# A Long-lived Fusogenic State Is Induced in Erythrocyte Ghosts by Electric Pulses

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*Abstract.* Treatment of erythrocyte ghosts in random positions in a suspension with membrane fusion–inducing direct current electric field pulses causes the membranes to become fusogenic. Significant fusion yields are observed if the membranes are dielectrophoretically aligned into membrane–membrane contact with a weak alternating electric field as much as 5 min after the application of the pulses. This demonstrates that a long-lived membrane structural alteration is involved in this fusion mechanism. Other experiments indicate that the areas on the membrane which become fusogenic after treatment with the pulses may be very highly localized. The locations of these fusogenic areas coincide with where the transmembrane electric field strength was greatest during the pulse. The fusogenic membrane alteration, or components thereof, in these areas laterally diffuses very slowly or not at all, or, to be fusogenic, must be present at concentrations in the membrane above a certain threshold. The loss of soluble 0.9–3-nm-diameter fluorescent probes from resealed cytoplasmic compartments of randomly positioned erythrocyte ghosts occurs through electric field pulse-induced pores only during a pulse but not between pulses or after a train of pulses if the probe diameter is 1.2 nm or greater. For a given pulse treatment of membranes in random positions in suspensions, an increase in ionic strength of the medium results in (*a*) a decrease in loss during the pulse, (*b*) no difference in loss between pulses, and (*c*) an increase in fusion yield when membrane–membrane contact is established. The latter two results (*b* and *c*) are incompatible with a fusion mechanism that proposes a simple relationship between electric field–induced pores and fusion.

MEMBRANE fusion induced by electric fields (2, 3, 15, 26, 27) offers several unique characteristics both for studies of fusion mechanisms and for applications in which delivery of membrane contents or entrapped loads to a target is a component of the experiment. These characteristics include the possibility of very high fusion yields (2, 3, 15, 26, 27), control of the moment of fusion to small fractions of a second (2, 3, 15, 19, 26, 27), fusion synchrony which is instantaneous as far as human perception can determine (19), and fusion induction without the use of exogenous chemicals. The first three of these characteristics make it possible to study or induce fusion at the single cell or single membrane level. Electric field–induced fusion has been demonstrated in many membrane systems suggesting wide applicability of this fusion method (2, 3, 15, 26, 27).

Membrane fusion by electric fields (electrofusion) has been previously achieved if membranes of different cells are in close contact with each other and one or more strong direct current pulses are applied to the medium to develop a high transmembrane strength electric field (500 to 1,000 V/mm) in the region of close contact (2, 3, 15, 26, 27). Membranes of cells floating in suspension have been brought into close contact by micromanipulation (1, 17), by using agents which aggregate the cells (11, 25) or by applying an appropriate continuous alternating current (AC) to induce a low strength alternating electric field (7 to 15 V/mm) in the suspension (2, 3, 15, 26, 27). The alternating electric field causes the cells to become aligned into long parallel rows of cells in contact with each other (the so called "pearl chain" formation). This alignment phenomenon is called dielectrophoresis (14). Parts of membranes of different cells can be in close membrane–membrane contact under certain experimental conditions (e.g., cells resting in a monolayer after gravity sedimentation onto a substratum (20) or grown to confluence while attached to a substratum [12, 22]).

Regardless of the method by which membrane–membrane contact is achieved, other laboratories have reported the use of an electrofusion protocol in which membrane–membrane contact is established before and maintained during the application of the fusion-inducing pulses. The electrofusion phenomenon and earlier reports of pulse-induced pore formation (8, 16, 18) have led to the proposal of a hypothetical mechanism that depends on this protocol (2, 3, 15, 26, 27). Thus pulses induce one or more pairs of concentric pores, one in each of the two membranes, in the membrane–membrane contact areas. In this mechanism, membrane fusion and pore induction occur simultaneously without communication developing between the cytoplasmic compartments

and the extracytoplasmic space (see Fig. 17 of reference 26). In a variation of this mechanism, cytoplasmic–extracytoplasmic communication exists during the interval after pore formation but ends upon fusion (see Fig. 10 of reference 3). In either version of the proposed mechanism, concentric pore pairs are portrayed to play an integral and simple role in the fusion mechanism.

We recently reported that a significant fusion yield could be obtained if fusion-inducing pulses are applied to suspensions of erythrocyte ghost membranes in random positions and then AC is applied immediately afterward to bring about membrane–membrane contact (19). This observation is not predicted by the currently proposed electrofusion mechanism (2, 3, 15, 26, 27).

We present here new data that (a) identify a pulse-induced structural alteration in the erythrocyte membrane which is not only fusogenic but is long-lived; (b) indicate that the fusogenic membrane alteration may be located within a very small fraction of the entire surface of the membrane; and (c) suggest that electric field–induced pores may not be directly or solely involved in this fusion mechanism. Portions of this work have been reported in preliminary form (21).

## Materials and Methods

The preparation of erythrocyte ghost membranes, labeling the ghost cytoplasmic compartments with fluorescein isothiocyanate–dextran (FITC–dextran)[1] labeling the ghost membranes with 1,1'-dihexadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI), devices for electric pulse generation, fusion chamber construction, protocols, dilutions of pellets for optimum visual observation, and sources of chemicals were as previously described (19) with the following modifications. Ghost membranes were prepared unlabeled and labeled with either DiI or FITC–dextran in 30 mM phosphate buffer (pH 8.5). To change ionic strength, membranes in pellets were resuspended, washed twice, and pelleted in either 20, 40, or 60 mM sodium phosphate (NaPi) buffer (pH 8.5). In separate experiments the cytoplasmic compartments were labeled with 6-deoxy-N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]-aminoglucose (NBD-G), Lucifer Yellow, and R-phycoerythrin. These probes had effective molecular diameters of ~0.9, 1.2, and 8.8 nm, respectively. All such labeled membranes readily lost their fluorescence to the background upon one or two slow freeze-thaw cycles. NBD-G and R-phycoerythrin were obtained from Molecular Probes Inc. (Junction City, OR) and Lucifer Yellow was obtained from Sigma Chemical Co. (St. Louis, MO). The pulse-induced leakage of each fluorescent soluble marker from the cytoplasmic compartments was monitored as follows. The number of cytoplasmic compartments in which the fluorescence remained distinctly above the background fluorescence (the incremental diminution of fluorescence from cytoplasmic compartments during each pulse was matched by a corresponding incremental increase in background fluorescence) after a given pulse treatment was counted and divided by the number of fluorescent cytoplasmic compartments in the same field of view before the pulse treatment. This ratio was subtracted from unity to get the fraction of membranes which completely lost the label. In all soluble fluorescent marker loss experiments, the pulses were applied to membranes in random positions in suspensions. In all fusion yield experiments, pulses were applied before membrane–membrane contact was induced. Also, as previously observed (19), pulse-induced shape changes occurred with all membranes ending in the perfect sphere geometry as pulses were applied. Fusion yields were calculated by counting all fusion events in which fluorescence from a DiI-labeled membrane moved to at least one unlabeled membrane in the same pearl chain and dividing by the prefusion number of labeled membranes (labeled and unlabeled membranes were mixed in the ratio 1:14). Pulses were applied at the rate of 1/s.

Two experiments were conducted to determine if the fusogenic property induced in the fusogenic areas by pulses could diffuse laterally in the plane of the membrane and to determine if localized parts of the membrane or all

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[1] Abbreviations used in this paper: DiI, 1,1'-dihexodecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; FITC, fluorescein isothiocyanate; NBD-G, 6-deoxy-N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]-aminoglucose.
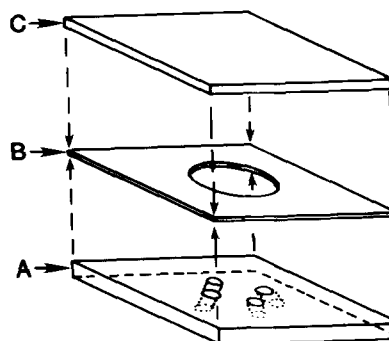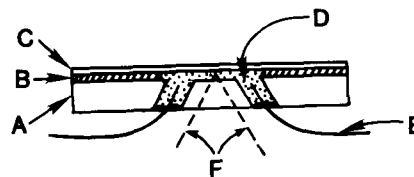
Figure 1. Chamber to permit axis of electric field lines from AC used align membranes to be parallel or perpendicular to axis of electric field lines from DC fusion-inducing pulses (see Fig. 2). Upper view is cross section; lower view is exploded perspective. Plexiglas (Rohm and Hass Co., West Hill, Ontario, Canada) sheet (2.4 mm thickness) in which four electrode holes (1.6 mm diameter) converge at 30° angle to center (center-to-center hole spacing: 3.2 mm). Chamber is formed by Plexiglas sheet (A). Parafilm (American Can Co., Greenwich, CT) sheet (B) with hole (5.5 mm diameter) to surround outer limits of electrode holes, forms a gasket between Plexiglas sheet and cover slip (C). Seal is made by application of heat to the Parafilm. Membrane suspension (D) is added to chamber through one of the electrode (E) holes. Lines (F) indicate path of light from phase condenser.

locations of the membrane became uniformly fusogenic. Both experiments used a fusion chamber design (Fig. 1) which had four ports for electrodes. The ports were located at the corners of a square. In the first experiment (Fig. 2), pulses applied through two electrodes in opposite corners of the square were followed, in the first case, 15 s, or in the second case, 300 s later, by alignment into membrane–membrane contact by applying AC through the same pair of electrodes, and fusion yield was scored. The same protocol was used in the second experiment with one change. The alignment of the ghost membranes into membrane–membrane contact was accomplished by AC applied through the pair of electrodes which did not carry the pulses. Because Brownian motion-induced tumbling was insignificant (see Discussion), membrane–membrane contact in the first experiment was established at or near locations (the poles) on the membranes where the pulse-induced transmembrane electric field strength was the highest. In the second experiment (Fig. 2), the membrane–membrane contact was established at or near locations (the equator) on the membranes where the pulse-induced transmembrane electric field strength was at or near zero. In all four cases, ghost membranes were suspended in 60 mM buffer (see above) and 10 pulses with a decay halftime of 0.8 ms were used to generate an electric field strength of ~700 V/mm. Regardless of conditions, separate experiments (data not shown) on electrofusion yields and pore-related loss of the cytoplasmic marker in membranes aligned by an alternating electric field for long periods (min) before fusion-inducing pulses were applied were no different than for short periods (seconds). The length of time membranes are exposed to the alternating electric field to induce pearl chain formation thus appears to have no effect of the processes involved either due to direct effects of electric fields or to indirect effects such as Joule-heating. Small shavings of octadecane (Sigma Chemical Co.), a low melting temperature (28–30°C) wax, were added to our fusion chamber and exposed to extensive numbers of pulses. As no evidence of melting could be observed, we concluded that significant heating did not take place.

A: DC Pulses    B: AC Alignment

I:

II:
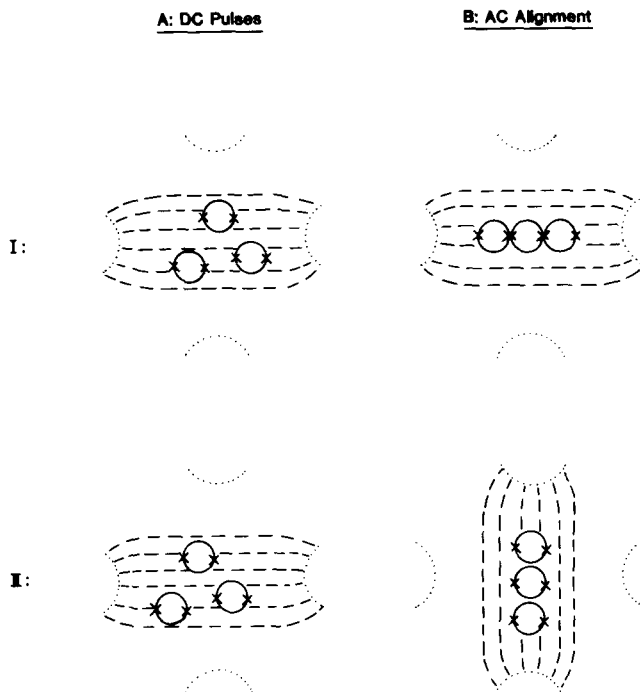
*Figure 2.* Principle of the experiment to determine size and lateral mobility of fusogenic sites in fusogenic area in DC pulse-treated membranes. Dimensions are not to scale. Membranes are indicated by solid circles. Field orientation is indicated by dashed lines. Edges of electrode holes are indicated by dotted lines. (I) Transmembrane field strength from pulse treatment in step $A$ is greatest at sites ($\times$) on membranes where contacts are made by passing an alternating current in step $B$ through same electrodes that carried the DC current pulses. (II) Axis of electric field from DC pulse treatment (step $A$) is perpendicular to axis of AC field application (step $B$): membrane–membrne contacts are made at the location where the pulse-induced transmembrane field strength was at or near zero. Observations were always made in center of chamber where electric field lines were most parallel.

## Results

Whether membrane–membrane contact was established 15 s or 120 s after the application of pulses, fusion yields were generally both proportional to pulse strength (i.e., peak electric field strength and decay halftime) and pulse number (Fig. 3).

Membranes brought into membrane–membrane contact at places on the membrane where the transmembrane electric field strength was expected to be highest during a pulse resulted in fusion yields of 18 ± 7 (SD)% or 8 ± 3 (SD)% for contact made 15 s or 300 s after the pulse treatment, respectively. In contrast, for membranes brought into membrane–membrane contact at places where the transmembrane electric field was expected to be at or near zero the fusion yields for 15-s or 300-s intervals between the pulses and membrane–membrane contact were 1.8 ± 1.7 (SD)% and 1 ± 0.8 (SD)%, respectively.

The loss of fluorescence from FITC–dextran–labeled cytoplasmic compartments of randomly positioned erythrocyte ghosts through pulse-induced pores was also proportional to pulse strength and number (Fig. 4). For a given pulse treatment, an increase in the ionic strength of the membrane suspension generally resulted in an increase in electrofusion yield, particularly for higher pulse numbers and longer decay
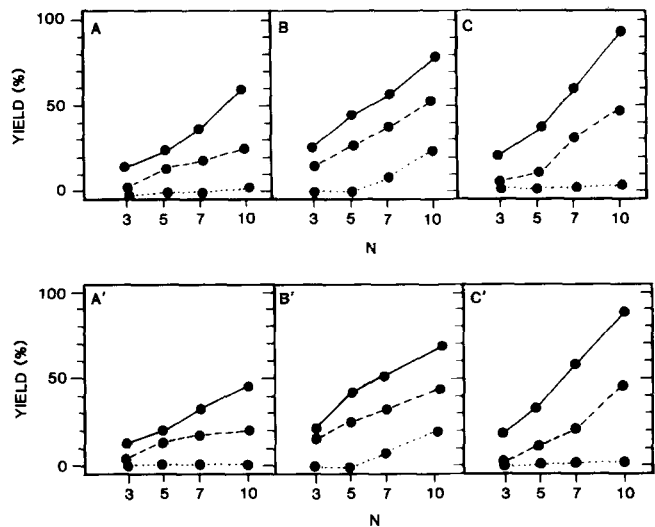


*Figure 3.* Fusion yield (%) as a function of three variables: buffer (NaPi, pH 8.5) strength ([$A$] 20, [$B$] 40, or [$C$], 60 mM), number of pulses ($N$), and decay halftime ($t_{1/2}$) of DC pulses. Decay halftimes: (...) 0.2 ms; (- -) 0.6 ms; (——) 1.2 ms. Peak electric field strength during pulse: 700 V/mm (yields at 500 V/mm, not shown, were proportionately lower). Membrane–membrane contact was established with AC either 15 s ($A$–$C$) or 120 s ($A'$–$C'$) after the final pulse in a sequence of 3–10 pulses applied while membranes were in random postiions.
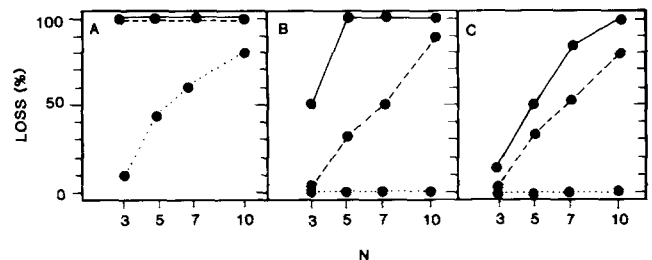


*Figure 4.* Loss of FITC–dextran from resealed cytoplasmic compartments of erythrocyte ghosts which were in suspension in random positions as a function of Fig. 3 variables and peak pulse field strength (losses at 500 V/mm, not shown, were proportionally lower). Loss (%) was scored as ratio of distinct fluorescent cytoplasmic compartments remaining after a pulse treatment to number of fluorescent cytoplasmic compartments present before the pulse treatment subtracted from unity and multiplied by 100.

half times of the pulse (Fig. 3), and generally a strong decrease in FITC–dextran loss (Fig. 4). The pattern of loss of NBD-G and Lucifer Yellow from labeled cytoplasmic compartments was similar to that for FITC–dextran (data not shown) except that ($a$) generally fewer pulses were required for a given loss and ($b$) unlike Lucifer Yellow, a fraction of the NBD-G-labeled membranes slowly lost a considerable amount of fluorescence up to 1–2 min after any pulse treatment. Ghost membranes labeled with R-phycoerythrin, on the other hand, never lost any fluorescence regardless of the pulse treatment.

## Discussion

The observation of significant fusion yields when membrane–membrane contact was established up to 5 min after the fusion-inducing pulse treatment of membranes in random positions (i.e., not held in close membrane–membrane con-

tact) showed that a long-lived fusogenic state was induced by the pulse treatment.

Membrane–membrane contacts made 15 s after pulses were applied resulted in high (18%) or low (2%) fusion yields depending on whether the membrane–membrane contact points were at or near the locations where the highest or lowest strength pulse-induced transmembrane electric fields were experienced, respectively. When membrane–membrane contacts were made 300 s after the pulses were applied, fusion yields were about half as high for the high field location (8%) but still low (1%) for the low field location. It is possible that the fusogenic structural alteration may not have fusogenic components that laterally diffuse freely in the plane of the membrane. It is also possible that the fusogenic components laterally diffuse from the locations where they were induced but must reach a minimum threshold concentration at locations where they were not induced before they can initiate fusion.

Brownian motion–induced tumbling of a spherical-shaped ghost membrane with a radius of 3.5 $\mu$m can be quantitatively estimated using Perrin's equation (13). Thus the average per membrane net tumble would be ~4.2 degrees of arc in 300 s. An angle of 4.2° rotated about one of the two lines forming that arc would intersect a circular area of 0.27% of the total area of a hemisphere. The area would have a radius of ~0.25 $\mu$m. Thus the data could be interpreted to mean that the fusogenicity at the circumference of this area is half of what it was at the center of this area. If fusogenic areas are induced at both locations (in the centers of both hemispheres) where the transmembrane-pulsed electric field strength was greatest and their movement is tied to membrane tumbling, then they represent a very small fraction of the total membrane surface area. This may explain why no fusion was observed when ghost membranes in a fusion chamber were treated first with pulses while in random positions in suspensions, and then the fusion slide was given a mechanical flick about an axis perpendicular to the axis of the electric field pulses before the membranes were brought into membrane–membrane contact with AC. It is possible, however, that the pulse treatment may have induced a disturbance in the medium such that ghost tumbling induced by Brownian motion would not occur exactly as predicted by Perrin's equation.

Our data (Fig. 4) show that a greater total loss of label occurs with increased total number of applied pulses or if the pulses have greater decay halftimes or develop a greater electric field strength in the suspension medium. The fact that FITC–dextran loss to the extracytoplasmic background occurs in steps and only simultaneous (within human perception) with the passage of each DC pulse (19) indicates that membrane pores are induced with large enough diameters, numbers, or lifetimes to permit a rapid and perceptible efflux of a fraction of the total label from the cytoplasmic compartment and then the pores quickly reclose. For a given pulse treatment a drastic decrease (Fig. 4) in loss of FITC–dextran (i.e., through pulse-induced pores which reached smaller maximum diameters, or were fewer in number, or shorter lived) occurred as the ionic strength was increased. However, significant loss of label between each pulse, separated by as much as 1–2 min, or after a train of pulses was never observed at any ionic strength. The fact that a greater loss of Lucifer Yellow or NBD-G occurred during each pulse suggests that the decrease in pore diameter down to or close to the effective

diameter of the probe takes longer. The fact that no loss of R-phycoerythrin could be detected with any of the pulse treatments indicates that the pulse-induced pores either never reached diameters >8.8 nm or reclosed so quickly that only insignificant amounts of the label could escape. Nevertheless, significant loss of fluorescent label between pulses or up to 1–2 min after a pulse was not observable for Lucifer Yellow. However, for NBD-G, pulse treatment of many membranes resulted in a slow continuous loss of the fluorescence. Therefore, after the pulse the diameter of the induced pores decreases to some dimension close to or smaller than the molecular diameter of the probe molecule (i.e., ~1.2 nm). Other studies (8, 16) also indicate that the diameter of pulse-induced pores in erythrocytes diminishes monotonically with time and that pores with initially smaller diameters close to a given diameter sooner than pores with initially larger diameters. Lieber and Steck (9, 10) showed that the pores induced in erythrocytes during the preparation of Dodge ghost membranes (4) are circular holes with a residual diameter of 1–2 nm and number one per membrane. The participation of this hemolytic hole in membrane fusion is unlikely because the probability of a hemolytic hole being present in a membrane contact area is extremely low compared to the high fusion yields observed.

It has been indirectly estimated that pulse-induced pores in intact erythrocytes have a total pore area which is, at most, ~$10^{-5}$ of the total membrane area, have diameters of at least 0.5–1.5 nm, and number from about $10^0$–$10^3$ per membrane (8, 16). Their geometry, location, and dependence on pulse waveform have not been characterized. In erythrocytes they can reseal in minutes or less or up to several hours depending on experimental conditions (18). The probability of head-on collision of free edges of pulse-induced pores as originally separate membranes are brought into membrane–membrane contact should, to a first approximation, be proportional to the pore area fraction of total membrane area (i.e., ~$10^{-5}$). If the above estimate of total pore area is valid and the intact erythrocyte membranes can be compared, in this case, with erythrocyte ghost membranes, then this is low by at least 3 orders of magnitude for pore collisions to account for observed fusion yields even if the total pore area is confined to a small area (~$10^{-2}$) of the total area of the membrane.

If pulse-induced pores laterally diffuse in the plane of the membrane at rates comparable to membrane lipids or membrane proteins, then (depending on assumptions of lateral diffusion coefficient, pore density and pore diameter, and contact area) a pore on one membrane could encounter a pore on another membrane at a finite time after membrane–membrane contact is established. Thus the pore–pore encounter probability could approach observed fusion yields. However, this would result in the observation, at least under some of our conditions, of fusion events occurring at times ranging up to several seconds or more after membrane–membrane contact is established. In contrast, all observed fusion events as monitored by the instantaneous movement of FITC–dextran to unlabeled cytoplasmic compartments were always simultaneous, within human perception, with either the application of pulses if membrane–membrane contact was established first or upon membrane contact (19) if pulses were applied first. Hence, the pores either (a) diffuse laterally in the membrane, at very slow rates, or (b) are laterally immobile (possibly due to cytoskeleton–membrane restrictions on lat-

eral mobility), or (c) do not exist in sufficiently large numbers to be fusion-initiating. Also a time-dependent acceleration in loss of FITC–dextran from randomly positioned ghosts after any pulse treatment was never observed suggesting that, if pores diffused laterally and collided with themselves on the same membrane, they did not coalesce into larger pores.

The diminution in fusogenicity of the fusogenic state is not likely to be due to repair based on metabolic processes because ghost membranes should be highly depleted of metabolic substrates and intermediates. While little is known about the effect of electric field pulses on lipids, a number of papers have been published on pulse-induced conformational changes in bacteriorhodopsin (23, 24) and model peptides (5, 6). These studies all used pulse field strengths up to 10–20 times higher than used in our study. In another study (7), mouse erythrocytes were loaded with $^{14}$C-labeled sucrose through pores which were produced by pulses that had order of magnitude similarity with our pulses, and were then injected back into mice. Since the survival of these pulse-treated erythrocytes was found to be similar to the normal half-life of the erythrocytes, it could be concluded that the pulse treatment resulted in little or no irreversible changes of biological significance.

The existence of an earlier body of literature on pulse-induced membrane breakdown has made the hypothetical involvement of pulse-induced pore formation in the mechanism of membrane electrofusion intuitively convenient although no experimental evidence for this involvement has yet been presented (2, 3, 15, 26, 27). In our experiments there is no question that both membrane pore formation and membrane fusion took place in the erythrocyte ghost membranes after the application of fusion-inducing pulses. However, the fact that (a) a long-lived (time scale on the order of minutes) fusogenic state was revealed when membrane–membrane contact was made to occur a finite time after the pulses were applied, (b) the observation of pore resealing in the tens to hundreds of millisecond time scale, and (c) the lack of a correlation between pulse-induced FITC–dextran loss and fusion yield when ionic strength is changed all indicate that pore formation as previously presented (2, 3, 15, 26, 27) may not be involved in a simple way in this electrofusion protocol.

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