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Facilitation of electrofusion of plant protoplasts by membrane-active agents

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Protoplasts isolated from a suspension culture of *Daucus carota* were subjected to electrofusion by means of a combination of dielectrophoresis to align the cells and brief d.c. shocks to induce fusion. The protoplasts were treated with agents known to alter membrane structure and function in order to define the factors that limit electrofusion. Lysophosphatidylcholine, dimethylsulfoxide and calcium chloride enhanced electrofusion; low temperature reduced fusion. However, lysophosphatidylcholine, calcium chloride, and low temperature all decreased membrane fluidity as measured by electron spin resonance spectroscopy of intact carrot protoplasts labeled with 5-doxylstearic acid. These spectra were taken in the presence of $Fe(CN)_6^{3-}$ and thus largely reflect the fluidity of the plasma membrane. Treating the protoplasts with pronase or proteinase K also facilitated electrofusion. The proteinase K effect was largely reversed by the specific protease inhibitor phenylmethylsulfonyl fluoride. Protoplasts labeled with fluorescein isothiocyanate-concanavalin A did not exhibit any perceptible capping or clustering of membrane proteins in response to protease treatment. Enhancement of protoplast electrofusion by proteases may be due to improvement of cell-cell contacts through the removal of membrane surface determinants. However, the possibility that proteases enhance electrofusion by causing aggregation of membrane proteins cannot be eliminated.

Introduction

Membrane fusion is a component of cellular processes as diverse as cell division, fertilization, and vesicle transport. The artificial induction of cell fusion is also essential in somatic hybridization. Thus understanding and controlling membrane fusion is of basic importance to cell biology and somatic cell genetics.

Many valuable studies have focused on fusion in natural systems [1–3]. However, the artificial induction of fusion in liposomes or cells facilitates studies of membrane structure and fusion. As a result, the action of chemical fusogens has been intensively studied. These fusogens include polymers such as polyethylene glycol [4]; fusogenic lipids, for example, lysophosphatidylcholine [5] and glycerol monooleate [6]; calcium ions [7,8]; and viruses or viral membrane proteins [9]. The general view is that fusion is a three step process. First membrane contact must be established. Contact probably involves neutralization of surface charges [10], migration of membrane proteins to

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Abbreviations: ESR, electron spin resonance; DMSO, dimethylsulfoxide; PMSF, phenylmethylsulfonyl fluoride; FITC-ConA, fluorescein isothiocyanate-concanavalin A; lysoPC, lysophosphatidylcholine; Mes, morpholineethanesulfonic acid.

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expose free-lipid domains [1,2,11], and possibly membrane dehydration as well [4]. Then the normal bilayer structure must be destabilized; possibly by formation of some non-bilayer lipid intermediate such as micelles [12], crystalline lipids [8], or hexagonal-II phase lipids [13] or simply through a localized catastrophic collapse of membrane structure [2]. Finally, the fused membranes must restabilize in their normal bilayer configuration.

Changes in membrane fluidity have frequently been found to be correlated with membrane fusion. In some cases an increase in membrane fluidity has been associated with an increase in fusion [14–17], which may enhance hydrophobic interactions between apposed membranes [18]. In other cases the induction of fusion is associated with a chemically induced decrease in membrane fluidity [8,19], possibly associated with localized crystalline lipid domains.

Recently, electric fields have been utilized to induce cell fusion [20]. Electrofusion, as developed by Zimmermann and co-workers, utilizes the dielectrophoretic force generated when cells are exposed to a nonuniform a.c. field to induce the formation of cell-to-cell contacts. Cell fusion is then induced by one or more brief d.c. pulses. Previous work in our laboratory has focused on the practical use of electrofusion for the formation of somatic hybrids in plants [21,22].

Electrofusion has a physical basis completely different from those of all other methods of inducing cell fusion. Thus studies of the mechanism of electrofusion may provide valuable general insights about membrane fusion. The experiments described here represent a preliminary investigation of the mechanism of electrofusion in plant protoplasts. Our approach has been to examine the effects on electrofusion of treatments known to modulate the chemically induced fusion of animal and plant cells. The effect of these treatments on the fluidity of the plasma membrane has been examined to probe possible structural intermediates formed during fusion. The cells used in this study, protoplasts derived from a carrot suspension culture, were used in a previous study of membrane fluidity and polyethylene glycol-induced fusion [7,23] and thus afford the opportunity for a direct comparison. The results of our work suggest that the membrane interactions that occur during electrofusion have many features in common with those that occur during chemically induced cell fusion. These may include the participation of some non-bilayer lipid intermediate. Modification of the structure or the distribution of membrane surface proteins is also important.

Materials and Methods

A non-morphogenic suspension culture of *Daucus carota* (kindly provided by Dr. Wendy Boss, Dept. of Botany, North Carolina State University, Raleigh, NC, U.S.A.) was maintained on ER medium [24] under constant light (5 $\mu E/m^2 s$) on a rotary shaker at 125 rpm. The culture was transferred weekly at a dilution of 1 : 20. Four to 7 days after subculturing, protoplasts were released by treating the cells with 2% Driselase in 0.4 M mannitol (pH 5.1) for 5 h. The digest was passed through a 62 μm mesh and then centrifuged (130 $\times g$ for 3 min). The protoplast pellet was washed three times with 10 ml of 0.4 M mannitol and was resuspended in 2–3 ml of 0.4 M mannitol and counted with a hemacytometer. For fusion, the protoplast density was adjusted to approximately $5 \cdot 10^4$ cells/ml.

For most fusions, 10–15 μl of protoplast suspension were introduced into a D.E.P. Systems (Metamora, MI, U.S.A.) Open Fusion Slide No. 2050S. This fusion chamber consists of a pair of platinum plates mounted parallel to each other on a glass microscope slide. The slot between the plates is 0.5 mm across and holds about 15 μl of liquid. So that fusion could be observed, the fusion chamber was mounted on the stage of an inverted microscope. A different chamber was used to investigate the effects of temperature on fusion. This chamber was built in our machine shop according to the design described by Watts and King [25] but with the electrode plates spaced 5 mm apart. This chamber could be introduced into an incubator, so temperature could be controlled during fusion. Fusion efficiency was similar in the two types of electrofusion chambers.

Electric fields used for fusion were generated using a Zimmermann Cell Fusion Power Supply (G.C.A. Procision Inc., Chicago, IL, U.S.A.). Cell-to-cell contacts were induced by dielectrophoresis with an a.c. field (160–200 V/cm, 600 kHz). After

2 min to allow for alignment of the cells, the a.c. field strength was increased to 200–250 V/cm. Fusion was then induced by applying 2 d.c. pulses (600–1000 V/cm, 50 μs in duration). After fusion the a.c. field was reduced to 30 V/cm. This weak a.c. field relieved the tension on the protoplasts, allowing them to coalesce, but prevented the protoplasts from drifting out of the plane of focus.

Fusion products were identified visually at 400-fold magnification and counted. The fusion efficiency was calculated from the fraction of cells observed after the experiment that had undergone fusion. When fusions were carried out in the D.E.P. Systems fusion chamber, only those cells that had been aligned into chains by the a.c. field were counted, unaligned cells were ignored. This procedure permits an accurate estimate of the response of the cells to the d.c. field alone. This approach was not possible when the Watts and King type chamber was used. Instead the protoplasts were transferred, after fusion, from the chamber to a microscope slide for counting.

For lysophosphatidylcholine treatment, 0.5 ml of packed protoplasts were resuspended in 1 ml of 0.1 mg/ml or 0.2 mg/ml lysoPC in 0.4 M mannitol. After 5 min at room temperature, the protoplasts were washed three times with mannitol (to remove the lysoPC from the medium) before fusion. In the case of the DMSO and $CaCl_2$ treatments, the protoplasts were washed and then resuspended before electrofusion in 0.4 M mannitol containing the appropriate concentration of DMSO or $CaCl_2$. In studies of the effects of temperature on fusion, the protoplasts were resuspended in media that had been previously warmed (or chilled). The fusion chamber was also prewarmed (or chilled), and fusion was then performed in an incubator.

Fluorescein isothiocyanate-concanavalin A (FITC-ConA) (Sigma Chem. Co., St. Louis, MO, U.S.A.) was dissolved at a concentration of 0.2 mg/ml in 0.4 M mannitol, 0.5 mM Mes (pH 6). Approximately 50 μl of protoplasts were resuspended in 1 ml of the FITC-Con A solution for 5 min at room temperature. The protoplasts were washed twice with 0.4 M mannitol and then observed by fluorescence microscopy with an inverted Nikon microscope and a Nikon B filter package.

For electron spin resonance (ESR) spectroscopy, carrot protoplasts were isolated and then washed three times in 10 ml of 0.4 M mannitol containing 0.5 mM Mes (pH 5.5). The protoplasts were treated with lysoPC (0.1 mg lysoPC/50 μl packed protoplasts) for 5 min prior to washing. The amount of lysoPC used was calculated to be the same, on a per-cell basis, as that used in the fusion experiments. In the case of $CaCl_2$ treatments, $CaCl_2$ was included in the wash medium. Approximately 0.1 ml of packed protoplasts (about $3 \cdot 10^6$ cells/ml) were mixed with 1 μl of 5-doxylstearic acid (Molecular Probes, Junction City, OR, U.S.A.) (12.5 mg/ml in ethanol) prior to the further addition of 50 μl of 10 mM $K_3Fe(CN)_6$. Then this protoplast suspension was drawn up into a 50 μl or 100 μl glass capillary and sealed. ESR scans were recorded on a Varian E-12 Spectrometer that had been modified to allow horizontal mounting of the sample. This modification prevents the cells from migrating out of the cavity as they settle due to gravity. The temperature was held constant, by means of N_2 flow into the scan chamber and was regulated with a Varian Variable Temperature Controller. The precise spectrometer settings differed for each experiment but were generally as follows: field set, 3400 G; scan range, 100 G; scan time, 8 min; time constant, 1 s; modulation amplitude, 4 G; receiver gain, $2 \cdot 10^4$; microwave power, 5 mW at a frequency of 9.125 GHz.

Results

Day-to-day variation in electrofusion

In the course of the experiments described in this paper, the efficiency of electrofusion (fraction of cells in contact that undergo fusion) differed considerably for experiments performed on different days. Examples of the extent of this variability can be seen if the control values for the different experiments shown in Table I are compared. Much less variation was observed for the fusion of successive aliquots taken from the same batch of protoplasts (see standard errors in Table I).

This day-to-day variability in electrofusion did not appear to be due to differences in the time at which the protoplasts were isolated, with respect to the suspension's subculture interval, or to minor

TABLE I

CHEMICAL TREATMENTS STIMULATE CARROT PROTOPLAST ELECTROFUSION

Carrot protoplasts were isolated in 0.4 M mannitol containing 2% Driselase, washed, and subjected to electrofusion. Chemical treatments (0.1 mg/ml lysoPC; 2.5% DMSO; and 0.1 mM $CaCl_2$) were by addition of the agent 5 min prior to fusion. The controls represent protoplasts receiving no chemical treatment. Data are presented as the mean ± S.E. for seven determinations.

Treatment	Percentage fusion
LysoPC	39.3 ± 1.7
Control	24.7 ± 1.3
DMSO	35.9 ± 1.4
Control	28.8 ± 1.9
Ca^{2+}	28.2 ± 4.0
Control	15.4 ± 2.3

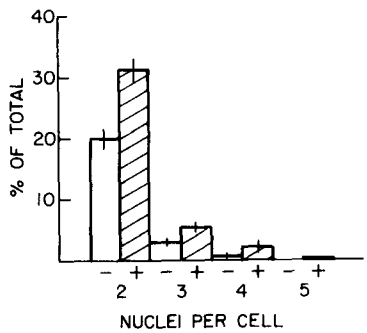

Fig. 1. Distribution of nuclei among electrofusion products as affected by lysoPC treatment. Carrot protoplasts, with or without a 5-min pretreatment in 0.1 mg/ml lysoPC, were subjected to electrofusion. The data are presented as the number of nuclei per cell, observed after fusion, as a percentage of the total number of cells. Shaded bars indicate treated protoplasts; open bars indicate control protoplasts. The error bars represent the S.E.

variations in how the protoplasts were isolated (data not shown). However, when the suspension was subcultured onto defective media, such as media containing aged vitamin stocks, it yielded protoplasts with greatly reduced electrofusion efficiencies. Good fusion was restored if the suspension was subcultured for several passages on fresh media. These observations suggest that the source of the variation is related to the physiological state of the cells and presumably reflects differences in the composition and or organization of their plasma membranes.

We have attempted to control this day-to-day variation by growing the suspension culture and isolating the protoplasts under as uniform conditions as possible. However, the day-to-day variation could not be entirely eliminated. Thus, we have always compared the results of successive experiments in relation to the efficiency of fusion in their respective controls.

Effects of lysoPC, DMSO, and calcium chloride on fusion

LysoPC, DMSO, and $CaCl_2$, which are known to enhance PEG-induced fusion of animal or plant cells, were all found to stimulate the electrofusion of carrot protoplasts (Table I). These effects are statistically significant at the 0.01 level or greater. The treatments also increased the proportion of multicellular fusion, again reflecting the enhancement of electrofusion susceptibility. As an example, Fig. 1 shows the number of nuclei per cell following the fusion of lysoPC-treated cells. Similar data were obtained with DMSO and $CaCl_2$.

In addition to stimulating fusion, the lysoPC and DMSO treatments also increased cell lysis. Lysis was observed during protoplast treatment and washing as well as during fusion. The extent of lysis was difficult to quantify, but most of the protoplasts subjected to electrofusion did survive. However, when the concentration of lysoPC was increased to 0.2 mg/ml, lysis was so extensive during treatment and washing as to preclude completion of the experiment. Increasing the length of the lysoPC treatment also increased lysis. For this reason lysoPC was given as a 5 min pulse and then was washed out of the medium.

Treatments with DMSO at concentrations of 1%, 2.5%, and 5% all increased fusion by approximately the same amount (data not shown). As was observed for lysoPC, increasing the DMSO concentration increased the extent of lysis. Treatment with 5% DMSO caused about half the protoplasts to lyse; lysis was even more extensive at higher DMSO concentrations.

The inclusion of 0.5 mM $CaCl_2$ in the fusion medium enhanced electrofusion (Table I) without increasing cell lysis. Higher concentrations of $CaCl_2$ could not be tested because they interfered with cell alignment by dielectrophoresis.

TABLE II

EFFECT OF TEMPERATURE ON THE EXTENT OF ELECTROFUSION

Carrot protoplasts were suspended in 0.4 M mannitol at various temperatures and subjected to electrofusion. Each experimental treatment (Exp.) is shown in relation to a control value obtained from the fusion of the same batch of protoplasts at room temperature (23°C). Fusion is expressed as the percentage of cells recovered after the experiment that had undergone fusion. Data represent the mean ± S.E. for three determinations.

	Temperature (°C)		% Fusion
A	Exp.	10	3.1 ± 0.7
	Control	23	18.1 ± 3.1
A	Exp.	20	14.8 ± 3.4
	Control	23	12.2 ± 2.3
C	Exp.	30	13.7 ± 2.1
	Control	23	14.2 ± 1.8

Effect of temperature on fusion

The effect of temperature on fusion is shown in Table II. The data are presented as pairs with the treated protoplasts compared with controls, which were taken from the same preparation of protoplasts but held at room temperature (23°C). Lowering the temperature to 10°C resulted in a significant decrease in the extent of fusion. Raising the temperature had no significant effect on fusion; however, protoplasts warmed to 30°C were very sensitive to lysis. Lysis was reduced if the electric field strengths were reduced; under these conditions a small enhancement of fusion was observed at the higher temperature (data not shown).

The inhibition of fusion at low temperatures is consistent with the known temperature-dependence of membrane breakdown voltage [20]. Membrane reorganization during fusion would also be expected to exhibit a similar response to temperature.

ESR measurements

Initially ESR measurements were attempted on isolated membranes obtained from the carrot suspension culture cells. However, we were unable to isolate purified plasma membranes in quantities sufficient for ESR analyses. Therefore, all subsequent ESR measurements were made on intact carrot protoplasts. As seen by comparing spectra (a) and (b) of Fig. 2, the ESR signal decayed over a period of 40 min. The spin label used here, 5-doxylstearic acid, may well have partitioned throughout all the membranes in the cells. Little cell lysis occurred during these ESR measurements; thus this signal loss is presumably due to the reduction of spin probe localized in the cytoplasm and in internal membranes or the internal face of the plasma membrane. Addition of the oxidant $K_3Fe(CN)_6$ to the external medium resulted in regeneration of ESR signal (Fig. 2). Because ferricyanide is a non-permeating anion, we argue that the ESR signal observed in the presence of ferricyanide is localized in the outer surface of the plasma membrane. This system was also used by Boss and Mott [7] and Boss [23] in their study of the effects of PEG and Ca^{2+} on the membrane fluidity of carrot protoplasts. In addition to observing signal regeneration by ferricyanide, Boss reports that the non-permeating cation La^{3+} induces large shifts in the ESR signal. This result provides further evidence that ESR

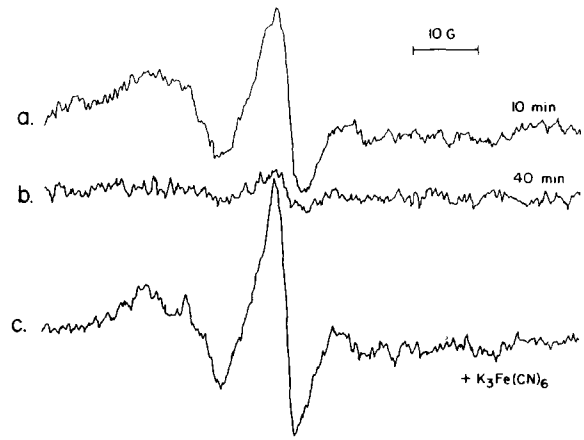

Fig. 2. Representative ESR spectra obtained for carrot protoplasts, labeled with 5-doxylstearic acid, as a function of time and ferricyanide addition. So that a time course for signal loss could be obtained, each scan was short (4 min). As a result only the major peaks are visible against the background noise. Carrot protoplasts were mixed with 5-doxylstearic acid. Aliquots taken from this suspension after 10 min (a) and 40 min (b), were used to generate ESR scans. Then 50 μl of 10 mM $K_3Fe(CN)_6$ was added to the remaining 50 μl of protoplast suspension and the final scan (c) was taken. The horizontal line indicates 10 gauss.

signals observed here arise from the plasma membrane and are not an average of all the cellular membranes.

Effect of temperature on the ESR signal

Fig. 3 shows representative ESR spectra obtained for carrot protoplasts at different temperatures. Decreasing temperature reduces membrane fluidity, and this decrease is reflected by an increase in the parameter $2A_{max}$ and a decrease in $2A_{min}$. Lowering the temperature also causes the loss of one of the peaks associated with the parameter $2A_{min}$ (vertical arrow Fig. 3). Presumably this loss results from clustering of the label in more fluid regions of the membrane; the resulting localized high concentration of label causes exchange broadening and loss of signal. Values of $2A_{max}$, obtained from the spectra shown in Fig. 3, have been used to produce the Arrhenius plot shown in Fig. 4. This plot displays more clearly the abrupt change in fluidity experienced by the membranes as they were cooled from 20°C to lower temperatures. We interpret this discontinuity to reflect a membrane phase transition.

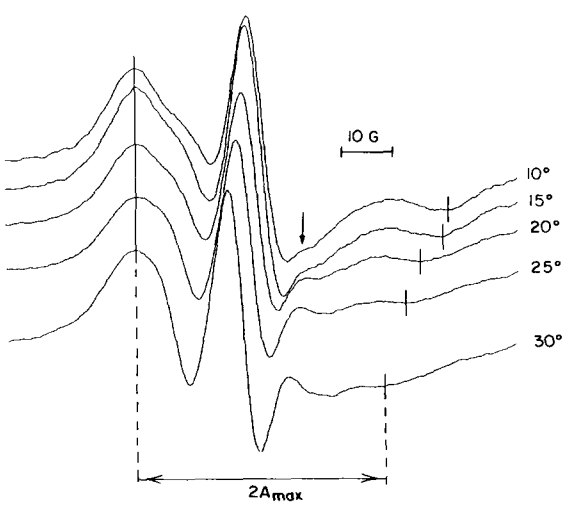

Fig. 3. ESR spectra obtained for carrot protoplasts held at different temperatures (10–30°C). Carrot protoplasts were labeled with 5-doxylstearic acid. Then ferricyanide was added, the sample was chilled (or warmed), and its spectrum was recorded. The vertical lines indicate the positions of the peaks used to calculate the parameter $2A_{max}$. The horizontal bar indicates 10 gauss. The vertical arrow indicates the position of the peak that is lost due to exchange broadening at low temperatures.

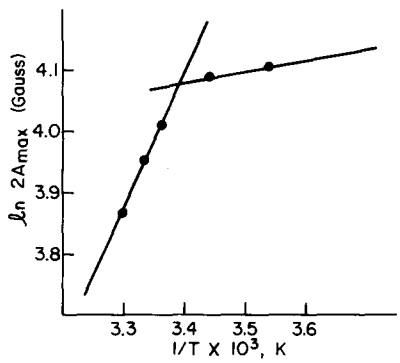

Fig. 4. Arrhenius plot of changes in carrot protoplast fluidity parameter as a function of temperature. Values of $2A_{max}$ obtained from the experiment shown in Fig. 3 were plotted against the reciprocal of temperature (K).

Effects of lysoPC and calcium on fluidity

Results of ESR measurements made on protoplasts treated with lysoPC and calcium chloride are tabulated in Table III. LysoPC treatment resulted in a small, but reproducible, decrease in membrane fluidity. Calcium chloride, at a concentration of 20 mM, caused a large reduction in fluidity. Some spin exchange broadening was also observed in the Ca^{2+}-treated cells, just as it was in low-temperature scans. However, no reduction in fluidity was observed for protoplasts treated with 0.5 mM $CaCl_2$. Boss and Mott [7] were also unable to detect a decrease in the membrane fluidity of carrot protoplasts in response to Ca^{2+}

TABLE III

ESR SIGNAL SHIFTS FOR CARROT PROTOPLASTS LABELED WITH 5-DOXYLSTEARIC ACID AND TREATED WITH lysoPC OR $CaCl_2$

Intact carrot protoplasts were suspended in 0.4 M mannitol containing 0.5 mM Mes (pH 5.0) plus either 20 mM $CaCl_2$ or 0.2 mg lysoPC (per 100 μl of protoplasts). The protoplast suspension was mixed with 1 μl of 5-doxylstearic acid and 50 μl of 10 mM $K_3Fe(CN)_6$. ESR spectra were recorded immediately. Data are presented as the separation between the two outer peaks, $2A_{max}$, for both treated and control protoplasts. Also shown is the signal shift (Δ) due to the treatment.

Treatment	$2A_{max}$ (G)	Δ (G)
20 mM $CaCl_2$	54.7	+5.9
Control	48.8	
LysoPC	51.6	+1.4
Control	50.2	

concentrations below 10 mM. The lack of response to lower Ca^{2+} concentrations probably reflects the relatively poor sensitivity of ESR when it is applied to plant protoplasts, whose large size (30–50 μm in diameter) creates signal-to-noise difficulties.

No ESR measurements were attempted on protoplasts treated with DMSO.

Proteases enhance fusion

We treated carrot protoplasts with either pronase or proteinase K in order to examine the role of membrane proteins on electrofusion. A 5-min treatment with 1 mg/ml pronase or a 3-min treatment with 0.25 mg/ml proteinase K significantly stimulated electrofusion (Table IVa). When the length of the pronase treatment was extended to 10 min or the concentration of proteinase K increased to 0.5 or 1.0 mg/ml, extensive protoplast lysis occurred during washing of the cells and subsequent electrofusion. Inclusion of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) along with proteinase K largely blocked the enhancement of electrofusion (Table IVb). In independent experiments, PMSF treatment alone was found to have no effect on fusion (data not shown).

TABLE IV

STIMULATION OF THE ELECTROFUSION OF CARROT PROTOPLASTS BY PROTEINASE TREATMENT AND ITS REVERSAL BY PMSF

a. Carrot protoplasts were suspended briefly in 0.4 m mannitol containing 0.5 mM Mes (pH 5.5) plus either 1 mg/ml pronase or 0.25 mg/ml proteinase K and then were washed with 0.4 M mannitol and subjected to electrofusion. Fusion was determined from the percentage of protoplasts with one or more cell-to-cell contacts (before fusion) that underwent fusion in response to the d.c. pulses. Each treatment was compared with the extent of fusion of control (untreated) protoplasts from the same batch of cells. The data are presented as the mean ± S.E. for eight determinations (proteinase K) or ten determinations (pronase).

b. Protoplasts were treated with proteinase K, as described above, but with the addition of 2 mM PMSF, and then were washed and subjected to electrofusion. The data are presented as the mean ± S.E. for three determinations.

	Treatment	% Fusion
a.	Pronase	13.8 ± 0.9
	Control	6.9 ± 0.8
	Proteinase K	16.6 ± 1.4
	Control	7.4 ± 1.3
b.	Proteinase K	11.3 ± 0.8
	Proteinase K + PMSF	4.6 ± 0.3
	Control	2.4 ± 0.3

FITC-Con A studies

Because the protease experiments indicated that membrane proteins may be important in electrofusion, we examined the effects of proteases and electric fields on the distribution of cell surface fluorescence of protoplasts labeled with FITC-Con A. Both protease-treated and untreated protoplasts could be labeled with this lectin. Some clustering of the label was observed in all the cells. Neither protease treatment nor prolonged exposure to a.c. fields was observed to alter the distribution of FITC-Con A. Capping or distinct clustering of the label was never observed.

Discussion

The experiments described in this paper show that lysoPC, Ca^{2+}, and DMSO promote electrofusion. LysoPC and Ca^{2+} were also found to reduce membrane fluidity. DMSO reduces fluidity, at least in other systems [8]. These observations might suggest an inverse relationship between fluidity and susceptibility to electrofusion. However, we found that low temperatures reduce both electrofusion and fluidity. Thus, the relationship between fluidity and electrofusion must be indirect. This circumstance parallels what is known for naturally and chemically induced fusions in a variety of other systems and indicates that both these types of fusion share some basic mechanistic features with electrofusion.

Early observations suggested that a fluid bilayer is essential for membrane fusion. For example, a positive correlation is observed between increasing temperature and fusion [14–17]. However, other work has shown that many fusogens, including Ca^{2+}, DMSO, and lysoPC [7,23], actually reduce membrane fluidity. It has been suggested that fusion takes place through the formation of a non-bilayer lipid intermediate whose presence can be associated with a reduction in membrane fluidity [19,26]. For example, Ca^{2+} and DMSO are known to bind acidic phospholipids and are thought to form crystalline lipid arrays

that are sites of membrane fusion [8,26]. Other non-bilayer lipid structures that have been proposed as fusion intermediates include micelles [12] and hexagonal-II phase lipids [13]. Micelles are believed to form in the membrane in response to lysoPC treatment [12] and hexogonal-II phase lipids have been observed in membranes treated with fusogenic lipids, Ca^{2+}, or dehydrating agents [13].

An alternative to such non-bilayer structural intermediates is suggested by Pinto da Silva and Nogueira [2] from an ultrastructural investigation of exocytosis. Their micrographs indicate that membrane fusion results from an abrupt transition between two noncontinuous states and probably reflects a catastrophic collapse of the structure of apposing lipid bilayers and their immediate reorganization as a single membrane.

Electrofusion has been proposed to result from the formation of field-induced transmembrane pores at the sites of cell contact [20]. Models of this process envision pairs of apposing pores whose lumens are filled with a dispersion of lipids [20]. This is analogous to the catastrophic membrane collapse of Pinto da Silva and Nogueira [2]. With respect to the poration model of electrofusion, the data presented here could be interpreted in two ways. By deforming the bilayer, Ca^{2+}, DMSO, and lysoPC may facilitate field-induced pore formation. Alternatively, the observation that electrofusion is stimulated by treatments that promote the formation of non-bilayer lipid structures suggests that a non-bilayer structure, other than pores, may be involved here as well. Such a possibility was recently suggested by Sowers [27] based on his observation that the electrofusion of erythrocytes can be achieved even when the d.c. pulses are given before aligning the cells with the a.c. field. Our observation that lysoPC, Ca^{2+}, and DMSO promote electrofusion may provide an experimental approach for distinguishing between these possibilities. If these compounds facilitate pore formation then they should reduce the voltage at which field-induced permeability increases occur. A lack of effect of these compounds on the membrane-breakdown voltage would provide evidence for the participation of a non-bilayer intermediate in electrofusion.

In addition to lipid-associated phenomena, membrane proteins must also be involved in cell fusion. The clustering of membrane proteins to form free-lipid domains has been proposed to be involved in cell fusion in many systems including fusion in response to lysoPC [28], DMSO [29], Sendai virus [30] and PEG [4]. Presumably, these free-lipid domains provide regions where tight membrane contact can occur [11].

Prior to this study, Zimmermann and coworkers reported that the electrofusion of animal cells is stimulated by pronase and dispase and is not blocked by PMSF [31]. They suggest that protease activity is not important in this response but that absorption of pronase onto the plasma membrane causes aggregation of integral membrane proteins and thus improves cell-cell contacts. Subsequently, Ohno-Shosaku and Okada [32] reported that the electrofusion of mouse lymphoma cells is stimulated by trypsin and that the response to trypsin is reversed by the inhibitors aprotinin and *p*-tosyl-L-lysine chloromethylketone. These observations suggest that protease activity does specifically enhance electrofusion. Similarly, we found that pronase and proteinase K enhanced electrofusion of carrot protoplasts and that the proteinase K effect was blocked by PMSF. We did not examine the effects of PMSF on pronase treated cells because there is no reason to believe that all the proteases in this crude enzyme preparation would be inhibited by PMSF, which inactivates only serine proteases.

Whether protease treatment induces clustering of membrane proteins or simply strips the membrane surface is unclear. In preliminary experiments we labeled protease treated cells with fluorescein isothiocyanate conjugated to concannavalin A. No capping or clustering of fluorescence was observed. However, the sensitivity of this technique is probably not sufficient to detect the occurrence of small clusters of proteins that are distributed over the whole cell surface. Ultrastructural studies may be necessary to resolve this question. In any case, enhancement of electrofusion by proteases suggests that closely apposed lipid bilayers are required for electrofusion just as they are for chemically and virally induced fusions.

Acknowledgments

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