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Escherichia coli membranes during electrotransformation: an electron microscopy study

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Structural changes undergone by *Escherichia coli* cell envelope membranes under the conditions of electrically induced gene (DNA) transfer (exponential pulse of about 13 kV/cm, $\tau = 5$ ms) were studied by freeze-fracture electron microscopy. Special device similar to that of Stenger and Hui ((1986) J. Membr. Biol. 93, 43–53), that allowed cryofixation of samples almost simultaneously with application of electric pulse, was employed to examine the cells within a short time (≤ 1 s) after the pulse. Extensive blebbing of cells was observed immediately after the pulse. At later times (30–40 s after the pulse) blebbing was not detected, instead infrequent cellular membrane fusion and formation of large membrane 'openings' or pores were observed. An attempt to relate the observed membrane changes with cellular viability and permeability to exogenous DNA failed. Challenge of cells with a plasmid DNA 10 s after the pulse application resulted in a dramatic loss (at least four orders of magnitude) of the number of transformants compared to cells pulsed in the presence of DNA. On the other hand the results on additional pulsing of cells prior to the main electrotransformation procedure suggested that the life-time of membrane defects is at least no less than 2 min. Possible ways to reconcile the results are suggested.

Introduction

Electroporation, a reversible permeabilization of cells by electric fields [1], is now widely used to introduce various kinds of molecules, including proteins and nucleic acids, into different eukaryotic and prokaryotic cells [2–6]. The latter is of particular importance for genetics, recombinant DNA technology, and biotechnology progress.

Internalization of exogenous compounds by the cells induced by the electric fields including DNA (electrotransformation) is thought to occur via membrane pores or 'openings'. As evidenced by the results of measurements of permeation of diferent compounds through erythrocyte ghost [7–9] and Chinese hamster ovary cell (CHO) [10] membranes, the size of these openings seems to be insufficient for the penetration of circular and supercoiled DNAs into the cell. However the frequency of electrotransformation does not at best exceed 10^{-3}–10^{-2} (that is only one out of 100 or 1000 viable cells takes up DNA productively). This suggests that the pores, sufficient for DNA penetration, are induced electrically only in a very small fraction of cells. It is likely that these cells would not contribute significantly to the results of the permeation measurements [7–10] and the pores would escape detection in ultra structure membrane studies, especially when ultra thin section electron microscopy is used. Indeed, no pores have been yet discovered * though significant alteration of eukaryotic membranes during their electropermeabilization to exogenous DNA such as disruptions of the membrane bilayer in erythrocytes [11], the increase in the density of microvilli and the formation of blebs in Chinese hamster ovary cells (CHO) [10], and some others [12,13] were observed. In hepatocyte [12] and CHO [10] cells membranes the formation of blebs was correlated with the increase in membrane permeability to small molecules.

Surprisingly no ultra structure membrane studies have been yet undertaken on microorganisms variety of

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which has been and is being used for electrotransformation (see, for instance, reviews [6,14]). Recently we have shown that electron microscopy methods and especially freeze-fracturing and freeze-etching could yield valuable information and direct visualization of the nature and the extent of membrane changes during Ca^2-induced DNA transfer into Gram-negative bacteria (*Escherichia coli* and *Pseudomonas putida*) [15,16]. Since the frequencies of cation- and electric field-induced DNA transformations are alike we expected that the same experimental approach would allow to detect membrane changes induced by the electric field which are pertinent to DNA transfer.

So the aim of this study was to characterize the ultra-structural changes undergone by the cell envelope membranes of *E. coli* cells under the conditions of electrically induced gene transfer using freeze-fracture electron microscopy. In some experiments ultra thin section electron microscopy was also used in parallel. An attempt was made to relate these changes with the cells viability and permeability to exogenous DNA.

Materials and Methods

The cells of *Escherichia coli* LE392 strain were grown and prepared for electroporation and electrotransformation according to Ref. 17 with a slight modification. The cells were washed in 10% glycerol only once (instead of twice) and resuspended in 1/100 of the initial volume of the latter. The cells thus prepared were either directly used in the experiments or rapidly frozen in 100-μl aliquots in liquid nitrogen, placed and stored in the deep freezer (at $-70\,^\circ$C).In the latter case the cell samples prior to experiments were withdrawn from the freezer and thawed on ice. Both sample types yielded similar results in the experiments.

Plasmid pUC18 and pBR322 DNAs were isolated according to Refs.18 and 19.

Electronics and sample chambers. The exponential decay pulses were generated by a home made device composed of a high-voltage generator, an oscilloscope and a RC circuit. The scheme of the device is commonly used in electroporation and electrotransformation studies and has been described in detail in many papers (see, for instance, Ref. 17). A 25 μF capacitor switched into the RC circuit and a 200 Ω resistance switched in parallel to the sample chamber ensured the time constant, τ, of 5 ms [17]. Since this parallel resistance was much smaller than the sample resistance, it provided also the relative invariability of τ with the possible changes in the sample resistance.

Two types of sample chambers were used. One was routinely used in electrotransformation and consisted of two cylindric stainless steel electrodes with a variable slot between them. It is described in detail elsewhere [20,21]. The pack with sample chambers of this type was maintained in an ice box. Just before applying the electric pulse a chamber was withdrawn from the box and rapidly mounted in the generator unit. Its temperature, controlled with a thermocouple, was 1–2 °C before the pulse and did not exceed 10 °C after it. This chamber unit allowed the subsequent cryofixation of samples within 30–40 s. after the application of the electric pulse. The second type sample chamber allowed almost simultaneous application of the electric pulse to the sample and its cryofixation. It is presented in the Fig. 1 and schematically resembles the one used by Stenger and Hui [11], but without time measuring electronic sensor. It combines the extra fast freezing device [22] and the 'sandwich' sample holder routinely used in freeze-fracture experiments [19,20]. The latter consisted of two small and thin copper strips connected to a pulse generator (Fig. 1) which were sprayed with platinum and fixed between the springy stainless-silver wire electrodes in such a way that a sample slot between the juxtaposed strips was about 0.3 mm thick. The wire electrodes connected to a pulse generator were mounted in a teflon holder which was attached to forceps. Starting temperature of this 'sandwich' sample chamber was 1–2 °C.

Application of electrical pulses to cells. 30 μl (in case of I type sample chamber) and approximately 2 μl (II type sample chamber) cell samples (about 10^{10} cells per ml) preincubated for 1 min on ice were pulsed once (if not stated otherwise) at a field strengthof 13 kV/cm and a pulse decay constant, τ, of 5 ms. All procedures were performed in the 'cold' room at 2–4 °C.

Electrotransformation. Cell samples (about $5 \cdot 10^9$ cells per ml) with added transforming DNA (0.5 μg per ml) were pulsed as described above and immediately placed into 10 V (in case of the I sample chamber) or 100 V (II type sample chamber) of the medium, containing 2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$,10 mM $MgSO_4$, and 20 mM glucose, and designated SOC-medium [17]. The suspension was diluted (when necessary) and plated on L-agar with a proper antibiotic (13 μg/ml of tetracycline, or 50 μg/ml of ampicilline). Cell viability was checked by plating the appropriate dilutions of the samples on L-agar without antibiotics.

Freeze-fracture experiments. Cell samples electroporated in the I type sample chamber were rapidly withdrawn from the chamber and placed between the two copper strips similar to those, shown on Fig. 1, but without the attached electrodes. The latter were then rapidly fixed to the extra fast freezing device (Fig. 1). Quenching and processing of the samples were according to Ref. 23. In each experiment two controls and seven samples were simultaneously fractured and processed as described in detail elsewhere [15]. The cells in the II type sample chamber already attached to the

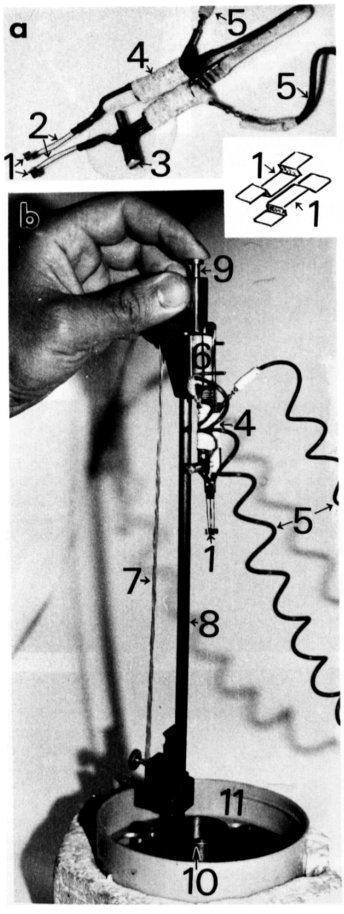

Fig. 1. 'Sandwich' sample chamber for electroporation (a); a plunger device for rapid cryofixation (b). 1, Copper strips (6 mm long and 2 mm wide); 2, spring stainless steel wire electrodes; 3, slot width regulating screw; 4, forceps; 5, wires to a pulse generator; 6, forceps holder of the plunger; 7, elastic spring; 8, plunger sliding track; 9, trigger; 10, a vial with liquid propane; 11, a bath with liquid nitrogen.

extra fast freezing device were pulsed and quenched almost simultaneously (the time between the pulse application and quenching did not exceed 1 s).

Ultrathin sectioning. Since the time of chemical fixation of samples with glutaraldehyde is about 15 s [10] ultra-thin sectioning was performed only on the cell samples electroporated in the i type sample chamber. The procedures of chemical fixation and ultra-thin sectioning are described in detail elsewhere [15].

Mathematical treatment of experimental results. Linear least-squares regression and Student's criterion (t-test) analysis were used for the approximation of experimental curves and for the determination of statistically significant difference between the results [24].

Results

First we examined the envelope membranes of *E. coli* cells electroporated under the conditions usually used for electrotransformation, that is in the I type sample chamber. All cell samples were quenched from 2 to 6 °C. The technique used allowed the chemical fixation of the samples within 15 s after the pulse [10] and extra fast freezing of samples about 30–40 s after the pulse (see Materials and Methods) The results of these experiments are presented in Fig. 2. Fracture faces of control, unpulsed *E. coli* cells (Figs. 2a,b) exhibited classical patterns of rough particles on the outer membranes and of smooth particle denuded areas (Figs. 2a,b, asterisks) together with the areas of segregated particles on cytoplasmic membranes (for a review, see Ref. 25).

Majority of cells electroporated under the conditions optimal for gene (DNA) transfer (in our conditions an electric pulse of 13 kV/cm and $\tau = 5$ ms) exhibited fracture faces similar to those of controls. However, in some cells (approximately in 2–3% of the cells examined) large membrane defects in the form of round openings or pores and disruptions of membrane continuity were seen (Figs. 2c–e). In addition infrequent (one among several hundreds of cells) fusion of membranes of adjacent cells could be also seen (Fig. 2f). The presence or absence of exogenous DNA in the samples had no detectable effect on the results even when rather high concentration of the latter (about 25 μg per sample) were used. No blebs have been detected on any of the fracture faces examined.

However on thin sections of the same samples occasional blebbing (Fig. 3e, arrowhead) could be observed. Since the chemical fixation arrested the cells earlier after the pulse, as compared to cryofixation, the results might reflect the transitory formation of blebs within 15 s after the pulse. Possibility of the artifact was unlikely since no blebs were observed in unpulsed cell samples. This prompted us to investigate the mem-

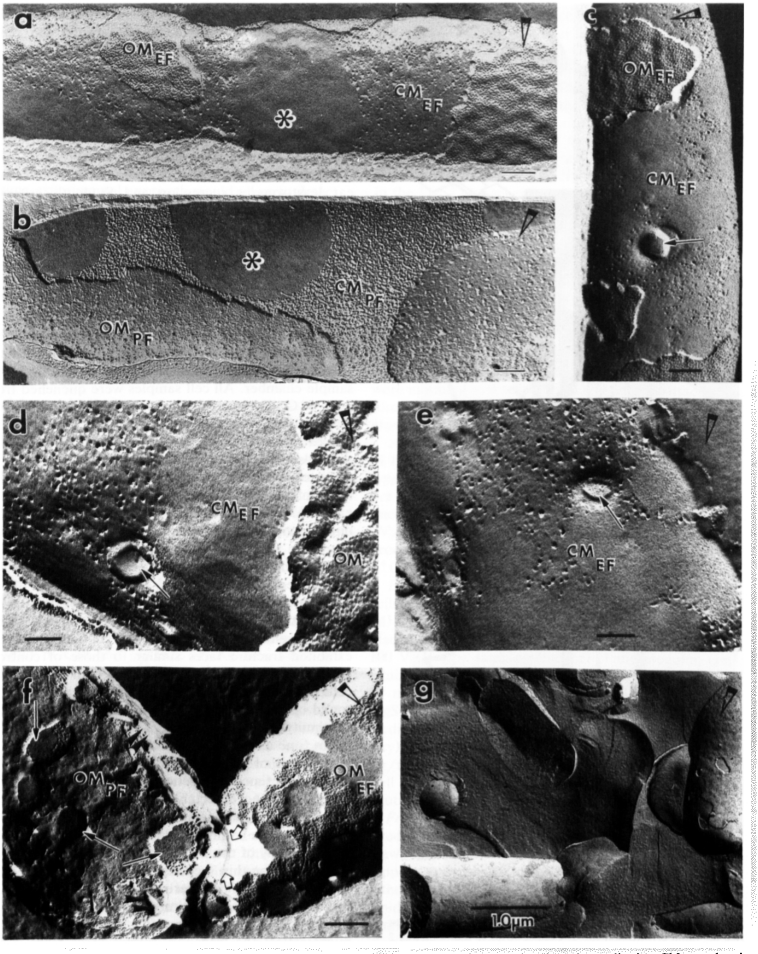

Fig. 2. Fracture faces of control (a,b,g) and pulsed (c–f) *E. coli* cells quenched from 2–6°C 40 s after the pulse application. CM, cytoplasmic; OM, outer membranes. Smooth particle denuded areas are shown with (*); round openings (pores) and surface defects (arrows), disruption of membrane continuity (double arrowheads) and infrequent fusion of cells (open arrows) are seen. Bars on all the micrographs, except Fig. 2g, designate 0.1 μm; open arrowheads designate the direction of shadowing.

brane changes at still earlier times after the pulse application.

Bearing in mind that freeze-fracture is a much more powerful and reliable approach in disclosing membrane defects, including pores, than thin sectioning, we attempted to minimize the time between the electric pulse application and cryofixation. So the second procedure for almost simultaneous electroporation and quenching in 'sandwich' [11] was employed (see Materials and Methods). Fracture faces of thus electropo-

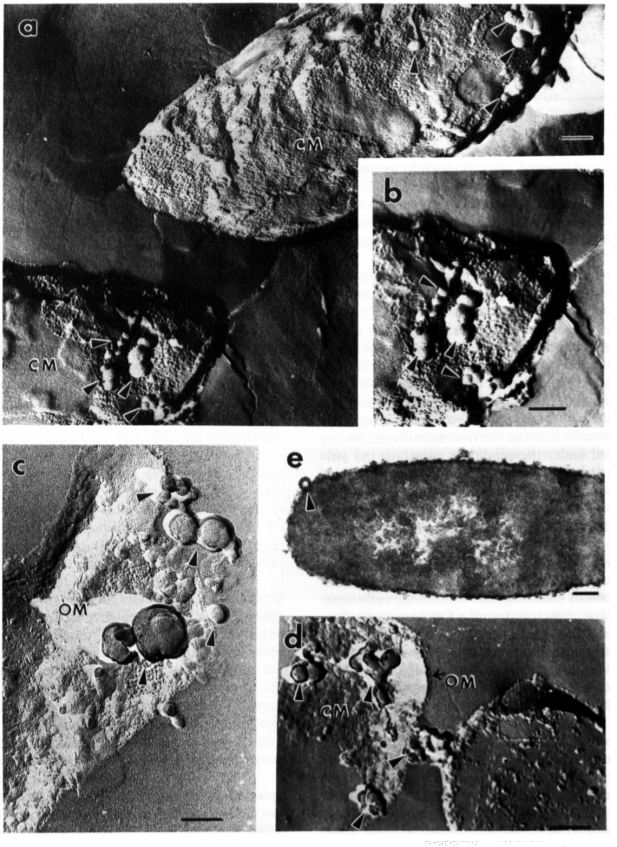

Fig. 3. Membrane defects of electroporated cells. (a–d) Fracture faces of cells quenched almost simultaneously with electric pulse application. (e) Thin sections of cells. Along with other defects frequent blebbing is seen (arrowheads). For controls, see Fig. 2g.

TABLE I

Transformation efficiencies in different sample chambers vs. $\bar{E}$

The results of a typical experiment are presented. n.d., transformants not detected under employed assay conditions.

Sample pulsed at $\bar{E}$ (kV/cm)	Efficiency of transformation (transformants/μg DNA)	
	'Sandwich' chamber	standard chamber
0	n.d.	0
6	n.d.	$2 \cdot 10^4$
10	$6 \cdot 10^3$	$1 \cdot 10^5$
13	$1 \cdot 10^5$	$5 \cdot 10^5$
16	$5 \cdot 10^4$	$8 \cdot 10^4$

rated cells indeed exhibited multiple blebbing (Figs. 3a–d). Blebs of varying sizes frequently arranged in bunches were easily seen on many cells (about 60% of cells examined). And again, as in thin section experiments, no blebs were detected in unpulsed samples fractured simultaneously with the pulsed samples (Fig. 2g).

Both types of electroporation sample chambers exhibited close electrotransformation efficiencies, the latter in the 'sandwich' sample chamber being only 5-times less than in the standard chamber at optimal conditions (at 13kV/cm pulse) (Table I). Besides, both efficiencies exhibited similar dependence on the electric field strength (Table I and Refs. 5 and 17). This suggests that the freeze-fracture results of samples pulsed in different chambers can be directly compared.

It is noteworthy that the electron microscopic results were obtained under the conditions providing the efficient electrotransformation of cells. To further relate the results of electron microscopy with the cells permeability to exogenous DNA and to evaluate the life-time of DNA-related membrane defects we used two experimental approaches.

In the first approach we investigated the ability of cells to be electrotransformed at different times after the application of electric pulse. That is the cells were first pulsed and then transforming DNA was added to them. This ability declined very sharply within the first 10 s after the pulse, the number of transformants at this time being at least four orders of magnitude lower than in controls, when the cells were pulsed together with DNA. No further significant decline was observed at 40 s after the pulse.

In the second approach we studied the effect of pretreatment of cells with an electric pulse on the cell viability and the ability for subsequent electrotransformation upon the second electric pulse. The experiments were performed in the following way. Cell samples were pulsed once but at different electric field strength (from 2 to 13 kV/cm) and left to recover on ice for 1 min. Then transforming DNA was added to the sample and in another minute the second electric pulse of 13 kV/cm was applied. Samples were further processed as described in electrotransformation section of Materials and Methods. The results of these experiments are presented in Fig. 4. They suggest that there are two types of the effect that a preliminary pulse exerts on the cell viability and the ability of cells for electrotransformation after the second pulse. Low field pulses of up to 2–3 kV/cm appear somewhat stimulating for both the ability of cells for subsequent electrotransformation and their viability after the second electric pulse. On the contrary, pulses of higher electric field strength (4–13 kV/cm) are inhibitory: the stronger the electric field strength, the stronger the inhibitory effect. This dependence can be satisfactorily approximated by straight lines with slopes significantly different for both curves, regression coefficient being larger for transformation ability curve.

Statistical significance of the difference in slopes has been estimated by the method of pair comparisons for each of five experiments and by comparing the regression coefficients of the two curves, constructed from the results of all five experiments. Both methods gave similar results and established the statistical significance ($P < 0.05$) of difference in slopes.

Thus the results indicate, firstly, that when the cell is subjected to a single electric pulse of strong electric fields (4–13 kV/cm) its ability to interact productively with DNA upon the second electric pulse is much

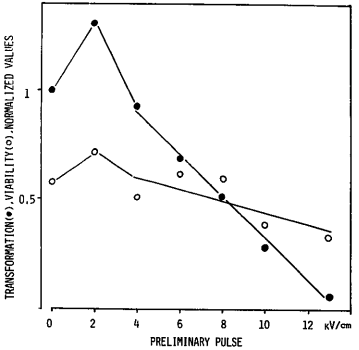

Fig. 4. The effect of preliminary treatment of cells with an electric pulse of different field strength on their viability and the number of transformants after the second pulse. Open circles, cell viability normalized to the initial number of cells (before any pulse at all); filled circles, the number of transformants, normalized to that of untreated control.

more severely damaged than its ability to survive after the second pulse. Secondly, since the cells were allowed to recover after the first pulse for about 2 min on ice, the results suggest also that the life time of certain membrane defects which appear critical to the cell viability and to the electrotransformation ability upon the second electric pulse, is at least not less than 2 min. On the contrary the permeability of cellular membranes to exogenous DNA, as suggested by the results of the first approach, vanishes within the first seconds after the pulse.

Discussion

We have examined the cell envelope membranes of Gram-negative bacteria *E. coli* under conditions providing the efficient electrotransformation of the cells. The evidence presented unequivocally indicate that certain dynamic changes in *E. coli* membranes take place within 1 s (or even less) to 40 s after the electric pulse.

It is likely that the first and immediate (within 1 s) consequence of the electric pulse is multiple blebbing of cells, accompanied by severe disruptions of outer membrane continuity.

Blebs are detected on both the OM and CM fracture faces (Figs. 3a–e). Nevertheless it is likely that they originate from the CM, and particularly from CM zones somewhat devoid of intermembrane particles (Fig. 3a). It is likely that the blebs are short lived structures, since they are not detected on fracture faces of the samples quenched 30–40 s after the pulse (Figs. 2c–e). However, they can be detected on thin section when the cell samples are fixed about 15 s after the electric pulse (Fig. 3e). It is noteworthy, that membrane blebbing in *E. coli* cells has been observed in many other cases such as during the cell division, T4 bacteriophage infection, the temperatures rising to 55 °C, or during the treatment of cells with high concentrations of divalent cations [15,26,27].

Quite remarkably similar electroinduced blebbing was detected in some mammalian cells [10,12,13] which are significantly different morphologically from the Gram-negative bacteria cells. Transitory nature of blebs suggests that they are of osmotic origin and reflect the immediate response of cytoplasmic membranes to the applied electric field. Indeed, experimental evidence obtained on bovine trachea epithelial cells, mouse embryo fibroblasts, rat fibroblasts and L-cells [13] indicates that electroinduced blebbing is promoted by some osmotically-dependent processes.

We suggest that blebbing might stimulate infrequent intercellular contacts of adjacent cells (Fig. 3d) which is known to promote subsequent fusion of their membranes [15,28]. It is likely also that the breakdown of blebs or their releasing from the cell surface might promote membrane fusion [29,30] and result in formation of extensive membrane pores or 'openings'. Both of these events were observed in this paper some 40 s after the electric pulse (Figs. 2c–f). The diameter of the pores varied from 30 to 100 nm. These diameters seem to be sufficient for entering the cell of both the linear and supercoiled DNA molecules.

Nevertheless, the question arises as to what membrane defects observed by electron microscopy actually determined the permeability changes for exogenous DNA molecules. The results of the experiments in which the cells were challenged with transforming DNA immediately after the electric pulse indicate that the transformation efficiency dropped drastically, the latter being at least four orders of magnitude less in 10 s after the pulse. Similar results were obtained during the electrotransformation of *Corynebacterium glutamicum* (Wolf, H. and Neumann, E., personal communication) and reported for *E. coli* during electrotransformation in the presence of Ca^{2+} and Mg^{2+} [31].

These results seem to suggest that only those membrane structural changes that occur within the first 10 s after the pulse are relevant to DNA penetration. However the cells treated with an electric pulse (a preliminary pulse) and allowed to recover on ice for 2 min exhibit both the reduced cellular viability and reduced ability for electrotransformation upon the second electric pulse compared to untreated cells (Fig. 4). Furthermore the ability of such cells for subsequent electrotransformation was more seriously damaged than the ability to survive after the second pulse.

This result suggests that either the number of cells able for electrotransformation is considerably reduced by the first (preliminary) pulse, or that these cells having retained the ability to interact with transforming DNA are more fragile in terms of viability than other cells of the population. The latter suggestion was shown to be the case in Ca^{2+}-stimulated transformation of *E. coli* [32]. The results presented in Fig. 4, suggest also that membrane damages relevant to DNA penetration might exist at least for 2 min at 0 °C.

So there seems to be a contradiction between the results on the life-time of DNA-related membrane defects. However, there are two ways to reconcile them. First is to suggest that though DNA-related membrane defects may still live for 2 min or even longer, their size rapidly becomes insufficient to accommodate DNA. The second is to suggest that though membrane defects remain large enough for DNA after 2 min or for even longer. DNA cannot enter the cell without the electric field. The results presented in this paper can not discriminate between these two suggestions. Nor there is conclusive evidence in the literature which confirm or reject movement of DNA into the cell via electrophoresis [33], electroosmosis [34], or via adsorption and subsequent diffusion [31]. We hope

that further experiments now in progress will clarify the situation.

Note added in proof

When the present paper was already submitted to BBA, a report by Chang and Reeze appeared ((1990) Biophys. J. 58, 1–12), in which with the help of a more sophisticated device for ultra-rapid freezing and pulse application, electroinduced pores of sizes similar to those demonstrated in this paper were discovered in erythrocyte membranes.

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