Electrically Stimulated Fusion of Different Plant Cell Protoplasts\textsuperscript{1, 2}

MESOPHYLL CELL AND GUARD CELL PROTOPLASTS OF VICIA FABA

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ABSTRACT

Cell fusion is induced between guard cell and mesophyll cell protoplasts of Vicia faba by electrical field application. The process of fusion is initiated by electrical breakdown of the cell membrane. Prior to the application of an external electrical field pulse which brings about reversible breakdown of the membrane, the cells (suspended in a low-conducting medium) are brought into close contact with one another by exposing them to an external alternating, nonuniform field (5 volts, electrode distance, 200 micrometers; 500 kilohertz). During this process, they form “pearl chains” which may become sufficiently long to form bridges between the electrodes. The process is reversible as long as this voltage is not exceeded.

Cell fusion is initiated as a result of an electrical field pulse of 50 microseconds duration and of sufficiently high intensity to induce irreversible electrical breakdown of the membranes. The process of fusion is completed within 40 minutes or less in the case of guard cell protoplasts, as well as in the case of fusion between guard cell and mesophyll cell protoplasts. The fused cells are spherical in shape, if the fusion product consists only of two or three cells.

The study of chemical- and virus-induced fusion is an area of research that has attracted an increasing amount of interest in the last decade (4, 11-13). Needless to say, the elucidation of the mechanisms involved in cell and membrane fusion would provide information on the dynamic properties of cell membranes and lead to an understanding of many biological phenomena in which membrane fusion occurs (e.g., fertilization, endo- and exocytosis, etc.). However, it is the possibility of somatic hybridization induced by cell fusion that really demonstrates the enormous potential of this phenomenon in the fields of biology, agriculture, and medicine (15).

Despite the great efforts on behalf of a large number of scientists and the numerous publications dealing with various fusogenic substances, such as lipids, polyethylene glycol, dimethyl sulfoxide, and fusogenic virus (7-8, 11-13), the molecular aspects of the process of membrane fusion are still not understood. One of the reasons for this may be the fact that experimental results are not easily explained and often contradictory, probably because the empirically developed experimental procedures used for cell fusion lack any defined physical or chemical background. Often extreme experimental conditions, such as high pH and high Ca\textsuperscript{2+} concentration, are required for cell fusion, and any fusion process initiated in this way proceeds neither in a synchronous manner nor does it lead to a high yield of fused binucleate cells.

Recently, Zimmermann and Scheurich (22) developed a quite different approach which they applied to mesophyll cell protoplasts of Vicia faba. The technique they used is based on the reversible electrical breakdown of cell membranes in response to a high external electrical field pulse of very short duration (a couple of \(\mu\)s) (9, 19, 20).

Close membrane contact between two cells, a prerequisite for fusion, is achieved by performing breakdown experiments in the presence of an inhomogeneous, alternating electrical field of low field strength. By simple physical laws, the cells are drawn into regions of high field intensity within the inhomogeneous field and, in the process, they form “pearl chains.” This phenomenon is termed dielectrophoresis (10). The fusion process is initiated as soon as electrical breakdown occurs in the membranes of two attached cells. This method of fusion yields a high proportion of synchronously fused cells. The process of fusion can be triggered in such a way as to yield a large proportion of binucleate fusion products.

Here, we report on fusion of cells of different types, i.e., between guard cell and mesophyll cell protoplasts of \(V.\) faba, with the aim to demonstrate that cell-cell fusion induced by electrical fields is not restricted to one cell type.

MATERIALS AND METHODS

Seeds of \(V.\) faba (cv. Hangdown, Weisskaimige) were germinated and grown in peat moss in a greenhouse maintained at 22°C (day) and 17°C (night) under natural conditions (May to June) and, thus, subjected to variable growing conditions. The plants were watered three times a week with tap water.

The leaves of 3- to 4-week-old plants were harvested after the plants had been stored in the dark for 24 or 48 h. Strips of epidermis were peeled off the leaves, floated in 0.23 M mannitol, and incubated for 60 min in a medium containing 0.23 M mannitol, 1 mM CaCl\textsubscript{2}, 4% (w/w) Cellulysin (pH 5.2, Calbiochem), as described by Schnabl et al. (17). After the removal of the epidermal and mesophyll cell protoplasts by centrifugation (300g, 10 min), the epidermal strips with the intact guard cells were washed with a mannitol solution (0.4 M, 1 mM CaCl\textsubscript{2}) and incubated for 1.5 h in a medium containing 0.4 M mannitol, 1 mM CaCl\textsubscript{2}, 4% Cellulysin (pH 5.2) at 30°C. During this period, they were gently shaken.

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\textsuperscript{2} Dedicated to the memory of Professor Noe Higinbotham.
After 1.5 h, the concentration of mannitol was increased to 0.5 M. Following a 3-h incubation in 0.5 M mannitol solution, the released guard cell protoplasts were washed out with a 0.6 M mannitol solution and filtered once through a sieve (25-μm mesh). The concentrated suspension of guard cell protoplasts was stored at 4°C and used within the next 3 h for fusion experiments. Mesophyll cell protoplasts were isolated and purified as described elsewhere (16, 22).

An account of the field techniques, including dielectrophoresis and electrical breakdown, is also given elsewhere (22). For the sake of clarity, a brief outline is presented here. In contrast to the effects in a homogeneous electrical field, uncharged cells in an inhomogeneous electrical field are subjected to a net force which represents the force of the field gradient acting on the induced dipoles within the cell. Because of the change in polarity of the induced dipoles, the net force persists even in an alternating electrical field, which means that electrolysis can largely be excluded (10).

The effect of this dielectrophoretic force is to move the cells towards the electrodes where the density of the field lines is greatest. Because the higher dielectric constant of the cell interior causes the field lines to become denser at each individual cell, the cells tend to form chains along the direction of field lines. This effect is known as "mutual dielectrophoresis" (10). In the experiments reported here, the dielectrophoretic voltage (amplitude, 0–25 v; sine wave, 500 kHz) was generated with a function generator supplied by the firm Toellner (type TE 7704). The cell suspension was introduced into a chamber with a capacity of 1 ml, which contained two parallel and horizontally mounted cylindrical electrodes made of gold-plated brass. The minimum distance between the two electrodes was 200 μm. A slide served as the bottom of the chamber. The gap between the electrodes was observed with a microscope. To prevent the solution from evaporating during the course of a longer experiment, it was covered with a glass cover slip. The function generator was connected to the electrodes by way of a 50-ohm resistor. A pulse generator (Hewlett-Packard, type 314 B) was switched in parallel to the electrodes and used to produce the pulse required for fusion. Inasmuch as the internal resistance of the function generator is low, the 50-ohm resistor serves as an external load for the pulse generator.

The single square pulse produced by the pulse generator was sufficiently high to produce dielectric breakdown in the cell membrane. In the course of this event, the electrical conductivity reversibly increases for a short period of time, during which a dramatic, but reversible, change in the permeability of the cell membrane occurs (for a more detailed account, see refs. 2, 6, 19, and 20).

To keep the electrical current and, in turn, the rate of electrolysis and any turbulence associated with the generation of heat to a minimum, the experiments were carried out in electrolyte-free medium. The osmolarity was regulated as necessary by addition of mannitol.

**RESULTS**

On application of the dielectrophoretic voltage (up to 10 v in amplitude) to the electrodes in the chamber, the guard cell protoplasts in the suspension begin to adhere reversibly to the electrodes and to each other. The resulting aggregates disintegrate very rapidly by Brownian motion and repulsion as soon as the alternating voltage is removed. Dielectrophoretic experiments with guard cell protoplasts were carried out in both 0.5 and 0.6 M mannitol solutions and the cells in the solution of higher osmolarity were observed to form much larger areas of contact. The osmolarity of the solution had no effect on the dielectrophoretic behavior of cells derived from plants that were grown under various conditions.

When close contact had been established, the dielectrophoretic voltage was reduced to an amplitude of 5 v and the fusion process was initiated by the application of a square pulse of 50 μs duration and an amplitude of 33 v.

Nearly all cells exposed to the field undergo fusion. Figure 1 shows the field-induced fusion of two guard cell protoplasts of *V. faba*. The fusion process takes 10 to 40 min. The tendency to fuse seemed to be increased at decreasing osmolarity where the membrane is under higher tension. On the other hand, the protoplasts seemed to be more sensitive to the single field pulse and more prone to burst at lower osmolarity.

Dielectrophoretic experiments with mixtures of guard cell and mesophyll cell protoplasts were carried out in 0.5 and 0.6 M mannitol solutions, respectively. At higher osmolarities the membrane tension and the mechanical stability of the mesophyll cell protoplasts was so low that, even at low field intensities (amplitude, 2–4 v) they were deformed in the direction of the field lines and subsequently disintegrated. To bring about fusion between guard cell and mesophyll cell protoplasts, voltage amplitudes of more than 30 v had to be used in the application of the single field pulse, whereas, in the control experiments, fusion between mesophyll cell protoplasts could be achieved with amplitudes of 12 and 15 v (cf. ref. 22). The process of fusion between a guard cell protoplast and a mesophyll cell protoplast usually takes about 10 to 40 min, that is, until a spherical aggregate has formed (Fig. 2).

[FIG. 1. Electrically induced fusion of two guard cell protoplasts of *V. faba*. Guard cell protoplasts were suspended in a solution consisting of 0.6 M mannitol and were collected at the electrodes by dielectrophoresis (sine wave, 500 kHz; amplitude, 10 v). After reduction of the alternating voltage to an amplitude of 5 v, a single square pulse was applied (33 v; 50 μs). Two guard cell protoplasts attached to each other by dielectrophoresis (a) and the time course of the fusion process (b–e) 20 s and 5, 20, and 40 min after application of the pulse, respectively, are shown. Scale: 1 cm = 12 μm.

3 Thus, using a flow chamber system, it is possible in future to obtain large quantities of fused cells (manuscript in preparation).
do not mix immediately. The time to complete fusion was constant within each preparation of protoplasts but varied between the different protoplast preparations by a factor of 4.

In a further set of experiments, suspensions containing both guard cell and mesophyll cell protoplasts of V. *faba* were used, but at higher suspension density. This resulted in the dielectrophoretic collection of a large number of pearl chains between the electrodes, which were composed of both types of protoplasts. Figure 3a illustrates a pearl chain consisting of mesophyll guard cell protoplasts and Figure 3b shows the process of fusion, following the application of a single field pulse.

Finally, it should be noted that electrically induced fusion in guard and mesophyll cell protoplasts occurs both in freshly prepared cells and in cells stored at 4°C for a period of 16 h in mannitol solution. The fused cells are viable for several days without any change in their osmotic or dielectrophoretic behavior.

**DISCUSSION**

The results demonstrate that guard cell protoplasts are capable of fusing with each other, as well as with mesophyll cell protoplasts. The only prerequisites are that attached cells are first formed by dielectrophoresis and that the breakdown voltage of the membrane of two attached cells is then exceeded on application of the single field pulse. Although the exact distribution of the field lines between the two electrodes is not known, it is fairly easy to predict that the breakdown voltage must indeed be exceeded in order to initiate the fusion process between two attached cells. Fusion is observed above an electrode voltage of 30 v at an electrode distance of 200 μm, i.e. at a field strength of 1500 v/cm, assuming a homogeneous electrical field. The corresponding apparent membrane voltage (V_m) can be calculated from the equation V_m = f·r·E, where f is the shape factor for spherical cells (1.5), r is the radius of the protoplasts, and E is the external electrical field (6). This equation assumes that the membrane is completely charged in response to the external field, E, and that breakdown occurs in the membrane area of closest contact, that is, in field direction. For guard cell (radius, about 8 μm) and mesophyll cell protoplasts (radius, 15–25 μm), the apparent membrane voltage is estimated to be about 1.8 and 4.5 v, respectively. The breakdown voltage of guard cell protoplasts was calculated to...
be 1.0 v, using the newly developed single particle analyzer (9, 19). The breakdown voltage for mesophyll cell protoplasts should be comparable (1, 20–22). On the basis of these estimates, and bearing in mind that the field strength can be much higher in the vicinity of the electrodes, it seems obvious that breakdown of the membrane is ultimately responsible for fusion. These considerations are further supported by the finding that the guard cell protoplasts require an external voltage pulse of about twice the intensity for fusion, as compared to the mesophyll cell protoplasts. The fusion of different cell types induced by an external electrical field suggests that the crucial event involved in triggering the fusion process is the change in the intrinsic electrical field within the membrane. This offers a simple mechanism by which both extracellular and intracellular fusion could be explained. Viruses and fusogenic compounds may well act in a comparable way by causing changes in the local intrinsic field within the membrane, an idea that has already been put forward by Gingell and Ginsberg (5). Because of the inhomogeneous field distribution within the membrane, it is quite conceivable that electrical breakdown is a natural event within the membrane. This would be caused by high local field intensities (of the order of $10^5$ to $10^6$ v/cm) and by the interaction of substances with the membrane (e.g. Ca$^{2+}$ ions), which would result in dramatic changes in the local field.

The resealing process that follows breakdown is very rapid, provided that the field application is carried out at room or elevated temperatures. Benz and Zimmermann (3) recently showed that, once breakdown has occurred, the resealing process in artificial lipid bilayer membrane made up of oxidized cholesterol is of the order of 2 μs at 20 C. They interpreted their results in terms of field-induced pores which are created in the membrane by electromechanical compression (1). These pores subsequently close by lateral diffusion of lipid molecules in the membrane plane (3). The diffusion coefficient was calculated to be of the order of $10^{-8}$ cm$^2$/s, a value that agrees well with values quoted in the literature. Therefore, the results suggest that pore formation and lipid diffusion are initiated by electrical breakdown. In light of the arguments and experimental evidence presented here, it seems likely that the diffusion of lipid molecules from one membrane to the other is facilitated during the fusion process because of the formation of pores, provided that the membranes are in close contact.

Finally, we point out that the fusion of guard cell protoplasts with each other or with mesophyll cell protoplasts could be of interest as a phenomenon in its own right. Fused guard cell protoplasts are sufficiently large to be impaled by electrodes, so that it would be possible to measure the membrane potential and the resistance with electrodes, and even cell turgor pressure, with the aid of the miniaturized pressure probe. Measurements of turgor pressure and turgor pressure relaxation curves in fused guard cell protoplasts are made possible because cells can now be mechanically supported by immobilization in a cross-linked alginate matrix (16, 18). It seems likely that fused and immobilized cell systems, such as those described here, will be used in the future to elucidate the transport function of stomata (14).

**LITERATURE CITED**

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