ELECTRIC FIELD-MEDIATED CELL FUSION

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ABSTRACT: The use of electrical fields to guide, hold and fuse cells is described. The electrical fusion process consists of two steps: the cells are collected to form pearl-chains between Pt electrodes by the action of dielectrophoresis, then a brief DC pulse is applied, such that the breakdown voltage of the membranes is briefly exceeded and cell-to-cell juncture of the membranes occurs around the pore formed by the pulse. Giant fused cells (diameter up to 100 m) can be formed by the electrically mediated fusion of mouse 3T3 fibroblast cells, provided that pronase is added just before field application.

INTRODUCTION

Cell-to-cell fusion plays an important role in many natural biological processes, including fertilization, endocytosis, exocytosis, and muscle fiber formation (Poste and Nicholson, 1978; Lucy, 1978; Baker and Knight, 1979). In vitro fusion, on the other hand, opens the fascinating possibilities of somatic hybridization which has useful potential for both medicine and agriculture (Köhler and Milstein, 1975; Olsson and Kaplan, 1980; Power, et al., 1978). Successful application of somatic hybridization requires that fusion techniques be available which can give a high yield of two-cell fusion. On the other hand, the fusion of many more than two cellular entities at a time in vitro will allow the formation of giant cells. Such giant cells can be made of more than 100 cells; these can have useful application for research using microelectrodes and microcapillaries to permit direct study of living membrane systems.

The fusion techniques reported in the literature which are based upon the use of chemical agents or of virus particles have disadvantages for some purposes (Poste and Nicholson, 1978; Lucy, 1978). For example, the kinetics of the fusion process is difficult to assess in such systems. Moreover, the yield of fused cells is very low in such processes, and it is difficult to incorporate preselected multiplet numbers (2, 6, etc.) of cells per aggregate.

Recently we have reported a new fusion technique based upon the application of very brief electrical pulses to cells in contact (Zimmermann and Scheurich, 1981a, 1981b; Zimmermann, Scheurich, et al., 1981). The technique is a combination of dielectrophoresis (DEP) (Pohl, 1973, 1978a, 1978b; Pohl and Pollock, 1982) and controlled electrical breakdown of the membrane (Zimmermann, et al., 1976). The procedure is

quite gentle, is synchronous, and can lead to a high yield of fused cells.

Of paramount importance, perhaps, is the fact that the electrically induced fusion does not necessarily strongly perturb the entire cellular surface, but only a small region of contact, and that for only a very brief instant. The size of the affected area can be made larger if desired. The size of the fused cellular aggregate can be controlled by the field arrangement used, and by the suspension density (Scheurich and Zimmermann, 1981). Moreover, by sequential loading of the fusion chamber, the technique can provide increased selectivity of fusion between two cell types rather than random fusion (Vienken, et al., 1981; Zimmermann and Vienken, 1982). Pronase treatment does, however, temporarily alter the cellular surface in this electro-fusion technique.

Electrical fusion can be initiated in all cells tested to date, including bacteria and plant protoplasts, yeast, sea urchin eggs, and mammalian cells (Zimmermann and Scheurich, 1981a, 1981b; Zimmermann, Scheurich, et al., 1981; Zimmermann, Richter, et al., 1981; Scheurich, et al., 1981; Scheurich and Zimmermann, 1981; Richter, et al., 1981). Giant cells prepared from human erythrocytes have been made by electrical fusion. We describe here the preparation of giant cells from permanent cell lines of mamalian cells. The preparation of giant cells by non-electrical fusion techniques has not been previously described, to our knowledge.

METHODS AND MATERIALS

The fusion chamber used here was that described earlier (Zimmermann and Scheurich, 1981a). It consists of two parallel cylindrical platinum electrodes mounted on a microscope slide, with a minimum spacing of 100 μm between the electrodes. The electrical circuitry was also quite similar to that described earlier. It consisted essentially of an AC signal generator to supply the requisite alternating voltage for dielectrophoretically controlling the juxtaposition of the cells (Pohl, 1973, 1978a, 1978b). There was also a square DC pulse generator for supplying the requisite direct current pulse for the fusion itself. The present circuit is provided with a fast (1 ms) switch and an oscilloscope to monitor the events. The fast switching between the application of the continual AC voltage and that of the DC pulse permits more precise knowledge than heretofore of the actual application of the electrical pulsing conditions to the cells, and avoids passing the high DC voltage into the AC signal generator (Pilwat, et al., 1981).

Mouse 3T3 cells, an established fibroblast line, were used in this study. These were grown in Eagle culture medium at 37 degrees C, then treated with proteolytic enzyme, trypsin, or mechanically separated, so as to provide a suspension of individual cells. These were then freed of the conductive medium and transferred into an iso-osmotic but relatively nonconductive medium in preparation for dielectrophoresis. For this purpose the cells were washed with 300 mM mannitol in deionized water, with the aid of repeated centrifugations and resuspensions until the resistivity of the suspending medium was at least 10 k ohm-cm.

The fusion process itself was conducted in the following manner. A suspension of the cells containing 1 mg/ml pronase was introduced into the fusion chamber, and an AC voltage applied (400 V/cm, 1 MHz) to produce dielectrophoresis. This caused the cells to accumulate on the two electrodes, producing pearl-chains of cells parallel to the field lines (Pohl, 1978a, 1978b; Pohl and Pollock, 1982) (Fig. 1). Once the cells had been thus aligned by DEP, the DC pulse was applied, and the

act of fusion followed visually by direct microscopic observations.

RESULTS

We shall here be concerned mainly with the fusion of large numbers of cells into one entity, i.e., with the formation of "giant" cells by electrically mediated fusion. For this desired result, close membrane-to-membrane contact between the cells is achieved by the use of dielectrophoretic force (Fig.1). Thereupon, only a brief DC breakdown voltage pulse of microseconds duration needs be used to effect fusion. This is so because the dielectrophoretic force pushes the cells together by a process known as "mutual dielectrophoresis" (Pohl, 1978a, 1978b).

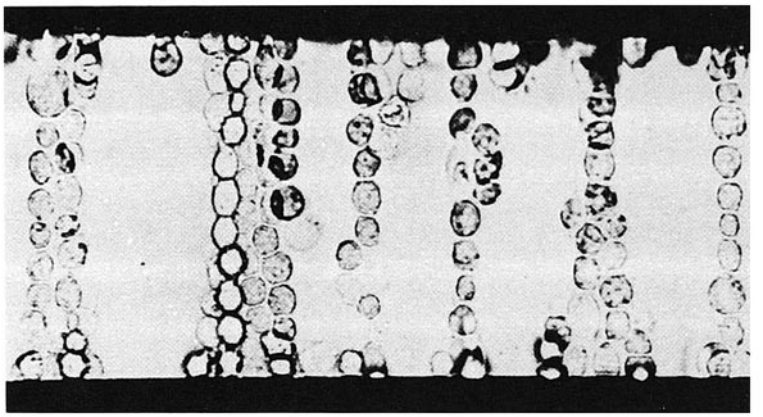

Fig. 1. Dielectrophoretic collection of mouse 3T3 fibroblast cells. The cells were suspended in 0.3 M mannitol solution and placed between the electrodes. After application of an inhomogeneous alternating field (400 V/cm, 1 MHz) the cells are arranged into pearl-chains.

To increase the likelihood of fusion, the area of touching must be increased over that of merely point contact between spheres. It is thus desirable to increase the dielectrophoretic force just prior to the application of the breakdown pulse. The AC voltage for the DEP is hence increased to a level just below (Lafon and Pohl, 1981) the breakdown voltage. Under these conditions, large membrane contact zones are formed by the flattening of the spheres. It is observed experimentally that extreme field strengths resulting in stretching of the cells, or of bud or vesicle formation, should be avoided. In particular, if the DEP force is sufficient to cause the unfused cells to elongate appreciably to form e.g. lemon or cucumber-like shapes, then fusion is unlikely during the subsequent breakdown pulse.

If on the other hand a critical pulse height is reached with an AC field applied, then many electric breakdown events are generated within the membrane. At the high frequencies (circa 1 MHz) used for the DEP, the time between cycles in which the breakdown voltage is reached is not sufficient for resealing of the membranes (Benz and Zimmerman, 1980; Zimmermann, Scheurich, et al., 1981). In consequence, more pores or enlargement of the pores occur than can be tolerated. This results in the mechanical breakdown of the individual suspended cells, that is, the irreversible destruction of the cell's plasmalemma. On the other hand, mechanical breakdown occurs in response to a single pulse only if the pulse is of long duration or if its field strength exceeds the critical value by several fold (Zimmermann, Scheurich, et al.,

1981). To produce optimum membrane contacts, conditions have to be used which on the one hand avoid irreversible destruction of the cell, yet can form large contact areas between cells without unduly stretching them. Once these conditions are established, the breakdown pulse can be injected to mediate fusion. The required field strength, E, can be calculated using the integrated Laplace equation derived for stationary conditions (Jeltsch and Zimmermann, 1979), viz:

$$V_m = 1.5\ rE\ \cos\theta \qquad (1)$$

where θ is the angle between the given membrane site and the field direction, V_m is the potential drop across the cell membrane, and r is the cell radius.

The preceding equation provides an estimate of the voltage drop across the membrane of a spherical cell in a homogeneous field. The breakdown voltage of 3T3 cells (average radius 8 μm) is determined to be 1.0 V by measurements using a single particle analyzer (Zimmermann, Scheurich, et al., 1981; Pilwat, et al., 1980; Zimmermann, et al., 1980). Thus, the field strength required to reach the breakdown voltage of the membrane area in the field direction ($\cos\theta = 1$) is calculated to be 0.9 kV/cm. Exposing the cells to this field strength will cause little or no fusion. Higher field strengths and longer pulse duration, the cells become mechanically destroyed.

However, fusion in high yield can be achieved if DEP and field pulse application is made in the presence of pronase (1 mg/ml) (Fig. 2). If such agents are added at least 30 s before application of the electric field, the cells can be subjected to rather higher field strengths and longer pulse durations than otherwise, and still undergo little or no irreversible destruction.

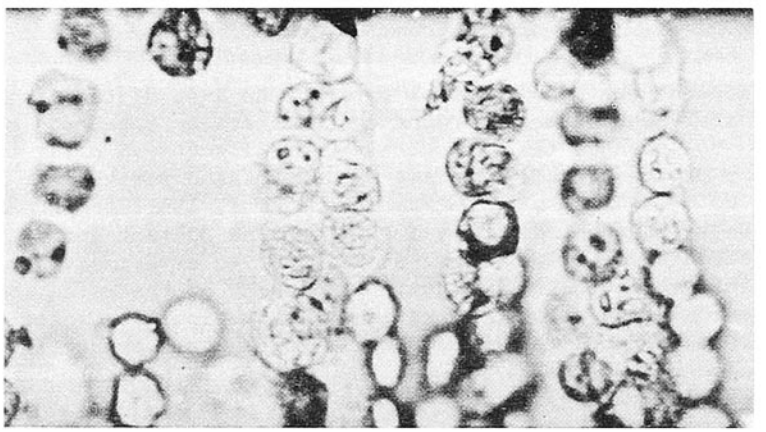

Fig. 2. Fused pearl-chains of mouse 3T3 cells 5 s after the breakdown pulse. The cells were suspended in 0.3 M mannitol solution containing 1 mg/ml pronase and collected by dielectrophoresis (400 V/cm, 1 MHz). Before application of a breakdown pulse with a field strength of 7 kV/cm and a duration of 50 μs the dielectrophoretic force was increased to 700 V/cm.

Moreover, the field strength of the AC voltage used for the DEP phase can now far exceed the critical field strengths required for breakdown and still not cause either destruction or undue stretching of the cells. Measurement of the breakdown voltage using the single-particle counter technique with and without the enzyme present shows that no appreciable change in this factor has taken place. Using a field pulse

of 7 kV/cm and 40 μs duration leads to fusion of up to 100% of the cells present in the field. Under these conditions, the fusion process resulting in the formation of larger cells takes place in about 3 min.

If many pearl-chains are arranged in parallel by using high suspension densities, fusion takes place not only mainly in the principal field direction, i.e. along a given pearl-chain of cells, but also between adjacent chains if pronase is present (Fig. 2). Under these conditions, giant cells of 10 to 100 or more originally individual cells can be obtained, having final diameters of up to 100 μm. Rounding up of such cells is readily observed (Fig. 3), particularly if they are transferred quickly back to a more physiologically favorable isotonic electrolyte solution (Zimmermann, Scheurich, et al., 1981; Zimmermann, Richter, et al., 1981; Pilwat, et al., 1981). Increasing the pulse duration, say to 60 μs, leads to mechanical destruction of the cells.

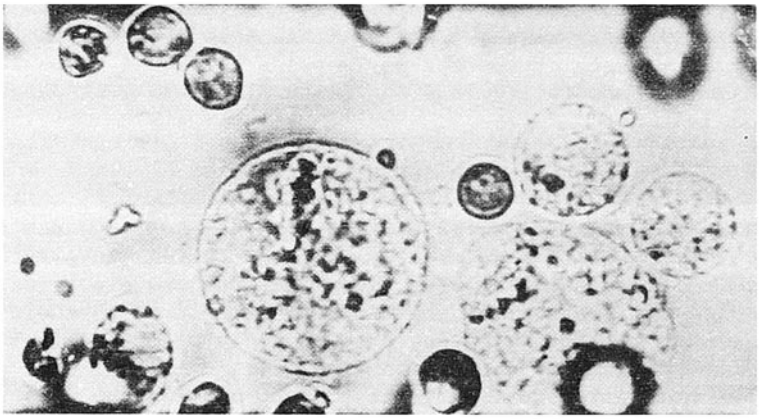

Fig. 3. Giant cell obtained by fusion of mouse 3T3 cells. The conditions for fusion were the same as in Fig. 2. After fusion such giant cells were transferred to an isotonic electrolyte solution.

The multinucleated cells apparently do not divide within the next several days. However, they are stable for even longer times, indicating that the cellular and membrane functions are not affected by the fusion process. The viability of the giant fused cells was also shown by using a vital stain, trypan blue. They could be subjected to slight osmotic stress without deterioration. Control experiments demonstrated that DEP conditions and the presence of pronase for about 10 min. did not alter the growth rate of the 3T3 cells.

Electrical breakdown of the membrane results -- as is well known-- in reversible change in its permeability, provided that the pulse length is not too long and the field strength not too high, as mentioned above (Zimmermann, Scheurich, et al., 1981a). Benz and Conti (1981), in particular, have shown recently that such electrical breakdown has little or no influence on the excitability of the squid axon. It should also be noted that electrically fused sea urchin eggs containing two to three nuclei can undergo fertilization and subsequent division (Richter, et al., 1981).

Now we turn to the readily observable and important phenomenon for the formation of giant cells, that of lateral cell fusion. It will be noted from Eq. 1 that it includes a cosine factor for the angle between the field direction and a radius from the cell center to the surface point on the cell. This implies that a critical breakdown field can be attained at angles greater than zero, but only if the potential at zero angle then exceeds the critical breakdown field. Thus, if the conditions

during fusion can be altered so as to permit the breakdown voltage to be exceeded by an increased amount without increasing permanent damage, more and more lateral fusion can be obtained.

The critical voltage can then be made to encompass wider angles on the cell surface while the maximal voltage effective at the poles of the cells still remains below that causing irreversible damage. This is in fact found for the action of pronase. The field stress permissible before irreversible damage occurs is increased by the presence of pronase (or related agents). In the presence of such agents, much lateral fusion can be brought about. Electrically mediated fusion can then produce truly "giant" cells in which fusion has occurred not only along lines narrowly restricted to the polar regions, but also laterally among adjacent pearl-chains.

It should be noted that the effect of the added enzyme is completely reversible. After the enzyme has been washed out from the cell suspension, the cells are again as sensitive to the field pulses and the alternating voltage as untreated cells. It is also observed that the cells, while in the presence of the enzyme, possess increased mechanical stability,at least temporarily, and can be handled with less danger of lysis than before.

DISCUSSION

At this point it is helpful to consider how and why such additives increase the success of electrically mediated fusion. In particular, by what means do pronase and related agents promote the electrical fusion of cells? Do the agents alter the membrane structure? Is their effect one of changing the intermingling of components of the cell membrane? Or is it one of altering the fusion kinetics?

We may note first that intimate contact between two adjacent cells to be fused is very probably necessary. In particular, we postulate that the process of fusion is optimal if the membrane surfaces themselves are in close contact. This implies that the process will be most successful if the tangent areas are free or relatively free of intramembranous particles such as proteins. A hypothesis introduced by Poste and Allison (1973), and later extended by Lucy and colleagues (Lucy, 1978; Ahkong, et al., 1978) proposes that the emergence of particle-free lipid domains must precede fusion when chemicals or virus particles are used to induce this process. We take a similar view of electrically mediated fusion (Zimmermann, Scheurich, et al., 1981; Zimmermann, Richter, et al., 1981; Zimmermann and Vienken, 1981). Such particle-free lipid domains are necessary to permit bridging between the cells at the two bilayers once electrical forces have initiated breakdown and generated channels between the layers. If proteins are present, the bridging process is hindered and the resealing of the single bilayer dominates, defeating the fusion process. The resealing time of the single bilayer is judged to be in the order of 1 μs from breakdown experiments on planar lipid bilayers (Benz and Zimmermann, 1980).

It appears necessary to postulate that by some as yet unknown mechanism the electric fields applied during the DEP phase and perhaps during the DC phase produce clean lipid domains in the regions of cell-to-cell contact. This is enhanced by the presence of pronase or related agents. It is possible that a very slight degradation of pronase, a proteolytic enzyme, or degradation of protein particles by pronase, can enhance the surface mobility of the intramembranous particles. It is perhaps more readily envisioned that the presence of an increased density of surface-adsorbed negatively charged protein moieties requiring aqueous counterions leads to a removal of such intramembranous par-

ticles from the cell-to-cell contact region due to the restricted solvent qualities of the medium in such confined regions. This enhanced rejection of intramembranous particles by the addition of negatively charged ones to the surface fits with what is known about the high fluidity or mobility of particles on the membrane surface.

The postulated emergence of lipid domains would also immediately explain the enhanced field stability of the cells to high fields in the presence of pronase. Generally speaking, electrical breakdown during a field pulse usually occurs in cell membranes at a lipid-protein junction, or within protein-rich regions. The resealing time of field-induced breaks in protein-rich regions is long, in the order of seconds to minutes, whereas the resealing time of lipid regions is in the order of 1 μs (Zimmermann, et al., 1976; Jeltsch and Zimmermann, 1979; Benz and Zimmermann, 1980; Zimmermann, Scheurich, et al., 1981). Thus, 1981). once breakdown has occurred, in a matter of about 10 ns (Benz and Zimmermann, 1980), the field lines will pass through into the cell interior, leading to probably adverse side effects on organelles and on the biochemical reaction pattern within the cell. In addition, if the resealing is slow, unfavorable osmotic processes may occur owing to the increased permeability of the membrane.

Both effects can lead to the eventual destruction of the cells. On the other hand, if breakdown and subsequent resealing occurs in a particle-free lipid domain, this occurs so very rapidly, because of high fluidity of the lipid domain, that resealing can occur even between pulses of a high-frequency AC field. This minimizes severe damage due to current passage. The cells can be exposed to higher field strengths and longer duration times, for the pores do not remain open long after the pulse has ceased. This aspect of increased field stability argues for the importance of the presence of particle-free lipid domains during electro-fusion.

The explanation suggested by this picture is consistent with current knowledge of breakdown (Zimmermann, Scheurich, et al., 1981).

We look forward to the application of such giant cells to a number of problems of interest to life scientists. For example, the giant cells prepared from various cell types can now be more easily studied with the aid of microelectrodes to examine electrical membrane properties, and to make such comparisons between normal and cancer cells. Preliminary studies have already shown that mouse myeloma cells and normal mouse lymphocytes can be made into giant cells by electric field-induced fusion.

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