Electroporation and electrophoretic DNA transfer into cells

The effect of DNA interaction with electropores

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ABSTRACT It has been shown recently that electrically induced DNA transfer into cells is a fast vectorial process with the same direction as DNA electrophoresis in an external electric field (Klenchin, V. A., S. I. Sukharev, S. M. Serov, L. V. Chernomordik, and Y. A. Chizmadzhev. 1991. *Biophys. J.* 60:804–811). Here we describe the effect of DNA interaction with membrane electropores and provide additional evidences for the key role of DNA electrophoresis in cell electrotransfection. The assay of electrically induced uptake of fluorescent dextrans (FDs) by cells shows that the presence of DNA in the medium during electroporation leads to a sharp increase in membrane permeability to FDs of $M_r < 20,000$. The permeability increases with DNA concentration and the effect is seen even if FD is added to the cell suspension a few minutes after pulse application. The longer the DNA fragment, the greater the increase in permeability. The use of a two-pulse technique allows us to separate two effects provided by a pulsed electric field: membrane electroporation and DNA electrophoresis. The first pulse (6 kV/cm, 10 μs) creates pores efficiently, whereas transfection efficiency (TE) is low. The second pulse of much lower amplitude, but substantially longer (0.2 kV/cm, 10 ms), does not cause poration and transfection by itself but enhances TE by about one order of magnitude. In two-pulse experiments, TE rises monotonously with the increase of the second pulse duration. By varying the delay duration between the two pulses, we estimate the lifetime of electropores (which are DNA-permeable in conditions of low electric field) as tens of seconds. The data suggest that the mechanism of cell electrotransfection is underlain by electrophoretic movement of DNA through membrane pores, the size of which is determined by interaction with DNA in an electric field.

INTRODUCTION

Electrotransfection of cells, i.e., transfection induced by electric field pulses, has become more and more popular as an effective technique for introduction of foreign DNA into cells of any origin (1). Various kinds of mammalian cells (2), insect (3), protozoan (4) and plant cells (5), intact bacteria (6), and yeasts (7) have been successfully transformed by means of this technique. The underlying mechanism of DNA electrotranslocation through the cell plasma membrane is not as yet understood, so experimental parameters are varied empirically. Understanding the mechanism is important for improving efficiency in experiments, such as expression cloning in mammalian cells (8), cell transformation in vivo for gene therapy (9), and obtaining transgenic cereals (10).

The first hypothesis for DNA electrotranslocation into cells was proposed immediately after the technique was invented by Neumann and co-workers (11). They assumed that DNA penetrates through membrane holes (pores) created by a high electric field. Later, this hypothesis was extended with additional stages, such as DNA binding to the membrane and subsequent lateral diffusion through the pore (12). It was also proposed that DNA can be transferred into cells with the flow of water during colloid-osmotic cell swelling upon electropermeabilization (13) or with electroosmotic water flux that takes place near a charged cell surface in a high electric field (14). The electrically induced DNA uptake by liposomes was shown to be mediated by a formation of membrane invaginations "swallowing" the DNA (15) that allowed the authors to assume the same process in cells. The possibility of electrophoretic transfer of DNA as a polyanion was also considered (16, 17), but only indirect evidence was provided for this hypothesis.

In the previous paper (18), we showed that electrically induced DNA transfer into cells is a fast vectorial process with the same direction as DNA electrophoresis in an external field. The results were inconsistent with the hypothesis of free DNA diffusion through long-lived electropores and contradicted osmotic and electroosmotic theories of DNA translocation. We suggested that DNA is transferred into cells directly during pulse application. However, it was unclear whether electrophoresis is a motive force of transmembrane DNA movement or if it only increases DNA concentration near the membrane, whereas translocation is accomplished by some other mechanism.

In the present work, we examine electrically induced permeability of cell membranes in the presence of DNA and efficiency of cell transfection by sequences of two unequal pulses. Using these approaches, we attempted to answer two questions: which force pushes DNA through the membrane and what kind of rearrangement in the structure of the plasma membrane mediates DNA translocation.

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MATERIALS AND METHODS

Cells and DNA

Experiments were carried out on simian Cos-1 cells (19), which were transfected with the plasmid pCH110 (Pharmacia-LKB, Uppsala, Swe-

0006-3495/92/11/1320/08 $2.00

den) that contained a bacterial β-galactosidase gene (*lacZ*) under the control of the SV40 ori and early promoter, providing for intracellular plasmid amplification and therefore sensitive detection of cell transformation. For permeation experiments, we also used calf thymus DNA and phage λ-DNA (both from Sigma Chemical Co., St. Louis, MO). The plasmid was isolated from *Escherichia coli* as described (20). The commercial DNA preparations were additionally purified with phenol-chloroform extraction and subsequent precipitation in ethanol. The linear form of pCH110 was obtained by restriction with EcoR1; λ-DNA was linearized by heating to 68°C followed by cooling on ice. Calf thymus DNA was fragmented by passage 20 times through a narrow syringe needle. The resulting fragments had a broad size distribution (from 5 to 100 kilobases [kb]), with a mean of 20 kb, as determined by agarose electrophoresis.

Electrotransfection

Cell transfection was performed using a single or dual pulse mode. Single-pulse experiments were carried out essentially as described previously (18). To apply a sequence of two unequal pulses to the chamber, we combined a setup of two high-voltage square-pulse generators G1 and G2 (both are laboratory-built), triggered by a time delay generator G3 (G5-54, USSR). G1 and G2 were connected to the chamber via high-voltage diodes to prevent their mutual interaction. If not mentioned otherwise, the parameters of two-pulse experiments were as follows (see Results): first pulse, 6 kV/cm, 10 μs and second pulse, 0.2 kV/cm, 10 ms. The delay between pulses was 100 μs except for two experiments, in which the delay was varied from 100 μs to 100 s. The chamber consisted of a U-shaped teflon spacer 2 mm thick and of two parallel electrodes of stainless steel with a working area of ~1 cm^2. The chamber was fixed by two contact screws in a special insulating holder. The chamber current during the pulse was measured as a voltage drop across the load resistor R_1 = 0.5 Ω and was recorded with a storage oscilloscope (S8-13, USSR). Since the shape of the second pulse deviated slightly from rectangular, the electric charge passed through the chamber in some cases was evaluated according to real pulse shape by numerical integration of the current over the pulse length. Estimated temperature rise in the chamber, ΔT, due to the Joule effect never exceeded 6°K.

In the majority of experiments, the cells were treated with electric pulses in phosphate-buffered saline (PBS) (Ca^{2+}- and Mg^{2+}-free), consisting of (mM) 140 NaCl, 5 NaH_2PO_4, 5 K_2HPO_4, pH 7.4. In the two-pulse experiments, we used low-conductance medium, consisting of (mM) 280 sucrose, 10 NaCl, 10 *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid, pH 7.4. Cells were washed with appropriate medium twice and resuspended in the same medium at a final concentration of 5×10^6 cells/ml. Aliquots of cell suspension (0.1 ml) were mixed with aliquots (1–3 μg) of plasmid, incubated 10 min at room temperature, and subjected to electric pulses. After 10-min incubation, cells were resuspended in 5 ml of Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (both from Gibco, Grand Island, NY), and seeded on Petri dishes of 60 mm diameter.

Transfection efficiency assay

Transfection efficiency (TE) was determined as a specific activity of expressed β-galactosidase per viable cell 48 h after transfection. Enzyme activity was assayed by standard colorimetric method with *O*-nitrophenyl-β-D-galactoside as a substrate (21). Transfection efficiency was expressed as $TE = 10^6 \times (OD_{420} - OD_{550})/(N \times T)$, where *OD*s are optical densities at corresponding wavelengths, *N* is the number of cells at the moment of fixation, and *T* is the time of incubation in hours (21). The data presented are the means of two independent experiments with two parallel assays each.

Assay of membrane electropermeabilization

The increase of membrane permeability as a result of electric treatment in some experiments was determined by the cellular uptake of low molecular weight fluorescent dye Lucifer Yellow (M_r = 457). This dye was added to the cell suspension at a final concentration of 1 mM before electric treatment. Fifteen minutes after pulse delivery, cells were washed with PBS twice, and the percentage of stained cells was determined with fluorescent microscope. To study membrane permeability for larger molecules, fluorescein isothiocyanate (FITC)-labeled dextrans (FDs) with molecular weights of 4,000, 20,000, 40,000, and 70,000 (FD4–FD70, all from Sigma Chemical Co.) were used. Before experiments, all the FDs were dissolved in PBS and purified from free FITC using spin-column chromatography on Sephadex G-15 (Pharmacia). The resultant stock solutions obtained were of 2 mM concentration for FD4, FD20, and FD40 and 1 mM for FD70. Aliquots of cell suspension (100 μl, 1–2 × 10^6 cells) were mixed with FD (to 0.5 mM), and in some cases with DNA, placed in the chamber, and subjected to electric pulse(s). Ten minutes later cells were resuspended gently in 0.7 ml DMEM with 15% fetal calf serum and incubated at 37°C for 30 min, allowing membranes to reseal. The FD not entering the cells was washed out carefully two times with DMEM and three times with PBS. Washing consisted of pelleting cells in Eppendorf tubes (600 *g*, 4 min) and gentle resuspension in 1 ml of fresh medium. Relative amounts of FD trapped in the cells were measured by a spectrofluorometer (model JY3D; Jobin-Yvon, Longjumeau, France) at 488 nm excitation and 520 nm emission wavelengths, respectively. In some experiments, FD was introduced into cell suspension 1, 10, and 100 s after the electric treatment. The level of fluorescence of cells subjected to all procedures except for electric treatment (usually 15–20% of total intensity) was subtracted.

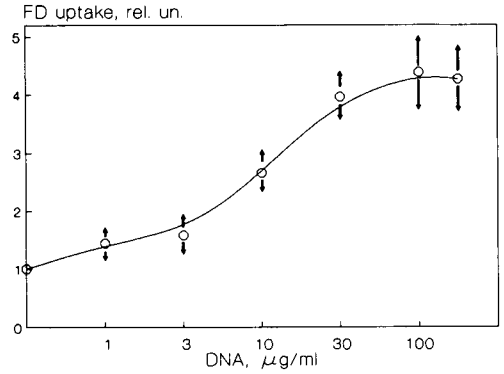

FIGURE 1 The dependence of FD4 uptake by cells on the concentration of calf thymus DNA in the pulsation medium. FD4 and DNA were added to cell suspension a few minutes before application of a single pulse of 1.5 kV/cm, 1 ms.

RESULTS

Effect of DNA on electrically induced uptake of FITC-dextrans by cells

The presence of DNA in the cell suspension during electric pulse treatment markedly increases the membrane permeability to molecules as large as dextrans. The dependence of FD4 uptake by cells on thymus DNA concentration in the pulsation medium, obtained at constant pulse parameters (1.5 kV/cm, 1 ms), is shown in Fig. 1. A reliable increase in cell fluorescence intensity is observed beginning at 1 μg/ml DNA. The midpoint of the sigmoid curve is at ~10 μg/ml, and saturation takes place at ~100 μg/ml DNA. In the preliminary experiments, we found that further increases in pulse ampli-

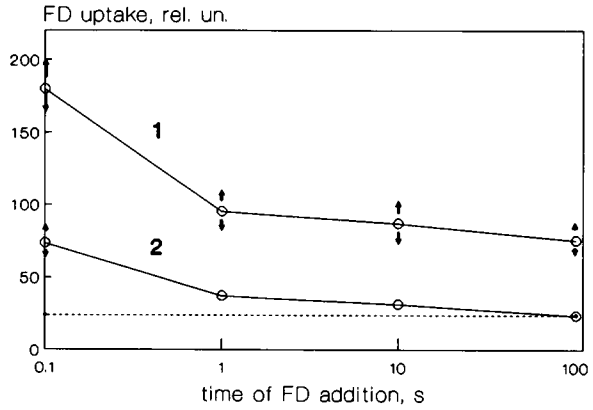

FIGURE 2 Cellular uptake of FD20 as a function of time of the dextran introduction after pulse application. Cells were subjected to a single pulse of 1.5 kV/cm, 1 ms in the presence of 0.1 mg/ml of calf thymus DNA (*curve 1*) and without DNA (*curve 2*). The values at $t = 0$ were obtained with FD20 added to the cell suspension before pulse application. The level of fluorescence of cells in the control (no pulse applied) is shown by the dashed line.

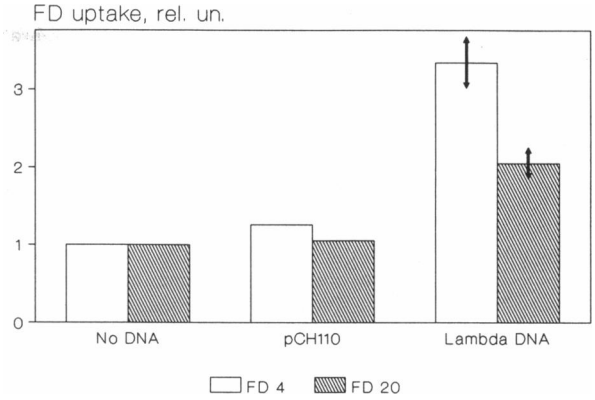

FIGURE 4 The effect of DNA size on electrically induced cellular uptake of FD4 (□) and FD20 (▨). The plasmid pCH110 (7.2 kb) or phage λ-DNA (48 kb) were added to the cell suspension in equimolar concentration: 1 and 7 μg/ml, respectively. The amounts of FD4 and FD20 entrapped by cells in the control (no DNA) are taken as unity.

tude and/or duration decrease the saturation level due to irreversible cell damage and loss of ability to reseal. Therefore, in single-pulse experiments, we did not exceed the pulse parameters mentioned above.

The DNA-dependent increase in permeability is a long-lasting membrane alteration. The effect of DNA also can be seen if FD is added not before but after pulse application. The amounts of FD20 taken up by cells when added at different time points after pulse treatment is shown in Fig. 2. The curve obtained in the presence of 100 μg/ml of thymus DNA (curve *1*) goes above the control (curve *2*) in a whole range of time intervals studied, without reaching the control level even 100 s after the pulse.

The relative amounts of fluorescent label entrapped by cells in the presence of DNA and in the control for FDs of four different sizes are plotted in Fig. 3. The effect

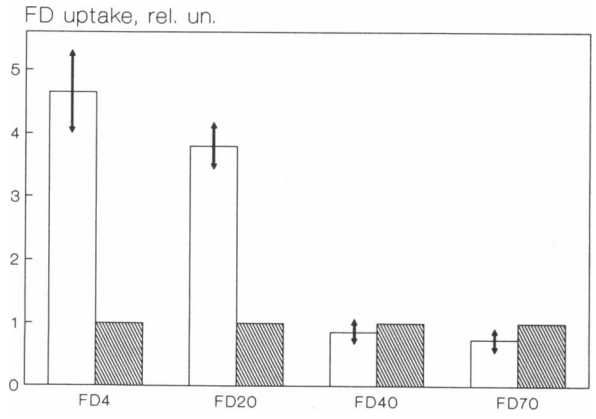

FIGURE 3 Normalized amounts of four different FDs taken up by cells on electric treatment by a single pulse of 1.5 kV, 1 ms in the presence of 0.1 mg/ml of calf thymus DNA. The control values assayed in the absence of DNA (▨) are taken as unity.

of DNA-dependent increase in plasma membrane permeability is observed only for "light" dextrans, FD4 and FD20. DNA does not influence (or even slightly decreases) the entrapment of larger dextrans, FD40 and FD70.

The rate of membrane permeability increase was found to be dependent not only on the size of FD but also on the size of DNA present in the pulsation medium. The relative amounts of FD4 and FD20 entrapped by cells on electric treatment in the presence of equimolar concentrations of pCH110 and phage λ-DNA (both linearized) are shown in Fig. 4. The highest increase of permeability is observed for light dextran FD4 in the presence of λ-DNA, the larger DNA used.

Electrotransfection in a two-pulse mode

The preliminary transfection experiments performed in a two-pulse mode have shown that the effects of two unequal pulses (see Fig. 5, *inset*) applied in tandem are nonadditive. The first pulse was short but of high intensity (4–7 kV/cm, 10–20 μs), whereas the second was of lower amplitude but much longer (0.2–0.5 kV/cm, 1–10 ms). It was revealed that the increase in TE by the second pulse when it follows the first one is much higher than the level of TE evoked by the second pulse alone. This suggests that the effects of these two pulses on cell membrane and the DNA situated nearby are different and that they create a sequence of events that favors DNA penetration into the cell.

To perform more exacting experiments, we scrutinized the parameters of each pulse. We found that the first pulse of 6 kV/cm and 10 μs provides efficient and reversible cell poration (98% of cells stained with Lucifer Yellow, with >90% viability). However, it is weak in electrotransfection; it induces <5% of the TE level obtained at 3.5 kV/cm, 100 μs (18). The second pulse of 0.2 kV/

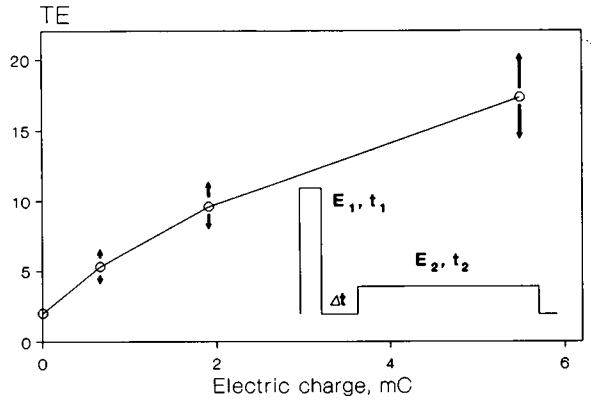

FIGURE 5 The transfection efficiency (TE) as a function of the electric charge passed through the chamber during the second (low-voltage) pulse. The duration of the second pulse was varied in this experiment, whereas the charge was evaluated as described in Materials and Methods. (*Inset*) The sequence of two unequal pulses that was used for separation of two different effects of electric field in electrotransformation. The first pulse of E_1 = 6 kV/cm and t_1 = 10 μs and the second pulse of E_2 = 0.2 kV/cm and t_2 varying from 0 to 10 ms were applied with the time gap Δt of 100 μs.

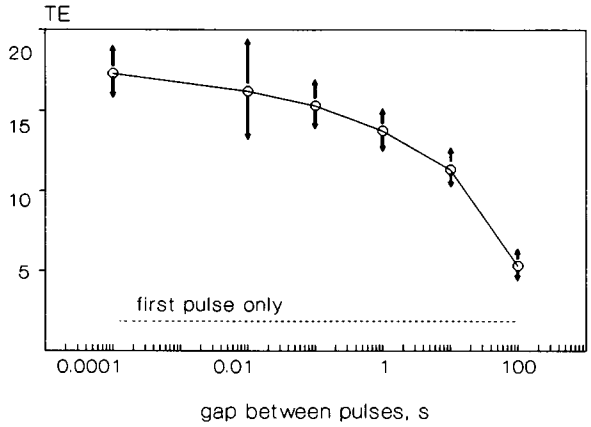

FIGURE 6 The dependence of transfection efficiency (TE) on the time delay between two pulses. The parameters of the pulses are as noted in the legend to Fig. 5. Cells were treated in a low-conductance medium (see Materials and Methods). The level of TE obtained with the first pulse only is shown by dashed line.

cm, 10 ms applied alone does not cause any poration or transfection, but when applied immediately after the first pulse, it enhances TE by one order of magnitude. Reversing the order of the pulses gives the same result as the first (high-voltage) pulse alone. The TE level rises monotonously with the amplitude of the second pulse, but above 0.4 kV/cm (10 ms duration), such a pulse is able to evoke a detectable level of transfection by itself. To reliably distinguish the specific effects of each pulse, in subsequent experiments the amplitude of the second pulse was chosen as 0.2 kV/cm.

The TE level as a function of electric charge passed through the chamber during the second pulse is shown in Fig. 5. In this experiment, we varied the duration (but not the amplitude) of the second pulse. However, due to a slight deflection of the pulse shape from rectangular, the electric charge in each run was calculated according to "real pulse shape" (see Materials and Methods). The TE dependence rises monotonously. The slope of the curve slightly declines with the increase of electric charge passed through the chamber.

In the second series of experiments, we studied TE as a function of time delay between the two pulses (Δt). As seen from Fig. 6, as the delay increases, TE declines, reaching one-half of its initial value at Δt = 10 s. Even at 100-s interval between pulses, the TE level is twice as high as that obtained by application of the first pulse alone. It is important to note that introduction of DNA into the cell suspension between two pulses (which usually takes 5 s) gave a negligibly low level of TE.

The two-pulse technique also allowed us to study the problem: Does DNA uptake by cells correlate to the entrapment of fluorescent dye? The FD4 entrapment by cells as a function of delay between two pulses in the presence of 0.1 mg/ml of thymus DNA is plotted in Fig. 7. The first pair of bars shows FD4 uptake on the first pulse only. The increase in FD uptake in the presence of DNA in this case is insignificant, probably due to short pulse duration. Note that in the absence of DNA the second pulse does not change FD4 uptake. We observed a more than threefold rise in FD4 uptake on the second (low-voltage) pulse application in the presence of DNA. At the same time, the amount of FD4 taken up by cells declines with increasing interval between pulses, similar to TE (compare with Fig. 6).

DISCUSSION

In the previous work (18), we showed that DNA translocation into cells using a high-voltage pulse takes <3 s, and DNA electrophoresis plays an important role in this

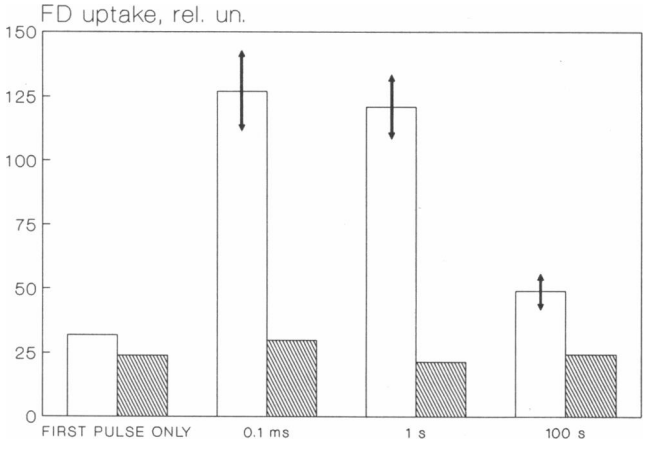

FIGURE 7 FD4 uptake by cells on electric treatment in a two-pulse mode as a function of the time delay between the pulses. FD4 uptake on electroporation in the presence of 0.1 mg/ml of calf thymus DNA is shown by open bars; the control values obtained with no DNA are shown with shaded bars.

process. We suggested that DNA is being transferred through the membrane directly during the electric field pulse. In the present work, we consider two more features of the process. (*a*) When the DNA crosses the membrane, it interacts with the membrane such that membrane permeability to indifferent water-soluble dyes becomes markedly higher. (*b*) If the membrane is subjected to a short electroporative pulse in the presence of DNA, subsequent DNA translocation through this membrane can be driven by a relatively low electric field, which is unable to form electropores by itself. These regularities have new implications for the mechanism of the electrically induced DNA uptake by cells.

Motive force of DNA electrotranslocation

Transfection of cells by sequences of two unequal pulses enables us to distinguish two different effects of the electric field: membrane electroporation and electrophoresis of DNA. Since the low-voltage pulse does not influence membrane permeability in the absence of DNA, even if it is applied after the high-voltage pulse, the sharp increase in TE in two-pulse experiments (Figs. 5 and 6) should be caused exclusively by DNA electrophoresis during the second pulse. The increase in TE by the second pulse is very similar to the observations made by Andreason and Evans (17), in which they reported a significant rise in transfection yield when cells were treated by a train of low-voltage pulses after a short electroporative pulse of high amplitude.

The TE dependence on the electric charge (Q) passed in the circuit during the second pulse (Fig. 5) rises monotonously, consistent with the hypothesis of electrophoretic DNA transfer. Under ideal conditions, when DNA transfer through a highly permeable membrane is driven by electrophoretic force only, the amount of DNA taken up by the average cell is proportional to the product $E \times t$ (more generally to $\int E\,dt$) or Q. With $Q = \int I\,dt = \int (U/R)\,dt$ and with $E = U/d$ = constant, $E \times t = QR/d$, where E is the electric field strength, t is the pulse duration, R is the resistance of the chamber, and d is the distance between electrodes. The linearity of TE(Q)-dependence should be observed at short pulse durations, when the DNA concentration in the cell is far from saturation.

Since relatively low (0.2 kV/cm) electric field provides an efficient DNA transfer through the electropermeabilized membrane, application of rectangular or exponentially decaying pulses of significantly higher amplitude (0.8–4 kV/cm—the range widely used for eukaryotic cells), in addition to forming pores, causes DNA translocation into cells by the same electrophoretic mechanism. Note that use of exponential pulses appears to be especially suitable, since these pulses have a high peak providing for poration and a long tail of relatively low amplitude, which is sufficient for effective DNA injection into the cell. Taking into account the vectorial character of DNA translocation, the factor of field orientation may also improve the efficiency. An advanced protocol reported recently by Tekle et al. (22), using repetitive bipolar pulses, eventually provides DNA injection from both sides of the cell.

However, the existence of electropores in the membrane and simultaneous application of low electric field is insufficient for DNA entry into cells. Addition of DNA into cell suspension in between the high "electroporative" and low "injecting" pulses gave extremely low TE. This strongly indicates that DNA influences electroporation and should be present during the first pulse also. Indeed, we observed increases in cell permeability on electric treatment in the presence of DNA (see Figs. 1–4). More exactly, this effect is not only due to the presence of DNA in the medium; as seen from the correlation between TE and the amount of FD taken up by cells in two-pulse experiments (compare Figs. 6 and 7), permeability rises due to DNA transfer into cells. In other words, DNA translocation leads to an alteration of electropores in the membrane. This effect can be easily explained in terms of a mechanical interaction between DNA and membrane due to the electrophoretic force, whereas it cannot be explained by free DNA diffusion through the electropores.

How does DNA cross the membrane by electrophoretic force?

The external electric field imposed to the cell in suspension does not penetrate through the initially intact membrane. As soon as electropores are formed, electric field crosses the membrane through these conducting structures (primary pores). The lines of electric field are concentrated in the pores, so the intensity of electric field E in the pore and in the nearby vicinity should be higher than that in the bulk. At appropriate field polarity, the polyanionic DNA experiences a strong attraction to the pore. The diameter of electropores has been estimated in the range of 1–10 nm (23, 24), whereas the size of DNA is usually larger. Cell electrotransformation by DNA as long as 150 kb has been reported (25). According to estimations (26) and direct measurements (27), the diameter of a statistical coil of such molecules is up to 2,000 nm (for 150 kb). However, DNA is a flexible molecule, susceptible to orientation and deformation in an electric field (28). At the same time, membrane electropores also appear to be flexible structures. The similarity in electroporation phenomena of cellular and pure lipid membranes (29) points to a primarily lipid matrix location of electropores, which have no rigid, strictly defined structure. We suggest that even if the pore size is smaller than the effective diameter of DNA, the DNA can enter the cell. According to estimates (30), electrophoretic pressure of DNA on the porated membrane is high enough to create a path in the membrane.

Our model suggests the translocation process consists of DNA alignment in an electric field, subsequent me-

chanical interaction with the pore, and simultaneous pore expansion. Such expanded pores should have longer lifetimes (Fig. 2). We also assume that the DNA molecule may prevent resealing of the membrane if it is part way through the pore when the field is turned off. Once the pores are formed in the presence of DNA, subsequent electrophoretic movement of DNA molecules through these pores is facilitated and can be driven by relatively low electric field (Figs. 5 and 6). Using the two-pulse technique, we have been able to estimate the lifetime of the pores that are competent to DNA permeation driven by low-intensity electric field. Varying the delay between two pulses, we showed that the pore lifetime is on the order of tens of seconds (Fig. 6). In principle, the slight decrease in TE with the increased delay between the pulses can be explained by randomization of cell orientation, but as shown in Appendix 1, this should be only a small fraction of the effect.

The larger the DNA present in the medium, the greater the increase in membrane permeability (Fig. 4). This result can be interpreted as an increase of the mean pore diameter on passage of larger DNA molecules. Pore widening in these conditions, however, is limited. In the experiment with thymus DNA (fragments of 5–100 kb length), we did not observe any rise in permeability for FD40 and FD70, whereas FD4 and FD20 were entrapped much more efficiently (Fig. 4). We may conclude that, given the accuracy of the time resolution of our method (see Appendix 2), the pore diameter in this case is less than the effective diameter of FD40 but larger than that of FD20, i.e., between 3.3 and 4.8 nm (31).

Within the model proposed, as a hypothesis, we may distinguish several different types of interaction between DNA and porated membranes (Fig. 8).

DNA penetrates through a single pore as a compact elongated globule (Fig. 8 *a*). Assuming that the pore size estimated above is the actual size of pores permeable for DNA, one may conclude that DNA thread of 2 nm diameter should cross the pore not as a round globule but as an elongated bundle. Similarly, if one of the ends of the DNA chain hangs loose from the globule, then it can be first pulled into the pore and then lead the whole globule through the membrane (Fig. 8 *b*).

DNA obeying the electrophoretic force may cut the membrane between two pores. Considering that FD40 molecules do not penetrate pores permeable for large DNA molecules, it is possible that a long DNA thread can enter two (or more) different pores (Fig. 8 *c*). In this configuration, when the electrophoretic force pulls DNA inside, the stretch of the molecule between two pores presses the membrane and finally is able to make a cut connecting the two pores, as a sharp thread can do. The longer the DNA, the more remote pores (or larger number of pores) will be engaged by one molecule (see data in Fig. 6). Since the thickness of the thread is 2 nm (in the case of circular DNA, doubled to ~4 nm thick), the resulting pore (cut between two primary pores) will be a narrow slit permeable to FDs of molecular mass ≤ 20,000.

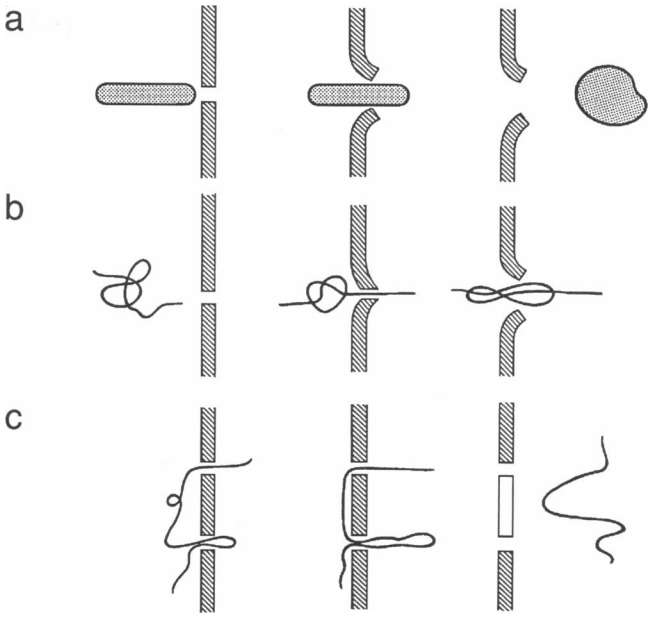

FIGURE 8 Possible ways of DNA interaction with electroporated membrane in a high electric field. (*a*) DNA coil is aligned in an electric field, and at the appropriate pulse polarity it moves toward the porated membrane. The DNA may interact with a single pore; the pore becomes wider upon DNA passage by the action of electrophoretic force. (*b*) Passage of the DNA coil can be initiated by penetration of one end of the thread, which then leads the whole molecule through one pore. (*c*) The DNA molecule can be involved in two pores (or more) and moving with electrophoretic force it cuts the membrane between these pores as a sharp thread can do (the nonshaded block represents the slit opened between the two pores).

Alternatively, the electric field pressing the DNA toward the membrane forms an invagination, which may result in an endocytosis-like DNA uptake. Electrically induced formation of endosome-like vesicles surrounding DNA has been shown in experiments with unilamellar liposomes (15). However, it appears that the long-lived alterations of membrane permeability, and the increase in TE on second (low-intensity) pulse application only when DNA was added before the first (high-voltage) pulse, make direct DNA interaction with electropore(s) a more likely explanation than endosome formation.

Summarizing the data presented above, we conclude that in our experimental conditions DNA electrotranslocation through the plasma membrane of mammalian cells is not a simple penetration via "ready-to-use" electropores. Electric field creates primary pores, whereas the subsequent process includes pore size adjustment as a result of mechanical interaction between the pores and the DNA driven by electrophoretic force.

APPENDIX 1

The decrease of TE with the time delay between the pulses can also be explained by the randomization of cell orientation during the delay.

Due to Brownian motion, cells may turn so that the electroporated part of the membrane will not be oriented normally to the electric field during the second pulse.

The rotational diffusion time constant τ_{rot} for the sphere of radius r is $\tau_{rot} = 4\pi r^3\eta/(3kT)$, which for $r = 8\ \mu m$, $\eta = 10^{-2}\ g\ cm^{-1}\ s^{-1}$, and $T = 273$ K is 569 s. The fraction $f(\Delta t)$ of cells retaining orientation at $\Delta t = 10^2$ s (see Fig. 6) is $f(\Delta t) = \exp(-\Delta t/\tau_{rot}) = 0.84$. Thus, the decrease of TE from 17 to 5 during $\Delta t = 100$ s is more than rotational diffusion can account for. This shows that resealing of the "competent" pores occurs.

APPENDIX 2

We cannot rule out that the pores are wide when DNA crosses the membrane but then shrinks rapidly. The technique used for assaying the rate of membrane electropermeabilization allows us to detect the existence of pores with lifetimes not shorter than the characteristic time of dye diffusion from the bulk solution into the cell. The average distance $\langle \Delta x \rangle$ between the dye molecules at a concentration of 0.05 mM is $\sim 0.015\ \mu m$, which is translatorily crossed by diffusion on average in $t_D = \langle \Delta x^2 \rangle / 6D = 0.85\ \mu s$. (Diffusion coefficient for FD40, $D = 4.4 \times 10^{-7}\ cm^2/s$ [31].) However, that is an underestimate, because it gives an average diffusion time for the first dye molecule through each pore. Apparently, it is not detectable in the experiment. The cell accumulates reasonable amount of dye when the dye penetrates to a depth of $\langle \Delta x \rangle = 0.5\ \mu m$ (i.e., when $\sim 20\%$ of cell volume is stained). In this case $t_D = 1$ ms, which can be taken as the time resolution for the method we used.

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