# Changes in membrane structure induced by electroporation as revealed by rapid-freezing electron microscopy

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ABSTRACT Cells can be transiently per- meabilized by exposing them briefly to an intense electric field (a process called "electroporation"), but it is not clear what structural changes the elec- tric field induces in the cell membrane. To determine whether membrane pores are actually created in the elec- tropermeabilized cells, rapid-freezing electron microscopy was used to examine human red blood cells which	were exposed to a radio-frequency electric field. Volcano-shaped mem- brane openings appeared in the freeze-fracture faces of electroper- meabilized cell membranes at intervals as short as 3 ms after the electrical pulse. We suggest that these openings represent the membrane pathways which allow entry of macromolecules (such as DNA) during electroporation. The pore structures rapidly expand to	20-120 nm <b>Waads</b> to <b>Hala</b> ng <b>Mass</b> first 20 ms of electroporation, and after several seconds begin to shrink and reseal. The distribution of pore sizes and pore dynamics suggests that inter- actions between the membrane and the submembrane cytoskeleton may have an important role in the formation and resealing of pores.

### INTRODUCTION

The introduction of large exogenous molecules into cells is required in a variety of experimental and practical situations, for example: delivery of drugs (Zimmermann et al., 1980; Kitao and Hattori, 1980); injection of proteins or second messengers for studying cellular chemical pathways (Russell, 1983) or receptor functions (Strand, 1987); and introduction of labeled or unlabeled antibodies for immunoassay (Yamaizumi et al., 1979). Insertion of DNA and RNA into cells has become a particularly important technique in molecular biology (Davis et al., 1986). Because the cell membrane is impermeable to most macromolecules, special methods are required to introduce these molecules into the cell. Commonly used methods include injection by micropipette (Graessmann and Graessmann, 1986), retrovirus infection (Williams et al., 1984), and calcium phosphate coprecipitation (Graham and Van der Eb, 1973), but each of these methods poses special problems and limitations.

A more widely applicable method of introducing molecules into cells is to transiently permeabilize the cell membrane by applying a pulse (or pulses) of high intensity electric field (Neumann and Rosenheck, 1972; Kinosita and Tsong, 1977; Benz and Zimmermann, 1981; Zimmermann, 1986; Sowers and Lieber, 1986; Neumann et al., 1982; Knight and Baker, 1982). Molecules can enter or leave the cell during this permeabilized state. Because this transient permeability was thought to result from the creation of membrane pores by the applied electric field (Kinosita and Tsong, 1977; Benz and Zimmermann, 1981; Sowers and Lieber, 1986), this process was called "electroporation". Recently, electroporation has been further improved by using a radio-frequency (RF) electric field (Chang, 1988, 1989*a*).

In spite of the fact that electroporation has become the most promising method of gene transfection (Chu et al., 1987; Potter, 1988), its basic mechanisms still remain largely unknown. Specifically, there is no evidence that physical pores are actually created in the cell membrane by the electric field (Stenger and Hui, 1986; Escande-Geraud et al., 1988). To gain a better understanding of electroporation, we developed a technique which utilizes rapid freezing followed by freeze-fracture electron microscopy (Heuser et al., 1979; Costello and Corless, 1978) to examine transient changes in the membrane structure of electroporated cells. The cell model used in this study was the human red blood cell. The electroporating properties of this cell have previously been extensively investigated (Kinosita and Tsong, 1977; Zimmermann et al., 1980; Bliss et al., 1988) and the red cell has also been used before for study of electrofusion (Sowers, 1984; Stenger and Hui, 1986; Chang, 1989a,b). Furthermore, the membrane structure of the normal red cell is very well known (Pinto da Silva, 1972; Weinstein, 1974) and it was expected to be less difficult to detect structural changes in its homogeneous, smoothly contoured membrane.

#### METHODS

Red blood cells collected from human blood were washed extensively and then resuspended in the poration medium, which was composed of

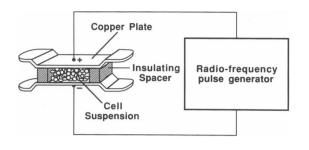


FIGURE 1 Apparatus for mounting red cells for electroporation and rapid freezing. Cells suspended in poration medium were sandwiched between two thin copper plates which served as both the sample holder and electrodes for the applied electric field.

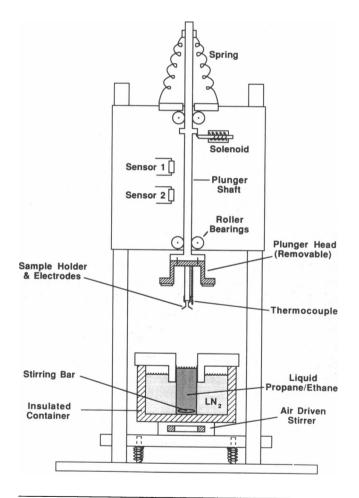


FIGURE 2 Apparatus for rapid-freezing electropermeabilized cells. The sample sandwich (shown in Fig. 1) was mounted at the tip of the plunger head. Sensors 1 and 2 monitored the position of the sample during the plunging action. Using signals generated from these sensors and a delay circuit, the electrical pulse could be applied at a predetermined period before the sample reached the coolant.

14 mM Na phosphate, 13 mM NaCl, and 150 mM sucrose (pH 7.4). This solution was slightly hypoosmotic in comparison to human serum, but it was found to preserve the red cell in normal biconcave shape better than an isoosmotic phosphate buffer. In the freeze-substitution experiment, 3% bovine serum albumin was added to the poration medium (to keep the suspended cells from separating during the warm-up stage of fixation).

Red cells suspended in the poration medium were sandwiched between two thin copper plates (Balzers Union, Hudson, NH) which served both as the sample holder and electrodes for the applied electric field (Fig. 1). Using a spring-driven propelling mechanism, the sample sandwich was plunged vertically into a liquid propane/ethane mixture cooled by liquid nitrogen (Fig. 2). Signals from two electronic sensors which monitored the position of the specimen during the plunging action permitted application of the porating pulse at various intervals before the specimen reached the coolant. The time delay between electrical pulsation and freezing was varied from 1 ms to many minutes.

Measurement of specimen capacitance indicated that the time required to freeze the entire sample (~40 layers of cells) was <3 ms (Fig. 3). If the freezing gradient is at least linear (Heuser et al., 1979), the single layer of cells near the copper plate surface could be frozen within 0.1 ms. Such a rapid freezing rate insured that the observed membrane structure is not distorted by large ice crystal formation and allowed us to capture transient morphological changes in the cell membrane.

The porating pulse used in this study was a 0.3-ms wide, DC-shifted RF pulse which oscillated at 100 kHz with an amplitude of 4-5 kV/cm (Chang, 1989a). We used an RF pulse instead of a direct current (DC) pulse because the RF field has been shown to be more effective than the DC field in permeabilizing and fusing cells (Change, 1989a,b). Also, the RF electric field in general provided better cell viability. This special electroporation device was designed and built in our laboratory.

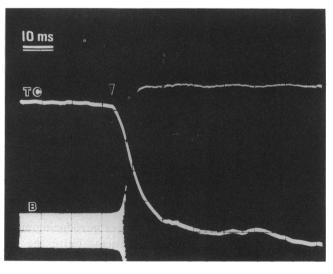


FIGURE 3 Oscilloscope traces showing the change of temperature and the physical state of water in the sample when the specimen was plunged into the coolant. The trace labeled "TC" shows the output of a thermocouple. The moment that the specimen came in contact with the coolant is marked by the arrowhead. Trace B shows the output of a bridge circuit which monitored the electrical capacitance of the specimen. The capacitance changed abruptly when the water in the sample froze.

The frozen samples were stored in liquid nitrogen and later processed either by freeze substitution (Ornberg and Reese, 1981) or by standard complementary-replica freeze-fracture electron microscopy techniques. Then, thin sections from freeze-substitution samples or the freezefracture replicas were examined using a transmission electron microscope (model 200-CX; JEOL USA, Electron Optics Div., Peabody, MA). To simplify the interpretation of the freeze-fracture views, contrast in micrographs was reversed to make the shadows appear as dark areas in the final print.

For light microscopy study, the suspended red cells were examined using DIC optics with a microscope (axiophot; Carl Zeiss, Inc., Thornwood, NY) and a video camera (Hamamatsu Corp., Middlesex, NY). Shapes of red cells after the application of the porating electrical pulse were recorded using a video recorder and analyzed later frame by frame.

#### RESULTS

#### **Electron microscopy**

The structure of the red cell membrane was already well known (Pinto da Silva, 1972; Weinstein, 1974). Control red cells prepared by our rapid-freeze, freeze-fracture method showed typical membrane structure (Fig. 4). When the red cell was permeabilized by an electric pulse, the structure of the cell membrane differed markedly from control membranes in having numerous pore-like structures on membrane E-face at 40 ms after the pulse (Fig. 5). The circular openings of these structures ranged from 20-120 nm in diameter. The E-face of the membrane curved into these openings, suggesting that these structures might be shaped like a volcano with its apex pointing away from the viewer, toward the outside of the cell (Fig. 5). Most of these openings ended as a planar disk of granular material typical of fractured faces through fluids.

The P-faces of the electropermeabilized red cell membranes also showed many pore-like structures complementary to those found in the E-face membranes (Fig. 6). Volcano-shaped membrane evaginations (50–120 mm in diameter) with their apices pointing outward were frequently seen. Thus, structures on the E-face and the P-face point to similar structural changes in the cell membrane after the red cells were permeabilized by the applied electric field.

The circular openings (as shown in Figs. 5 and 6) were observed only in red cell membranes that were porated by a high intensity RF electric field; they were not observed in any of the control cell membranes. Furthermore, these circular openings were the only major discernable changes in the structure of the cell membrane after electroporation, so they must represent the poration sites in the electropermeabilized cell membrane. It is still not clear from freeze-fracture micrographs, however, whether a particular opening is the true opening of the electropore, or whether it represents the cross-fractured

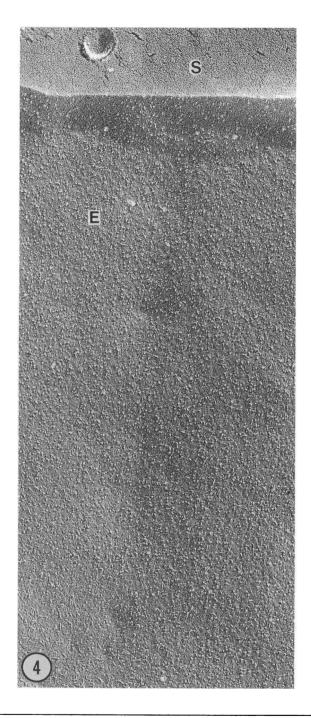


FIGURE 4 External (E) membrane face of a control human red blood cell frozen between copper plates by the plunge method and then freeze fractured. The frozen extracellular medium is labeled S. 60,000X.

neck of an elongated membrane evagination which may have a smaller opening at its apex.

Views in the plane perpendicular to the membrane were needed to visualize the actual pore opening. We therefore examined thin sections cut from freeze-substituted specimens that were rapidly frozen under conditions identical to those used in the freeze-fracture study. Boundaries representing the original fractured surface of the rapidly frozen specimen could be recognized (Fig. 7) and cells close to this boundary showed no visible ice crystal damage. Cells further from the boundary, on the other hand, were not as well frozen; in fact, ice damage could be easily seen in cells several cell layers further away from the boundary. The poorer freezing further from the copper plates was due to the simple physical fact that when the specimen was plunged into the liquid propane-ethane mixture, the cells adjacent to the copper plates would be the most rapidly cooled and frozen, whereas cells further from the copper plate would be frozen more slowly and therefore have more ice crystals (Heuser et al., 1979). In our experiments it was mainly the first layer immediately adjacent to the copper plate which was fractured.

The task of using the freeze-substitution method to examine the detailed structure of membrane pores was complicated by the fact that the thickness of our thin sections ( $\sim$ 70 nm) was greater than the diameters of many of the smaller circular openings observed in the freeze-fractured membranes. Nevertheless, we did occasionally observe discontinuities in the cell membranes of electroporated red cells (Figs. 8 and 9). Components of cytoplasmic proteins was found adjacent to these discontinuities, which suggests that the contents of the red cell had begun to escape through the membrane pores. Such discontinuities would be representative of the larger, circular membrane openings observed by freeze-fracture.

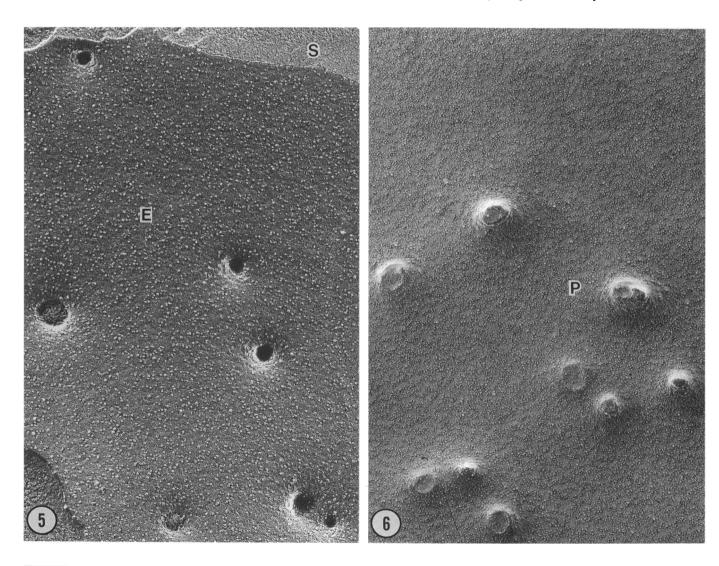


FIGURE 5 and 6 (Fig. 5) External (E) membrane face of an electropermeabilized human red blood cell frozen at 40 ms after the application of a pulse of an RF electric field. 60,000X. (Fig. 6) Protoplasmic (P) membrane face of an electropermeabilized human red blood cell frozen at 220 ms after the application of the electrical pulse. 60,000X.

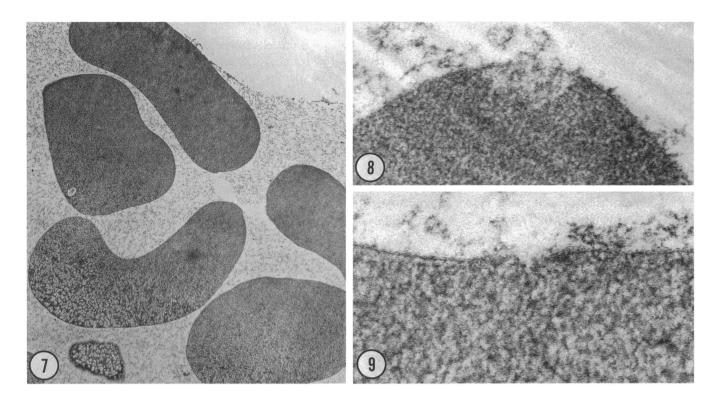


FIGURE 7-9 (Fig. 7) Thin section through freeze-substituted red cells frozen at 220 ms after the application of the electrical pulse and then freeze-fractured before freeze substitution. The top surface was the original fracture face. The cells next to the fracture shows no sign of ice crystal damage. 9,000X. (Fig. 8) Higher magnification view of a freeze-substituted red cell frozen at 220 ms after the application of the electrical pulse, showing a membrane discontinuity which may represent electropores. 60,000X. (Fig. 9) Membrane discontinuity shown in another freeze-substituted red cell. A small amount of ice crystal damage is manifested by the clumping of the hemoglobin. 60,000X.

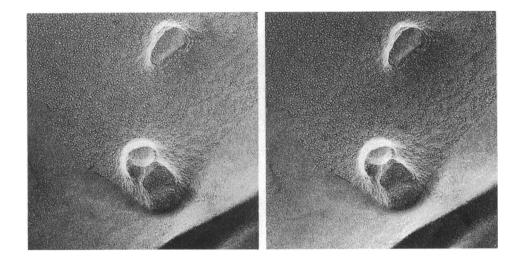


FIGURE 10 Stereo micrographs showing two pore-like membrane evaginations on the freeze-fractured P-face of an electropermeabilized red cell, frozen at 220 ms after applying the electrical pulse. Particle-free areas on the apices of evaginations are sites of membrane cross-fracture. 60,000X.

Although we found that the poration sites induced by the electric pulse generally appeared as circular membrane openings, their detailed shape still varied somewhat from site to site. Most of the membrane openings appeared as very deep pores (Figs. 10, 11, a and b). Occasionally, we observed shallow evaginations that could represent membrane areas which were deformed by the applied electric field but failed to form complete pores (Fig. 11, c and d). In fact, discontinuities in the membrane fracture plane which could represent discontinuities in the bilayer structure could be seen at some of their apices. Other shallow evaginations had a normal complement of intramembrane particles and lacked any visible discontinuity (Fig. 11 e). A second, less frequent type of pore opening appeared flatter and lacked a sharp curvature at its rim (Fig. 11 f). The sizes of the membrane openings at any particular time were not uniform. The

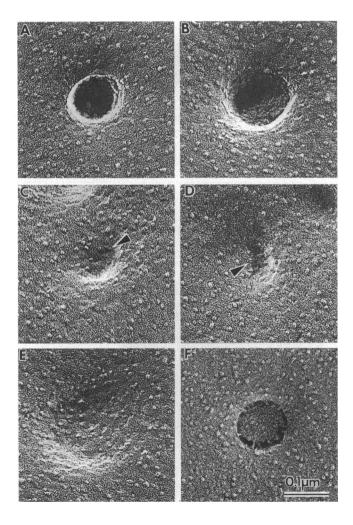


FIGURE 11 Various types of pore-like structures found on E-faces of red cells at 40 ms after an RF electrical pulse. Description of pore types is given in text. 120,000X.

diameters of the openings in ten membrane fracture planes from cells frozen at 40 ms after the electrical pulse varied from 20–120 nm (Fig. 12).

The membrane pore induced by the electric field is presumably a dynamic structure whose shape and diameter might change with time. This is, indeed, what we observed when we examined the membrane structure of cells frozen at different times after exposure to the RF electric field (Fig. 13). At t = 0.5 ms (here t is the time delay between the electrical pulsation and the time when the specimen was submerged by the coolant), the membrane appears to be smooth and without any unusual structural changes; it is virtually the same as the membrane of the control red cell. Cell membranes frozen at a slightly later time (i.e., t = 1 ms) also had a similar appearance. These observations suggest that, if electropores are formed in the first millisecond after application of the electric field, the diameter of these early pores must be very small (less than the resolution of the freezefracture EM,  $\sim 2$  nm). The earliest time that we could detect any significant structural changes in the membrane was at t = 3 ms (Fig. 13 b), where deep, pore-like membrane openings (20-40 nm in diameter) were found in a few E-face membranes of the electropermeabilized red cells. At a later time (t = 20 ms), circular membrane openings became more abundant and the size of the openings appeared to increase. At t = 40 ms, pore-like membrane openings could be observed in almost all fractured membranes, and their diameters had expanded to 20-120 nm (Fig. 13 c). These openings now resembled the volcano-shaped pore-like structures in the E-face and P-face membranes discussed above.

Circular openings apparently induced by the electric field were also observed in membranes frozen at later

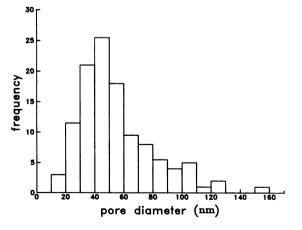


FIGURE 12 Histogram showing the distribution of pore opening diameters at 40 ms after an RF pulse. Data represent measurements from 10 different fractured red cell membranes.

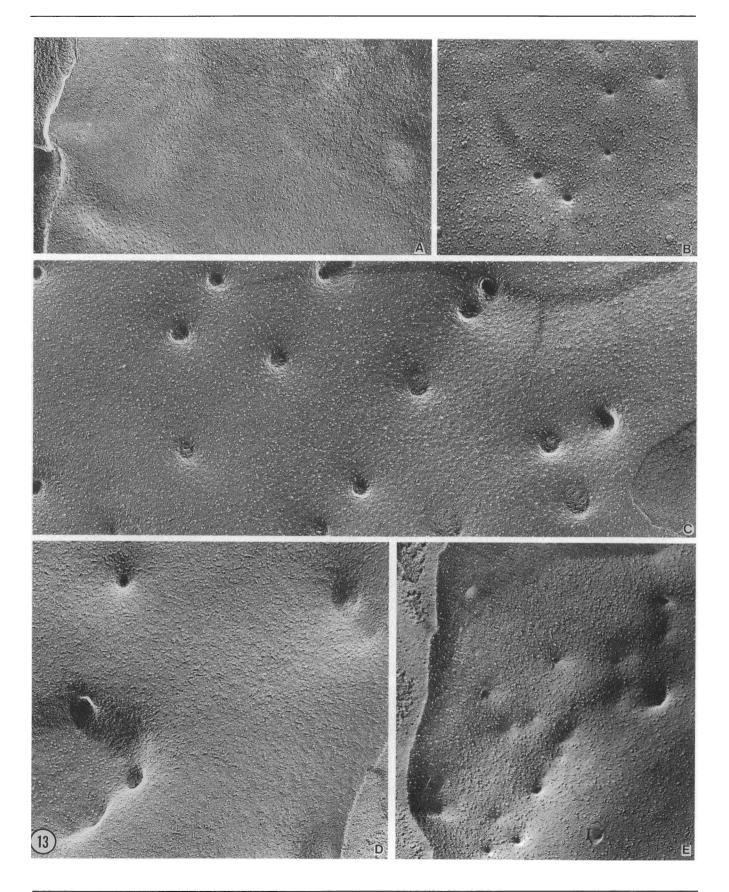


FIGURE 13 Micrographs showing the structure of the cell membranes (E-face) of red cells frozen at different times (t) after the application of the porating electrical pulse. (a) t = 0.5 ms, (b) 3 ms, (c) 40 ms, (d) 5 s, and (e) 10 s. 60,000X.

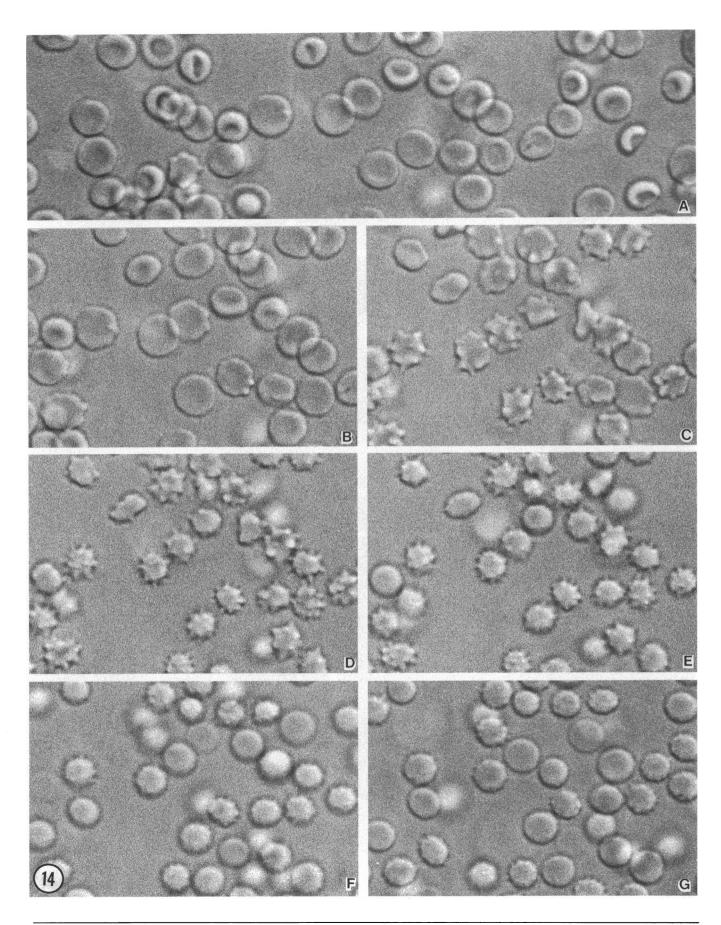


FIGURE 14 Light micrographs of human red blood cells recorded from the same field at various times (t) after electroporation with an RF electrical pulse. (a) control cells, (b) t = 0.2 s, (c) 1 s, (d) 4 s, (e) 10 s, (f) 30 s, and (g) 60 s. The micrographs were taken using DIC optics.

times. At t = 220 ms, circular membrane openings similar to those observed at t = 40 ms could be found in most red cells. Although the shape of these openings appeared to be conserved during this period, the median diameter of the openings seemed to increase, although the upper limit of the diameter remained almost unchanged. At t = 1.7 s, membrane openings like those shown in Fig. 13 c can still be found. At t = 5 s, most of the openings had become more shallow (with a larger area of membrane curving into the opening), and their diameters appeared to be smaller (Fig. 13 d). At t = 10 s (Fig. 13 e), the deep pore-like structures had almost disappeared and were replaced by numerous pit-like indentations in membrane, which may be remnants of previous membrane evaginations. These pits might still have openings in their centers, but such openings would have to be <3 nm in diameter because they could not be detected in the replicas.

The pit-like indentations gradually disappeared with time. At t = 20 s, the membranes of most red cells appeared almost like that of control red cells (not shown). However, some small volcano-shaped membrane openings occasionally could be found in a few red cells, suggesting that not all electroporated cells reseal at the same rate.

# Light microscopy

We used a DIC microscope equipped with video attachment to record the changes of cell shape after the red cells were exposed to an RF electric field that was identical to the one used to porate cells in the EM study. These observations addressed the question of the overall time course of the permeability changes in whole red cells, which we hoped to correlate with the time course of the structural changes in the rapid-frozen red cell membrane. The red cells began to change shape almost immediately after the application of the RF pulse; within a fraction of a second after the electrical pulsation their shape changed from the normal biconcave shape into a more irregular outline and began to shrink (Figs. 14, a and b). At ~1 s, most cells had become spiny shaped and they were smaller (Fig. 14 c). This shrinkage continued for a few seconds (Fig. 14 d) until the diameter reached a minimum at  $\sim 8-10$  s after the electrical pulsation. At about the same time, some cells gradually rounded up to become spherical in shape (Fig. 14e) and their diameter also slowly increased. By 30 s, most cells had become smooth spheres (Fig. 14 f) and their diameters continued to increase though the rate was very slow. The spiny cells were gradually converted into round cells, and a few cells were found to be lysed at 60 s (Fig. 14 g). By analyzing the video images frame-by-frame, we measured the change of the red cell diameter as a function of time after the exposure to the RF field (Fig. 15).

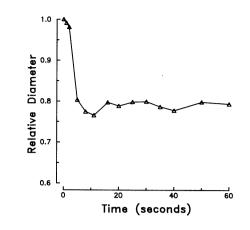


FIGURE 15 Variation in the diameter of red cells as a function of time after electroporation with an RF electric pulse, as measured from videotapes.

Judging from the results of the video microscopy study, nearly 100% of the red cells were porated by the applied RF electrical pulse. The observed rapid changes of cell shape and cell diameters suggest that there was a fast exchange of intracellular and extracellular material after the electroporation. Because our poration medium was hypoosmotic, one would expect that the cell should swell due to a net inflow of water, but the cells shrank instead. This shrinkage must be caused by a rapid escape of cellular content, presumably hemoglobin molecules. The outflow of hemoglobin apparently lasted only for a few seconds, after which the cells stopped shrinking. At later times, the cells gradually swelled, which may be due to a resealing of the membrane pores to the point of becoming too small to allow hemoglobin molecules to enter while water can still pass through.

## DISCUSSION

This study is the first direct morphological examination of the structural changes in cell membrane induced by an electroporating field with a millisecond time resolution. The results of our electron microscopic examination suggest that the process of electroporation is related to the creation of volcano-shaped openings in the cell membrane. Although it is still difficult to determine whether the observed openings represent the true orifices of the pores, or the cross-fractured necks of pores, there is little doubt that these volcano-shaped structures are the sites of electropores. First, these circular openings were not observed in the freeze-fracture views of control cell membranes. Second, these circular openings represent the only discernable changes in the membrane structure which could be consistently found after the cells were electropermeabilized. Third, these openings were the only structures in the electropermeabilized cell membranes that could provide a large enough pathway to allow the fast escape of hemoglobin molecules. Finally, our EM examination suggest that the circular openings disappeared in ~10 s, which corresponds very well with the resealing time of the membrane pores estimated from the egress of cellular content (presumably hemoglobin) as observed by light microscopy. More definitive information about the actual size and shape of the orifice of the pore might be obtained by examining serial ultrathin sections of red cell membranes prepared by freeze substitution; such study is now in progress in our laboratory.

Results of this direct examination of the electropermeabilized membrane have several important implications. Before the present investigation, it was not clear whether discrete physical pores were really formed in the electropermeabilized membrane. It had been suggested that the "electropores" could be membrane "defects", "cracks," or "crater-like structure" (Neumann et al., 1982; Stenger and Hui, 1986; Neumann, 1989), or that they could be partially randomized membrane structures (Zimmermann and Vienken, 1982) that might appear as transient blebs (Escande-Geraud et al., 1988). Because the appearance of discrete pore-like structures were the only observed change in the cell membrane after electroporation, our findings may eliminate several alternative explanations of electroporation and point to the volcanoshaped structures (Figs. 5 and 6) as the site of electropores.

Furthermore, this study reveals the dynamics of pore formation and pore resealing. Our findings suggest that the evolution of the membrane pore can be roughly divided into three stages: in the first stage (consisting of the first few milliseconds after the electrical pulsation), pores are being created and rapidly expanding. In the second stage (from a few milliseconds until several seconds), the pore structures remain basically stable, with the exception that some of the smaller ones may continue to expand a little. In the third stage, pores begin to shrink due to a resealing process. At first they probably shrink into partially resealed pores, which are smaller in size and may have a much longer lifetime than the transient membrane openings observed in the second stage. Studies on the transport properties of electropermeabilized red cell membrane have suggested the existence of small pores (~1 nm in diameter) which last for many minutes (Kinosita and Tsong, 1977) but these would have been too small to be detected by our methods.

Our observation that the membrane openings appeared to undergo a significant expansion in the first few milliseconds after the electrical pulsation is particularly interesting. Previous measurements of electrical properties of electroporated cells suggested that some electropores could be formed within microseconds (Kinosita and Tsong, 1979; Kinosita et al., 1988). Because the electrical properties of a membrane are mainly determined by its permeabilities to ions, such fast-forming pores could be very small. For instance, a pore with a diameter on the order of 0.5 nm would easly allow most small ions to pass through (Hille, 1984). Our observations suggest the electropores which allow large molecules (such as hemoglobin or DNA) to pass through may take longer time to develop.

The application of an intense electric field could have a chain of effects on the structure of cell membrane that might include: (a) primary effects, including dielectric breakdown due to the induced membrane potential (Zimmermann et al., 1980) and the structural fatigue caused by mechanical stress (Chang, 1989a); (b) secondary effects, including effects created by the movements of ions and mobile molecules (including water) after the cell membrane is permeabilized by the electric field, which might include local heating and membrane stress caused by material flow; (c) tertiary effects, which are due to changes in the cell as a result of membrane permeabilization, including cell swelling or shrinking and disruption of cytoskeletal structures due to changes in the local ionic environment.

Observations in the present study suggest that the process of electropore formation is not determined solely by the primary effects. Because our applied electrical pulse was only 0.3 ms long, the continuing changes in the membrane pores must depend on factors that were created by the initial breakdown of the membrane, such as movements of ions and water molecules, and the instability of the bilayer structure due to the formation of the initial pores. In red cells the transport of hemoglobin molecules may be particularly important in influencing the shape of the membrane pores. Our observed coincidence between the lifetime of the volcano-shaped membrane openings and the time course of egress of hemoglobin molecules suggests that the flow of hemoglobin could be one of the forces shaping the pores. Thus, the secondary effects are likely to play an important role in the electroporation process.

In most earlier work on electroporation, it was assumed that membrane pores were formed by a mechanism called "reversible breakdown" which was based on observations in lipid bilayers (Zimmermann and Vienken, 1982; Chernomordik et al., 1983; Glaser et al., 1988). Recently we pointed out that electropores in the cell membrane could be caused by an "irreversible breakdown" of the membrane in a local region (Chang, 1989*a*). The findings in this EM study seem to be consistent with this more recent view. The red cell membrane is attached to a network of cytoskeletal (or membrane-skeletal) proteins consisting mainly of spectrin, membrane actin, and ankyrin (Bennett, 1985; Cohen and Branton, 1979). The dimension of the holes of this network is in the order of 40–100 nm (Steck, 1989; Liu et al., 1987). If electropores are formed by an irreversible breakdown of the cell membrane in local regions, and their structures are stabilized by the attachments to the cytoskeletal proteins, one would expect that: (a) the maximum size of the pore complex should be in the order of 40-100 nm; and (b) the pore could initially expand very quickly until it reaches a stable size comparable to the holes in the cytoskeletal network. These predictions are in good agreement with our experimental findings. Not only do the dimensions of the volcano-shape membrane openings match the size of the holes in the cytoskeletal network, the dynamics of pore evolution also indicates that the volcano-shape structures became stabilized when they reached a size comparable to that of holes in the cytoskeletal network. Hence, results of this study support the notion that electropores are limited by the membrane-cytoskeletal interactions.

The existence of large, transient, pore-like structures offers an important clue to solving a major puzzle in electroporation. Previously, most of the understanding about the properties of electropores were based on transport studies, in which minutes were usually required to conduct the measurements. Because the membrane pores could have undergone a partial resealing by then, it is not surprising that the measured diameter of the electropores was only on the order of 1 nm (Kinosita and Tsong, 1977). It would be difficult to explain how macromolecules, such as large circular DNA (which may be several micrometers long and >6 nm wide) could enter the cell through such small pores. Findings from the present study suggest that electropores during the first few seconds after the electrical pulsation could have an opening as large as 20-120 nm, which would be large enough to allow gene fragments to pass through. The lifetime of these pore-like structures  $(\sim 10 \text{ s})$  is also long enough to allow long molecules (such as DNA) to diffuse into the cells.

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