200 V/cm (△) are shown. Other conditions were the same as in Fig. 1.

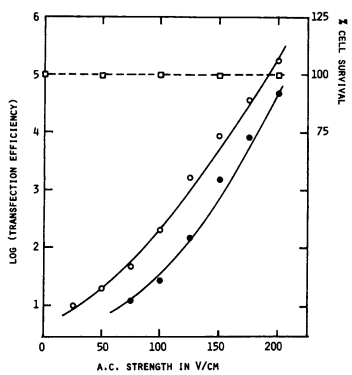

FIGURE 2 Transfection efficiency and percent cell survival as functions of field strength. A square waveform ac field of strength up to 200 V/cm was used in the experiment. Two frequencies were used. The 1 Hz (*data in* ○) was the optimal frequency, and the 5 Hz (*data in* ●) was slightly off the optimal frequency (see Fig. 1). Percent cell survival (□) in all cases were near 100%. The initial temperature of electroporation was 4°C and temperature rise due to the applied field was <2°C.

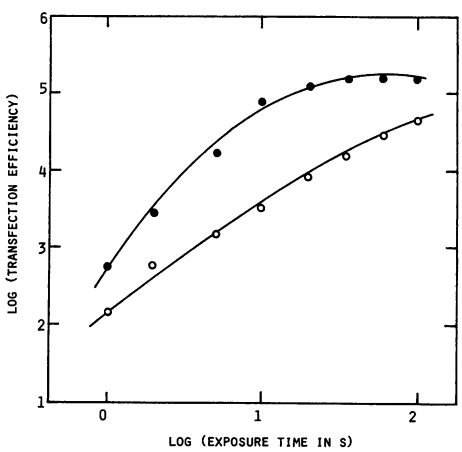

FIGURE 4 Dependence of transfection efficiency on the exposure time to the applied ac fields. Two field strengths, 150 (○) and 200 (●) were used in the experiment. Other conditions are the same as in Fig. 1.

efficiency leveled off after 30 s, although with the lower strength field of 150 V/cm, the efficiency continued to increase after 100 s exposure time.

Time sequence of DNA addition

DNA was added before or after the ac treatment of *E. coli*. The result shown in Fig. 5 indicates that transfection efficiency reached its maximal level if DNA was added at least 30 s before the ac field was turned on (200 V/cm square wave, duration 30 s). If on the other hand, DNA was added 1 min after the ac field was turned off, transfection efficiency dropped to the control level.

Other properties

A long exposure of a cell suspension to a low-frequency ac field might change the pH of the suspension because of the electrolytic reactions. The DNA transfection observed could arise from changes in solution pH. To show that this was not the case, the electrotransfection experiment was performed at three pH values, 7.4, 4.1, and 9.3. As shown in Fig. 6, the transfection efficiency was highest at pH 7.4. Acidification to pH 4.1 or alkalinization to pH 9.3 of cell suspension reduced the efficiency of the transfection.

Another control experiment was to ensure that the high values of transfections for the ac field–treated samples compared to control samples were not due to Joule heating of the samples. DNA transfection experiments were performed at several temperatures ranging from 4 to 37°C. Fig. 7 shows that increasing the temperature of the cell suspension reduced rather than increased the efficiency of the transfection. In other words, the transfection was electrically induced rather than thermally induced.

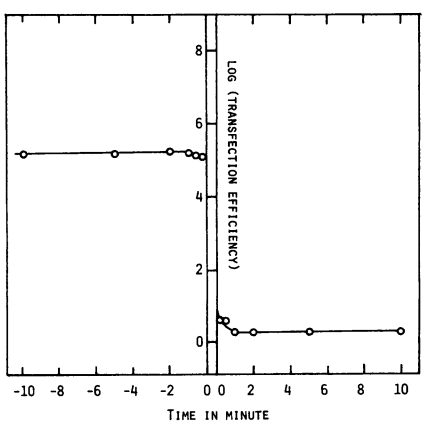

FIGURE 5 Effects of time sequence of DNA addition on transfection efficiency. A 200 V/cm, square wave ac field of 30 s duration was used in the experiment. Other conditions were the same as in Fig. 1. A negative time means that DNA was added before the ac field was turned on. A positive time means that DNA was added after the ac field was turned off. The transfection dropped to the control level if DNA was added 1 min after the ac field was turned off. The center column in time indicates the ac exposure time.

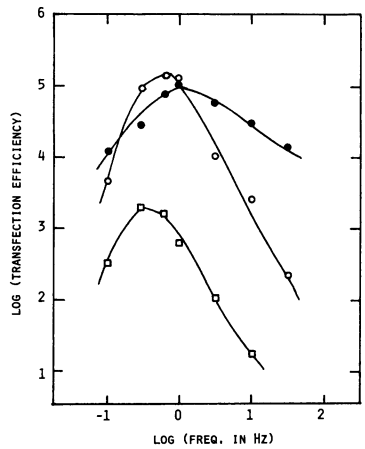

FIGURE 6 Effects of pH of the suspending medium on transfection efficiency. A square waveform ac field of 200 V/cm, 1 Hz was used. The exposure time was 30 s. Data for three pH values, 7.4 (○), 4.1 (●), and 9.3 (□) are shown. Other conditions are the same as in Fig. 1.

DISCUSSIONS

Comparison of high intensity dc and low intensity ac transfection

For a nearly identical system, the transfection of *E. coli* (JM105) by plasmid pBR322 DNA, a single 8 kV/cm – 1 ms square wave electric pulse resulted in the transfec-

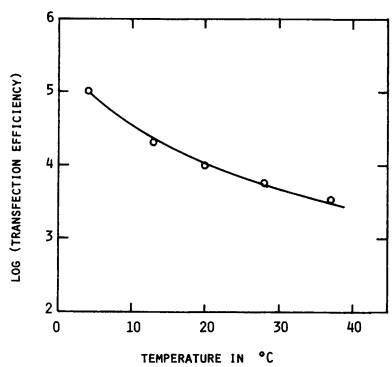

FIGURE 7 Dependence of transfection efficiency on the temperature of cell suspensions. A square waveform ac field of 200 V/cm, 1 Hz was used in the experiment. Logarithm of transfection efficiency was plotted against the final temperature of the sample. Other conditions are the same as in Fig. 1.

tion of $2 \times 10^8/\mu g$ DNA (26). The efficiency is, thus, much higher using the high-intensity dc pulse than that using the low-intensity ac field. The disadvantage of the high-intensity electrotransfection is that 25–50% of cells are killed. This could pose a serious problem because the electric pulses may selectively kill a population of cells of particular interest. The low-amplitude, low-frequency ac stimulation apparently did not have such a problem. However, the significance of the present results goes beyond the 100% survival rate of cells. The fact that a field strength as low as 50 V/cm could facilitate DNA transfection at all indicates that in the ac experiments DNA could not enter by simple surface diffusion through membrane electropores, as was previously proposed (26). Implantation of membrane pores by a dc electric pulse requires a field intensity of several kilovolts per centimeter. Such a field can generate a maximal transmembrane potential, $\Delta\psi_m$, of 0.4 to 1.2 V (33). When an oscillating electric field is used, the maximal transmembrane potential generated for a spherical cell can be calculated by the Schwan Equation (see 34, Marszalek, P., D. S. Liu, and T. Y. Tsong, submitted for publication),

$$\Delta\psi_m = 1.5RE/[1 + (\omega\tau)^2]^{1/2}, \quad (1)$$

where

$$\tau = RC_m(r_i + r_e/2). \quad (2)$$

In the equations, R is the radius of the cell, E, the applied field strength, ω, the angular velocity of the ac field, τ, the dielectric time constant of the cell membrane, C_m, the membrane capacitance, r_i, the resistivity of the cytoplasmic fluid, and r_e, the resistivity of the external medium. For a cell the size of *E. coli,* under the low-frequency conditions used, $(\omega\tau) \ll 1$ (Marszalek, P., D. S. Liu, and T. Y. Tsong, submitted for publication), the equation is simplified to

$$\Delta\psi_m = 1.5\,RE. \quad (3)$$

Eq. 2 is not strictly applicable to *E. coli* because the shape of an *E. coli* is rodlike ($\sim 2\ \mu m \times 0.7\ \mu m$) rather than spherical. For an upper bound first approximation, we can use Eq. 3 to estimate $\Delta\psi_m$ using R of 1 μm (4). When this is done, a 200 V/cm field gives a $\Delta\psi_m$ of 0.030 V and a 50 V/cm field of 0.008 V. This magnitude of transmembrane potential is nearly two orders of magnitude lower than the critical potential (0.4–1.2 V) for inducing electroporation (33). In the high-intensity dc experiment, the $\Delta\psi_m$ was 1.2 V, which is at least 40 times higher than that of the present study. However, as mentioned, electric fields that generate transmembrane potential in the range of millivolts are capable of activating membrane transport systems (30, 31, 34).

Comparison of results and some properties of the electroconformational coupling mechanism

As mentioned, a low level, spontaneous transfection was observed without an ac stimulation ($<10/\mu g$ DNA). Thus, some uptake mechanisms must exist for the plasmid DNA in the *E. coli* membrane. An ac field could simply amplify such mechanisms by either its electrophoretic forces (35), or the electroosmosis effect (36). Although, the applied field intensity was low, a DNA is a polyionic molecule, and an electrophoretic or electroosmotic force may be sufficiently large to facilitate its entry and increase the probability of the DNA transfection. A third possibility is the electroactivation of a membrane transport mechanism similar to that we proposed previously to interpret the electric activation of Na, K-ATPase (34, 37). In this mechanism, a membrane structure (a DNA binding site, a pore protein, or a lipid domain) is postulated to be electrically active because of its charges and electric dipoles. Let us consider a simple transport system.

$$\begin{array}{ccc} & DT \rightleftharpoons T'D & \\ D_{out} \nearrow & \uparrow\downarrow \quad \uparrow\downarrow & \nwarrow D_{in} \\ & T \rightleftharpoons T' & \end{array} \quad (4)$$

For convenience, we assume that the molar electric moment of the two conformational states of the transporter, T and T', differs by ΔM, with T being more stable at a low field and T' more stable at a high field. Several conditions must be met if Scheme 4 is to respond to an oscillating field to transport DNA from outside (D_{out}) into the cytoplasm (D_{in}). First, the binding site of T for DNA must be facing out and that of T' facing in. Second, the affinity of T for D_{out} must be greater than the affinity of T' for D_{in}. Third, the kinetic characteristics of the transport system must be able to synchronize with the driving force, i.e., the ac field (34, 37). When these conditions are met, an ac field will enforce the system to turn clockwise and, consequently, facilitate the transport of DNA across the cell membrane.

The model predicts that there would be a frequency window and an amplitude window for the transfection (34, 37). The frequency window was observed, but not the amplitude window. The failure to detect an amplitude window could mean that it exists at a field strength >200 V/cm. There are some technical difficulties in using a higher ac field because of Joule heating and electrolytic reactions due to long exposure time.

The transfection results using different waveforms (Fig. 1) are also consistent with the properties of the electroconformational coupling model. More than a 10-fold increase in transfection efficiency when ac was

changed from a sine wave to a square wave cannot be explained by the difference in the power input of the two waveforms. According to a recent analysis of the electroconformational model by Markin, V. S., T. Y. Tsong, B. Robertson, and R. D. Astumian (38) the conditions for an effective energy transfer to occur in Scheme 4 is

$$r_{conf} \gg r_{elec} \gg r_{bind}, \quad (5)$$

where the three rate constants are, respectively, for conformational transition of the transporter, for the rise of the electric field, and for the binding of DNA to the transporter. r_{elec} is much greater for a square wave than for a sine wave and for a triangle wave, and the efficiency for transfection is accordingly much higher by using the square wave than that using the other two waves.

The result of Fig. 6 gives further support to the electroconformational coupling mechanism for the low intensity ac transfection of cells. In the high-intensity dc electrotransfection experiments (26) DNA, which was added 10 min after the electric field was turned off, was still able to transfect. This is because those aqueous pores induced by the large amplitude dc fields were not fully reversible, and it took ~1 h for the cells to repair the electroperforated membranes. However, data in Fig. 6 show that within 1 min after the field was turned off, the effects of the ac field on cell membranes disappeared, i.e., the transfection rate was reduced to the background level. This could mean that it would take <1 min for a membrane transport system shown in Scheme 4 to relax to its zero field equilibrium when the ac field was turned off. Thereafter, DNA transfection was reduced to the background level. We caution, however, that whereas the electroconformational coupling model may explain many features of the experimental results, it must be considered a tentative model. We should also point out that *E. coli* has a peptidoglycan/lipopolysaccharide cell wall that precedes the plasma membrane. How such a cell wall may influence the entry of DNA is still not clear.

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