brief communication

Determination of electric field threshold for electrofusion of erythrocyte ghosts

Comparison of pulse-first and contact-first protocols

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ABSTRACT Rabbit erythrocyte ghosts were fused by means of electric pulses to determine the electrofusion thresholds for these membranes. Two protocols were used to investigate fusion events: contact-first, and pulse-first. Electrical capacitance discharge (CD) pulses were used to induce fusion. Plots of fusion yield vs peak field strength yielded curves that intersected the field strength axis at positive values (pseudothresholds) which depended on the protocol and decay half time of the pulses. It was found that plots of pseudothreshold vs reciprocal half time were linear for each protocol; when extrapolated to reciprocal half time = 0 (i.e., $t \rightarrow \infty$), these lines intersected the ordinate at values of the field strength considered to be the true electrofusion thresholds. In this fashion, the contact-first protocol gave an electrofusion threshold of 46.5 ± 11.5 V/mm for hemoglobin-free ghosts (white ghosts) and 40.9 ± 8.8 V/mm for ghosts with fractional hemoglobin (pink ghosts), while the threshold for the pulse-first protocol applied to pink ghosts was determined to be 93.4 ± 11.0 V/mm. Although the thresholds depended on the electrofusion protocol, plots of critical field strength vs reciprocal time had the same slopes, i.e., ~24 Vs/mm. The results suggest that the fusogenic state induced by an electric pulse in either the contact-first protocol or the pulse-first protocol (long-lived fusogenic state) may in fact share a common mechanism, if the two states are not actually identical.

INTRODUCTION

In recent years, electroporation and electrofusion have become important tools for biomedical research (Kinosita and Tsong, 1977; Zimmermann, 1982; Neumann et al., 1982; Potter et al., 1984; for excellent reviews see Sowers, 1989 and Neumann et al., 1989). Several models for the mechanisms of electroporation and electrofusion have been introduced based on the theoretical analyses of membrane electrocompression or breakdown (Crowley, 1973; Abidor et al., 1979; Sugar and Neumann, 1984; Dimitrov, 1984), but relatively little is known as yet about the definitive mechanisms responsible for either process. For example, it is not immediately obvious that electrofusion as a function of field strength should follow any mathematical relationship that gives information about the fusion mechanism, because it is not intuitively obvious why electrofusion yield should map one-on-one with a relationship that describes molecular membrane changes as a function of electric field strength or energy delivered.

The transfer of energy from an electrical field to the membrane necessarily requires that molecules in the membrane be responsive to electric fields (Tsong and Astumian, 1987). Such molecular transducers must be able to change the membrane in some physico-chemical respect so that at least temporarily, the membrane develops an alteration that makes electroporation or electrofusion possible. In any case, an energy or electric field threshold is implied that must be attained before the membrane alteration takes place. It has been reported that an inverse relationship between pulse strength and pulse length exists for membrane electroporation and electrofusion. The electric field threshold could be derived from this relationship. For example, in electroporation of CHO cells, this threshold is estimated to be ~30 V/mm (Rols and Teissie, 1990). It corresponds to a critical transmembrane potential (~270 mV) according to Cole (1968), and this, in turn, is a source of potential energy for molecules. If specific membrane molecules are involved, this critical potential would apply directly to them and could in principle result in the transfer of energy from an electric field to the membranes.

One potentially confounding possibility is that electrofusion can be accomplished through more than one mechanism. Electrofusion was first described in cells that had been brought into physical contact by dielectrophoresis before the application of a fusion-producing pulse, via the contact-first protocol (Neumann et al., 1980; Scheurich et al., 1980; Senda et al., 1979). Subsequently, it was discovered that electrofusion could be produced by a pulse-first protocol, in which cells are electropulsed before dielectrophoresis. This suggested the possibility that an alternate mechanism from that operating in the contact-first protocol might exist (Sowers, 1987; Teissie and Rols, 1986). The question still

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remains as to whether fusion induced with the pulse-first protocol is mediated mechanistically the same way as is fusion obtained with the contact-first protocol.

In this study, electrical CD pulses were applied to erythrocyte ghosts to develop curves (each at a fixed decay-half time, $t_{1/2}$) for fusion yield vs applied field strength; each curve was found to intersect the pulse field strength axis at a positive value. These intersections, referred to previously as pseudothresholds when applied to cell electroporation because they depended on the duration of applied rectangular pulses (Rols and Teissie, 1990), could be plotted against reciprocal $t_{1/2}$ varied in a series of experiments to give straight lines whose slopes could be measured. The results of this study suggest that the fusogenic states induced with the contact-first and pulse-first protocols may be induced via a common mechanism, even though the nature of such a mechanism is still uncertain.

MATERIALS AND METHODS

Rabbit erythrocyte ghosts were prepared by following a routine method (Dodge et al., 1963). A fraction of the erythrocyte ghosts was labeled with a lipophilic fluorescent dye, $DiIC_{18}$ (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, obtained from Molecular Probes, Inc., Eugene, OR), following the procedure of Sowers (1984). Described briefly, 2.5 ml of whole blood from New Zealand white rabbits were washed in isotonic phosphate NaP_i buffer (40 ml, pH 7.4) and then centrifuged at 300 *g* for 10 min. The supernatant was removed and the pellet was resuspended with 40 ml of hemolysis buffer (5 mM NaP_i buffer, pH 8.5) to cause hemolysis; this suspension was kept on ice for 20 min, after which it was centrifuged at 8,000 *g* for another 20 min. The resulting pellet was washed with buffer (20 mM NaP_i buffer, pH 8.5) and centrifuged again, followed by labeling with DiI. This step resulted in pink erythrocyte ghosts. White erythrocyte ghosts were obtained by following the same procedure with one more step for hemolysis after the first hemolysis step. Pink and white ghosts contain an average of 2.7% (0.15 mM) and 0.23% (0.013 mM), respectively, of the total hemoglobin present in intact red blood cells (Dimitrov and Sowers, 1990). These operations were conducted at 0–4°C.

All electrofusions were performed with an apparatus based on capacitor-discharge (CD). The electrofusion chamber used in the experiments contained two Pt wire electrodes separated by 2 mm as previously described (Sowers, 1984). Pulse strength was specified by the peak electric field strength generated in the chamber (range used: 50–800 V/mm); pulse duration was altered by varying the capacitances and resistances in the electrofusion circuit, and was specified in terms of pulse decay half times ($t_{1/2}$; range used: 0.25–0.95 ms). Electrofusion was performed at 22–24°C under an Olympus IMT inverted epifluorescence microscope, and fusion yield counts were made immediately afterwards.

Fusion yield was determined using fluorescence microscopy as follows. DiI-labeled and unlabeled ghost membranes from the same preparation were mixed in the ratio of 1:5, respectively, in a 20 mM NaP_i buffer (pH = 8.5). The ghost population density was determined with an hemacytometer and diluted to 10^7 ghosts/ml for all experiments. Almost all erythrocyte ghosts in a particular set up could be brought into close contact via dielectrophoresis (Pohl, 1978) for 75 s with a 60 Hz AC field of 20 V/mm peak amplitude. The exponentially decaying DC pulses were applied to the ghosts before (pulse-first protocol) or after (contact-first protocol) alignment. Immediately after application of the fusogenic pulse the fused and unfused ghosts were counted, so that fusion yield (FY) could be calculated from the relationship FY (%) = $[N_m/(N_s + N_m)] \times 100$, where N_m = the number observed of fused fluorescent ghosts, and N_s = the number observed of unfused ghosts (Sowers, 1984).

Fusion yield vs applied pulse strength for each value of $t_{1/2}$ indicated that as $t_{1/2}$ was increased, a leftward shift of the fusion yield curve occurred. By computer-fitting (Sigma Plot, Jandel Scientific, Corte Madera, CA) the fusion yield data according to a third-order polynomial equation ($FY = a + bx + cx^2 + dx^3$), an intersection point (E_p) on the field strength axis could be determined for each $t_{1/2}$; this point is analogous to the previously referred to pseudothreshold in the electroporation of CHO cells (Rols and Teissie, 1990). The relationship E_p vs $1/t_{1/2}$ was found to be linear and the true threshold, E_T, was obtained from the extrapolation of the straight line regressed to the data.

RESULTS AND DISCUSSION

It is not necessary that cells be in contact at the time of application of the fusogenic pulse for them to be capable of fusing; it is known that pulses comparable in strength to those used normally (contact-first protocol) can induce a fusion-ready state that will result in membrane fusion when cells are subsequently brought together by dielectrophoresis. Because this fusion-ready state appears to last for a relatively long while (up to 5 min) it has been referred to as the long-lived fusogenic state (Sowers, 1987; Teissie and Rols, 1986; Montane et al., 1990). This application of pulses before dielectrophoresis is called the pulse-first protocol (Sowers, 1989). In previous work performed by others high membrane population densities ($> 10^8$ ghosts/ml) were used, which necessitated a background correction for fusion yield because of a significant frequency of chance ghost-to-ghost contacts at the time of pulsing (Sowers, 1987). For the present study, the pulse-first protocol was modified in two ways: (*a*) only one CD pulse was used, and this was enough to cause a significant fraction of the ghosts to fuse upon being brought into contact with dielectrophoresis; and (*b*) 10^7 ghosts/ml were used in this study, resulting in negligible background fusion (0–3.5%). Although low membrane population densities produce better results, the density of 10^7 ghosts/ml was considered optimal for those experiments using the pulse-first protocol. This is because experiments using even lower population densities would require either the application of higher field strength or a longer period of AC field during membrane dielectrophoresis, which would increase the experimental complexity and reduce fusion yield in the pulse-first protocol. Having adopted a density of 10^7 ghosts/ml for use with the pulse-first protocol, we also used it with the contact-first protocol,

because this would allow for direct comparisons between the two methods.

Typical examples of the relationship between fusion yield and pulse field strength for the two protocols are given in Fig. 1; the examples shown are only for a $t_{1/2}$ = 0.72 ms, and illustrate the determination of pseudothresholds (E_p) from the abscissa intersect of best-fit curves developed by computer from a third-order polynomial (described in Materials and Methods). It can be seen in Fig. 1 that under similar conditions, increasing either the field strength or $t_{1/2}$ can result in a gain in fusion yield. It can be seen also in Fig. 1 that the fusion yield for the pulse-first protocol is significantly lower than that for the contact-first protocol, a general observation that obtains for the other $t_{1/2}$ as well (data not shown); this is in agreement with results reported by others (Sowers, 1987; 1990). Using the pulse-first protocol to induce cell fusion, three possible phenomena, not significant with the contact-first protocol, could lower the fusion yield: (*a*) rotational diffusion of the cells (i.e., the random rotation of cells that can take place before close cell contact occurs some time after application of the fusogenic pulse); (*b*) lateral diffusion of fusogenic sites due to fluidity of the membrane, so that these sites drift further away from membrane areas that will achieve contact with each other during dielectrophoresis; (*c*) misalignment between cell membranes during dielectrophoresis so that zones of the membrane with fusogenic sites (i.e., where the pulse field was applied perpendicular to the membrane surface) do not coincide with zones of cell-to-cell contact. The first two reasons in practice appear unimportant, at least under certain conditions, because calculation of rotational diffusion for the size ghosts that we used suggested a minimal effect of this

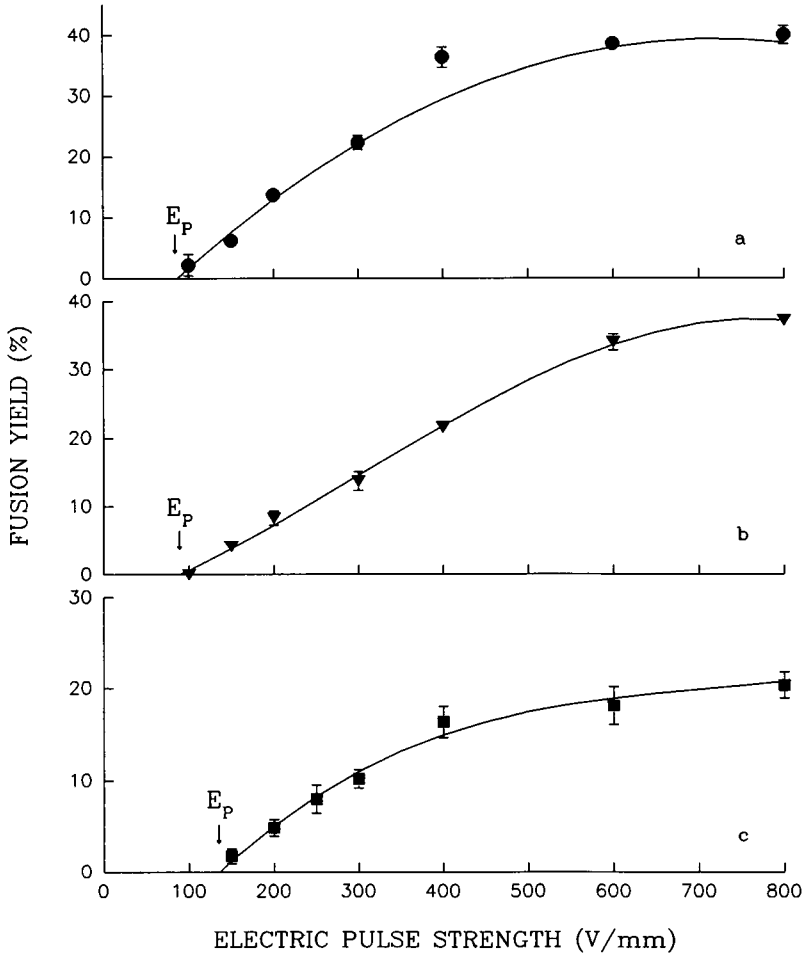

FIGURE 1 Determination of pseudothresholds for electrofusion: representative data for pulse decay half-time of 0.72 ms. Fusion yield vs peak electric field strength was plotted for the contact-first protocol (*a*, pink ghosts or *b*, white ghosts) and the pulse-first protocol (*c*, pink ghosts). A best-fit curve based on a third-order polynomial was used to find the abscissa intersect of the curve to give the electrofusion pseudothreshold, E_p, for each case.

variable. Previous work by others (Sowers, 1987) has shown that the application of electric fields at angles obtuse to pearl chains resulted in fusion yields as predicted by the Cole equation (1968), which shows no dependence on time, i.e., does not allow for diffusion of hypothetical fusogenic sites from one area of the cell to another. Thus, it is possible the phenomenon of misalignment is the major reason for the loss of fusion efficiency seen with the pulse-first protocol.

As was done previously to estimate true electroporation thresholds in CHO cells (Rols and Teissie, 1990), plots of the pseudo-thresholds, E_p, for the different fusion yield curves vs $1/t$ were used to determine the true electric field threshold for electrofusion. However, in the previous electroporation study a train of 10 rectangular pulses was used, whereas in the present case, CD pulses defined by different values of $t_{1/2}$ were employed. A notable observation was the linear relationship between E_p and reciprocal pulse duration for electrofusion. As seen in Fig. 2, E_p vs $1/t_{1/2}$ plots as a straight line of the same slope (23.6 Vs/mm) for both pink and white ghosts and regardless of whether the protocol was contact-first or pulse-first; this is true not withstanding that the threshold for the contact-first protocol ($\sim$43 V/mm) is significantly lower than that for the pulse-first protocol ($\sim$93 V/mm). The threshold value for electroporation of CHO cells was found to be $\sim$30 V/mm (Rols and Teissie, 1990) using the same graphic analysis (i.e., E_p vs $1/t$). The threshold membrane potential (V_m) corresponding to that value can be calculated using Cole's relationship; likewise, the V_m values for electrofusion of rabbit erythrocyte ghosts can be estimated. The results, shown in Table 1, indicate that electroporation in CHO cells and electrofusion in rabbit erythrocytes fused with the contact-first protocol, have the same threshold membrane potential. For instance, the threshold field for the contact-first protocol using pink ghosts yields a threshold V_m = 210 mV, which is consistent with the previous report that this transmembrane potential can change the permeability properties of lipid vesicles (Teissie and Tsong, 1981). This finding is furthermore consistent with electrofusion and electroporation having identical mechanisms, at least for the contact-first protocol; this point has been brought up before without direct evidence (e.g., Neumann, 1989) and even here the evidence remains circumstantial.

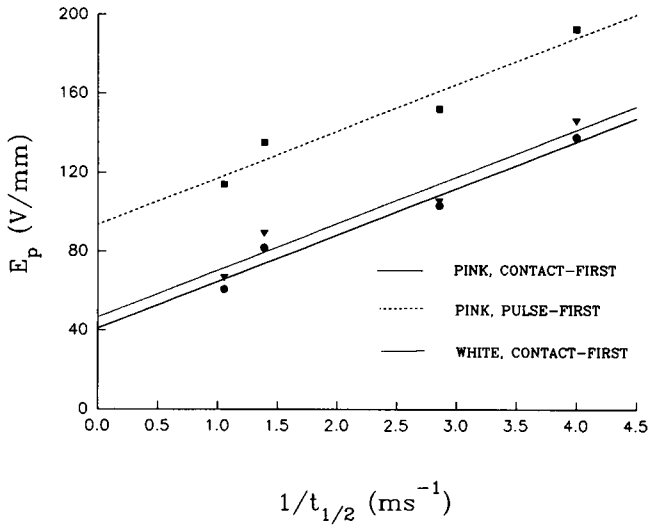

FIGURE 2 Linear regression of pseudothreshold vs $1/t_{1/2}$. Best-fit lines were drawn through the data points and extrapolated to the true threshold (Y-intercept) for each of the three types of experiments. (Best-fit parameters, r^2: pink ghosts, contact-first, = 0.96; white ghosts, contact-first, = .93; pink ghosts, pulse-first, = .94.)

An important thing to consider is the linear relation between pseudothresholds (E_p) and $1/t$. This can be expressed in simple form as

$$E_p = a(1/t) + E_T, \quad (1)$$

where a is a constant and E_T is the true threshold. For the study on electroporation, rectangular electric field pulses result in the applied field E being constant during the application of the pulse. In the present study, this is not the case, as the field decays exponentially with time. The apparent relationship we find is

$$E_p = a'(1/t_{1/2}) + E_T, \quad (2)$$

where a' is a constant in general different from a but E_T is the same threshold as in Eq. 1. The two Eqs. 1 and 2 imply that the pseudothresholds for both electroporation (as determined by Rols and Teissie, 1990) and electrofusion are increased in the same manner, i.e., linearly, as a function of the inverse of the pulse duration. The same kind of linear relationship has been reported for the release of potassium and hemoglobin from human red blood cells by electric pulses (Riemann, et al., 1975); it was found in the electrically induced uptake of fluorescence-labeled dextrans by mouse fibro-

TABLE 1 **Transmembrane voltages corresponding to true electrofusion thresholds: comparison of different protocols**

	E_T(V/mm)	V_m(V)
Pink ghost contact-first	40.9 ± 8.8	0.21
White ghost contact-first	46.5 ± 11.5	0.24
Pink ghost pulse-first	93.4 ± 11.0	0.50
Electroporation CHO cells*	30	0.27

Threshold data for electroporation of CHO cells are also included. See text for details.
*See Rols and Teissie (1990).

blasts for both rectangular and exponential-decay electric pulses (Liang et al., 1988; Kubiniec et al., 1990); and this relationship was preserved also in the electrofusion of human erythrocytes (Sowers, 1989).

It is of interest that if certain assumptions are made both Eqs. 1 and 2 can be viewed as equivalent expressions, even though one is based on rectangular pulses and the other on CD pulses; the linearity of Eq. 1 accordingly would be preserved when using CD pulses instead of rectangular pulses. Equivalency of the forms of both Eqs. 1 and 2 could be maintained if $t_{1/2}$ itself could be transformed in linear fashion to t, that is, $t_{1/2} = k\,t$, where k is a constant. In this way, a rectangular pulse of constant field, E, applied for a duration, t, would be effectively equivalent to a CD pulse of peak pulse strength E but of decay half time $t_{1/2} = k\,t$. It is easy to show using simple electrical theory that this requirement is met when $k = 1.39$ if it is assumed that the mechanism implied in both electroporation and electrofusion couples or transduces in the same way either the total energy delivered to cells or the total charge movements induced by the applied electric field. In the case of charge movements, this is so because the total charge moved through a constant resistance by a rectangular pulse of field strength E and of duration t would be the same as delivered by a CD pulse of maximum field strength E and having a decay half time $t_{1/2} = 1.39\,t$. This transform could be absorbed into the slope term a' in Eq. 2, so that $a' = a\,1.39$. If the mechanistic variable is instead the total energy delivered to the system, it is also easy to show that the two equations can again be reconciled by $t_{1/2} = 1.39\,t$. The physico-chemical process that causes cell fusion to be triggered by an electric pulse is uncertain at present. We do not know what coupling process or processes are at work, however, so there is no prior reason to expect any particular relationship. When the fundamental underlying processes are uncertain, experimentally-determined relationships become especially important. Even in the absence of a proven mechanism, it is possible on the basis of observations to form tentative conclusions regarding some features of the mechanisms involved; these conclusions can form the basis for future investigations.

Finally, if we assume that a' in Eq. 2 is a coupling constant between the electrical pulse and its effect on the cell membranes, the identity of slopes for pseudo-threshold vs reciprocal decay halftime seen in Fig. 2 for both the pulse-first and contact-first protocols suggests strongly a similar mechanism for both protocols; even though we cannot identify the mechanism(s) responsible for electrofusion with any certainty, it seems to us highly unlikely that the virtual identity of the slopes is merely coincidental. On the other hand, the different true thresholds would seem to imply dissimilar mechanisms. To address this point, we suggest that the true threshold values obtained by our approach are only maximum limiting values. In the case of the contact-first protocol, the ghosts are already in contact at the time the fusogenic pulse is applied, and potential inefficiencies are minimized. The threshold value estimated in this fashion is probably very close to the actual threshold applicable to a membrane component that responds to an applied electric field to yield electrofusion. Thus, in pulse-first protocol the apparent threshold, E_T, represents a limiting value whereby certain inefficiencies set by experimental conditions, not directly related to the fusogenic pulse, prohibit fusion yield from ever reaching the value seen for contact-first protocol. The prediction is that E_T for the pulse-first protocol will approach that for the contact-first protocol as the time interval between pulse application and cell-to-cell contact is reduced.

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