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Electric Pulse-Induced Fusion of Mouse Lymphoma Cells: Roles of Divalent Cations and Membrane Lipid Domains

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Summary. Mouse leukemic lymphoblasts (L5178Y) brought into close contact by dielectrophoresis underwent cell fusion following the application of electrical pulses in the presence of electrolytes. The electrically fused cells became spherical after switching off the dielectrophoretic field. Fusion between a cell vitally stained with Janus Green and that with Neutral Red resulted in the homokaryon with a mixed color. Intracellular potentials simultaneously recorded from the two cells located on both sides of the homokaryon were identical. The fusion efficiency was remarkably dependent upon temperature, displaying a discontinuity at about 11°C in the Arrhenius plot. The extracellular application of phospholipase-A_2 or -C suppressed the fusion yield. Thus, it appears that the phospholipid domains play a crucial role in the electric pulse-induced cell fusion. Treatment of the cells with proteolytic enzymes markedly enhanced the fusion yield, presumably due to removing the glycocalix and/or giving rise to fusion-potent, protein-free lipid domains. The presence of millimolar concentrations of divalent cations (irrespective of Mg^{2+} or Ca^{2+}) as well as of micromolar concentrations of Ca^{2+} (but not Mg^{2+}) was prerequisite to the resealing of membranes suffered from electrical breakdown upon exposure to electric pulses. In addition, extracellular Ca^{2+} (but not Mg^{2+}) ions at more than micromolar concentrations were indispensable for the cell fusion.

Key Words cell fusion · electrofusion · dielectrophoresis · calcium · magnesium · membrane lipid · lymphoma cells

Introduction

Membrane fusion is an essential step in a variety of cellular processes, such as exocytosis, endocytosis, fertilization, mitosis and myogenesis. Experimentally induced cell fusion by Sendai virus, polyethylene glycol and other chemical agents has provided a useful model for elucidating the mechanisms underlying the fusion processes (Okada, 1969; Lucy, 1978; Poste & Pasternak, 1978). However, some drawback to these fusion techniques is that the cell fusion is achieved under unphysiological conditions, in addition to its relatively low yield. Recently, a novel technique for cell-to-cell fusion with the aid of electric pulses, electrofusion, has been developed (Zimmermann & Pilwat, 1978; Senda et al., 1979; Neumann et al., 1980; Teissie et al., 1982; Zimmermann, 1982). With this technique cell fusion can be achieved without adding unphysiological fusogens, and the process is synchronous and instantaneous. Thus, this technique offers a new approach toward the analysis of fusion mechanisms. In addition, if the high yield of cell fusion were reproducibly attained and if the fused cells were highly viable, this method would be widely applicable to a variety of fields.

In the present study, the experimental conditions to achieve electrofusion of lymphoma cells with high efficiency and high viability have been examined. The results show that the involvement of Ca^{2+}, Mg^{2+} and membrane lipids is essential for electrofusion. Some of the results have been reported in preliminary form (Ohno-Shosaku & Okada, 1984; Okada et al., 1984).

Materials and Methods

Cells

Mouse lymphoma L5178Y cells were cultured in Fischer medium supplemented with 10% bovine serum. The cells were centrifuged twice, and resuspended at about 1.5×10^6/ml in the fusion medium. The cell suspension was stored by chilling on ice for 30 min to 8 hr until use. Unless otherwise stated, the experiments were carried out with these "stored cells," because fusion yields and cell viability were not virtually affected by the storage of the cells in the control fusion medium on ice. In some experiments, the cells were kept at room temperature (22 to 27°C) and provided for experiments within 75 min ("fresh cells"). For vital staining, the cells were preincubated with Janus Green (0.12 mg/ml for 30 min) or Neutral Red (0.24 mg/ml for 30 min), and then washed with a dye-free fusion medium. When necessary, the cells were pretreated with dispase (10 μg/ml for 20 min) or pronase E (50 μg/ml for 5 min), and stored on ice after washing with an enzyme-free fusion medium.

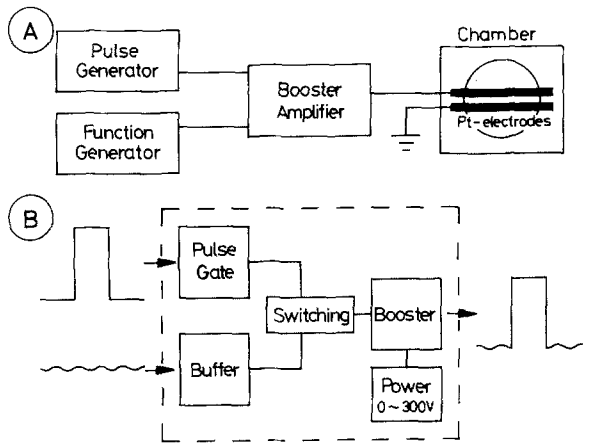

Fig. 1. Apparatus for electrofusion of the cells under dielectrophoresis. *A*) Arrangement of apparatus. *B*) Block diagram of circuit of a booster amplifier employed to reinforce the output voltage and reduce the output impedance

Media

The control fusion medium (a specific resistivity of about 1.1 kΩ cm) was composed of 0.3 M mannitol, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 5 mM Tris-HCl (pH 7.0 to 7.4). To change the Ca^{2+} concentration, an appropriate amount of $CaCl_2$, ethylene glycol bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) or Chelex beads (Bio-rad Labs.), on which ethylendiaminetetraacetic acid (EDTA) is coated, was added to the fusion medium devoid of Ca^{2+}. Effects of other divalent cations were studied using a Mg-free fusion medium or adding 0,1 mM $SrCl_2$, $MnCl_2$, $CoCl_2$, $BaCl_2$ or $NiCl_2$ to a Ca-free fusion medium containing 1 mM $MgCl_2$. To change the anion composition, 5 mM Tris-HCl were replaced by 5 mM Tris-H_2SO_4 or phosphate buffers at a constant pH. In some experiments, the fusion medium containing 7.5 or 15 mM KCl (a specific resistivity of about 160 or 100 kΩ cm) or NaCl was used.

Experimental Setup

The experimental setup employed is illustrated in Fig. 1*A*. The fusion chamber was similar to that employed by Zimmermann and Scheurich (1981), in which two parallel platinum wires of 0.2 mm diameter were placed at a distance of 0.2 mm. The alternating sine waves for cell dielectrophoresis were generated by a function generator (Kikusui 455), and the square pulses for induction of cell fusion by a pulse generator (Nihon Kohden SEN-3201). In order to attain electrofusion even in highly conductive solutions containing a considerable amount of electrolytes (specific resistivity down to 100 Ω cm), the output voltages from these apparatus were reinforced (up to 300 V) and the output impedance was reduced (down to several ohms) by using a booster amplifier, a circuit block diagram of which is shown in Fig. 1*B*. Using a switch device installed in the booster amplifier, alternating fields were automatically disconnected during the pulse application.

Experimental Procedures

Fusion experiments were carried out under a phase-contrast microscope (Nikon MTD) and monitored on a television screen (Tokyo Densi 9M 20A). After adding the cell suspension, the fusion chamber was left without agitation on the stage of the microscope until the cells settled down to the coverglass in the bottom (usually for several minutes). Gentle attachment of the cells to the coverglass enabled us not only to obtain distinct phase-contrast images but also to prevent the cells from adhering to the platinum electrodes after subsequent application of field pulses. On the bottom of the chamber the cells were allowed to adhere to each other by dielectrophoresis (Pohl, 1978) with alternating electric fields. Fusion was then induced by applying 2 to 4 successive rectangular pulses at an interval of about 2 sec. Unless otherwise noted, the cells were exposed to dispase (10 μg/ml) for 10 to 40 min before and during the field application to facilitate fusion (Pilwat et al., 1981; Scheurich & Zimmermann, 1981; Zimmermann et al., 1982). The yield of fusion was determined by counting the cells participating in fusion among 100 to 250 viable cells. Cell viability was estimated by Erythrosin B-exclusion test or by the characteristic phase-contrast images of irreversibly damaged cells; that is, their dark cytoplasmic feature and loss of halo around the cells (e.g., arrowheads in Fig. 3). Preliminary observations indicated that both methods yield virtually identical results. Intracellular recordings from the fused cells were performed using a standard microelectrode technique (Okada et al., 1977). The concentration of free calcium ions in the fusion media, $[Ca^{2+}]_o$, was measured with Ca^{2+}-selective microelectrodes made with Simon's neutral ligand sensor (Oehme et al., 1976), as reported elsewhere (Ueda et al., 1983). The Ca electrodes showed optimum responses between 10^{-3} and 10^{-7} M $[Ca^{2+}]_o$ with a slope of about 25 mV/decade and satisfactory selectivity coefficients against Mg^{2+} (about 10^{-6}). The experiments were usually made at room temperature. If necessary, however, the temperature of the fusion medium was reduced to desired levels by circulating ice-cold water around the fusion chamber and measured with a resistance thermometer (the tip diameter about 1 mm) dipped in the fusion medium.

Chemicals

The chemicals employed in the present experiments were as follows: EGTA (Nakarai Chemicals, Ltd.), Chelex beads (a gift from Dr. S. Kurihara, Jikei University), A23187 (a gift from Eli Lilly), dispase (containing 50% (wt/wt) of calcium acetate: Godo Shusei), pronase E (Kaken Chem.), trypsin (E. Merck AG), protease-Type I, IV and XI (Sigma Chemical Co.), α-chymotrypsin (Sigma), aprotinin (Sigma), *p*-tosyl-L-lysine chloromethylketone hydrochloride (TLCK, Nakarai), phospholipase-A_2, -C and -D (Sigma), chlorpromazine hydrochloride (CPZ, Sigma), CPZ sulfoxide (a gift from Smith-Kline & French Labs.), trifluoperazine dimalate (a gift from Yoshitomi Pharmaceut.) cytochalasin B (Sigma), colchicine (Nakarai), Erythrosin B (Sigma), Janus Green (Wako Pure Chemical, Ltd.) and Neutral Red (Wako). A23187 and TLCK were dissolved in ethanol, and cytochalasin B was in dimethylsulfoxide (DMSO). The addition of ethanol or DMSO alone of the dose employed (1 or 0.5%) did not affect the dielectrophoresis and electrofusion of the cells.

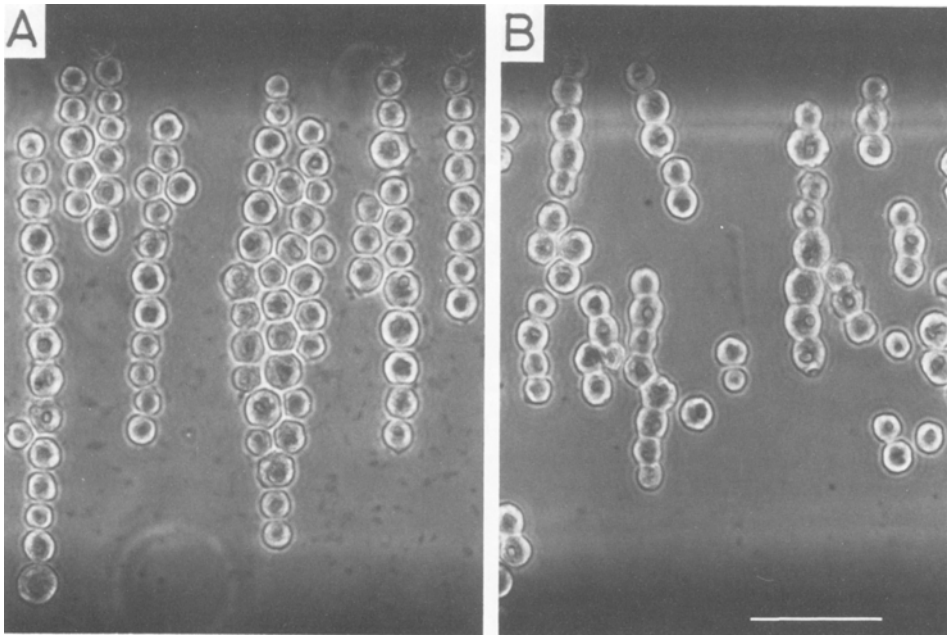

Fig. 2. Phase-contrast micrographs of stored L5178Y cells in the control fusion medium containing 1 μg/ml dispase. *A*) Under dielectrophoresis with sine waves (0.8 kV/cm, 100 kHz). *B*) 5 min after the application of a train of electric pulses (5, 6, 7 and 8 kV/cm, 20 μsec). Bar, 50 μm. Shadows on top and bottom of the micrographs are platinum electrodes

All data are expressed as the mean ± SEM (*n*: number of observations).

Results

Electrofusion of the Cells under Dielectrophoresis

In order to establish a close cell-to-cell adherence, which is prerequisite to subsequent cell fusion, dielectrophoresis of the cells was made as reported previously (Pohl, 1978; Zimmermann et al., 1981*b*; Zimmermann, 1982; Zimmermann & Vienken, 1982). The minimum intensity of sine waves with a frequency of 100 kHz was 0.8 kV/cm for both the fresh and stored L5178Y cells. Under the alternating electric field the cells aligned themselves with each other in parallel to the field lines and closely adhered, forming beads-on-a-string-like aggregates (Fig. 2*A*). Since the cells were detached immediately after switching off the dielectrophoretic field, it appears that irreversible cell adhesion or cell fusion was not brought about by this field application. Dielectrophoresis was not affected by the storage of cells on ice.

Cell fusion was then triggered by applying field pulses. A train of four pulses of 5, 6, 7 and 8 kV/cm with a duration of 20 μsec was optimum for fusion of the stored cells. In the case of the fresh cells, two pulses of 3.3 and 5 kV/cm with a duration of 10 μsec was as effective as four pulses of 5 to 8 kV/cm with 20 μsec, though the reason is not yet known. After switching off the dielectrophoretic field the fused cells gradually became rounded. Fusion observable under a phase-contrast microscope took place within several minutes (Fig. 2*B*). The time to attain rounding up of the fused cells depended not only on the number of cells which formed a fused cell but also on whether the cells were "fresh" or "stored." The homokaryons composed of two or three fresh cells became spherical within 30 min (Fig. 3, single arrows). It took longer to round up those composed of more than four cells. Most of those cells remained in rod-like shape for over 1 hr, although they gradually shrank (Fig. 3, double arrows). In the case of stored cells, however, it took more than 100 min to round up. This fact may be related to the observation that the smaller the values of intracellular ATP/ADP ratio of protoplasts which partake in electrofusion, the longer is the time needed to round up (Verhoek-Köhler et al., 1983).

The fused cells composed of both the cells pre-

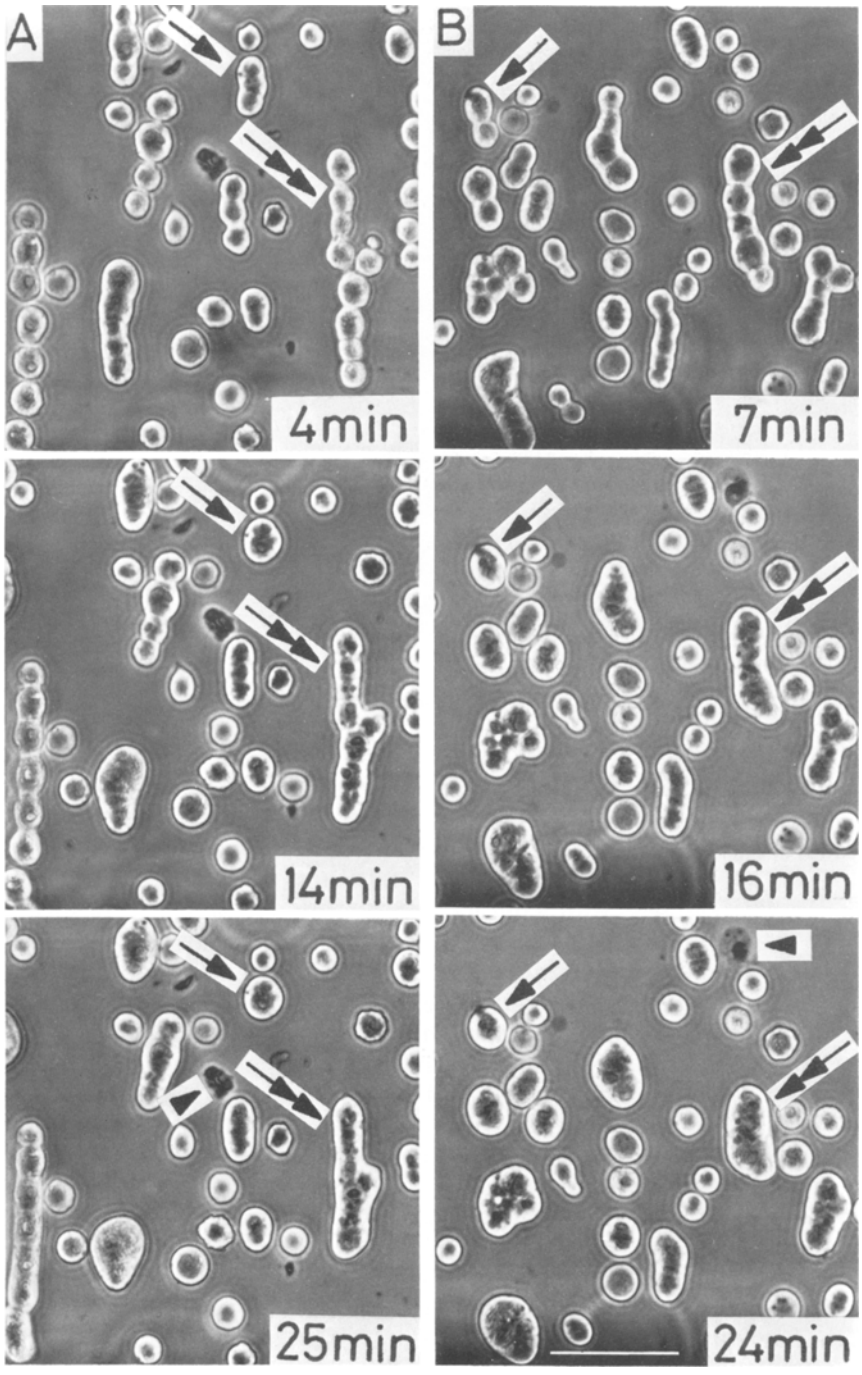

Fig. 3. Two sets of micrographs (*A*, *B*) which show the time course of rounding up of the fused cells after switching off the electric fields. The fresh cells were exposed to an alternating field and electric pulses (3.3 and 5 kV/cm, 10 μsec) in the control fusion medium containing 50 μg/ml pronase E and washed with the enzyme-free control fusion medium. (Arrows and arrowheads, *see* text)

treated with Janus Green (blue color) and with Neutral Red (red) exhibited an intermixed color (green) after several minutes. Thus, it appears that the cytoplasmic constituents can intermingle within the morphologically fused cell. The cytoplasmic continuity can also be evidenced by the following electrophysiological experiment. Two microelectrodes inserted into the two cells which underwent morphological fusion showed the same magnitude of the membrane potential (−5 to −10 mV in a control fusion medium). When a square current pulse (*I*) was injected into one cell, the resultant potential change (ΔE) occurred in the other cell (Fig. 4).

Effect of Temperature

It has been reported that the efficiency of electrofusion of plant protoplasts is relatively independent of the changes in temperatures between 20 and 40°C

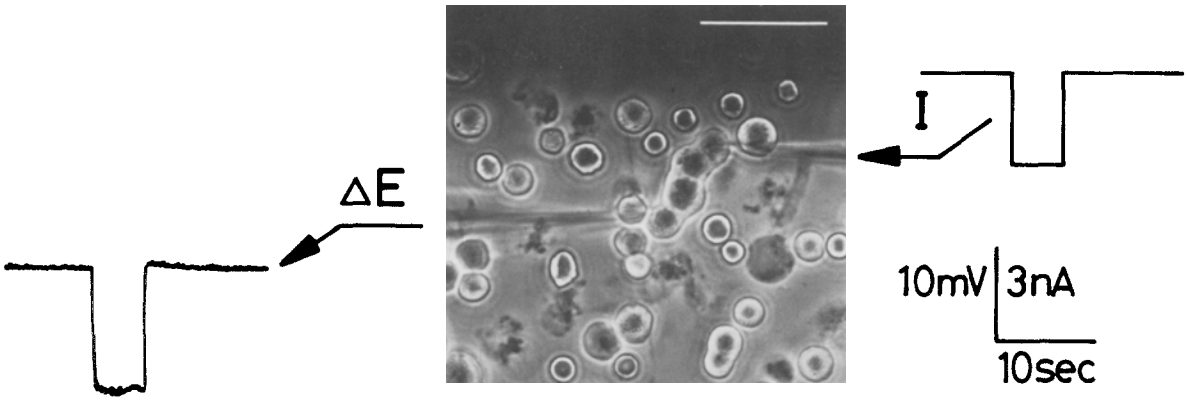

Fig. 4. Electrophysiological evidence for cytoplasmic fusion of lymphoma cells exposed to electric pulses. A micrograph shows the morphologically fused cell impaled with two microelectrodes in the control fusion medium after switching off the electric fields. A shadow on top is a platinum electrode. Bar, 50 μm. Two microelectrodes recorded the same magnitude of intracellular potential (−7 mV). From one of the microelectrodes a square current pulse (*I*) was introduced, and the potential change (ΔE) was measured with another microelectrode

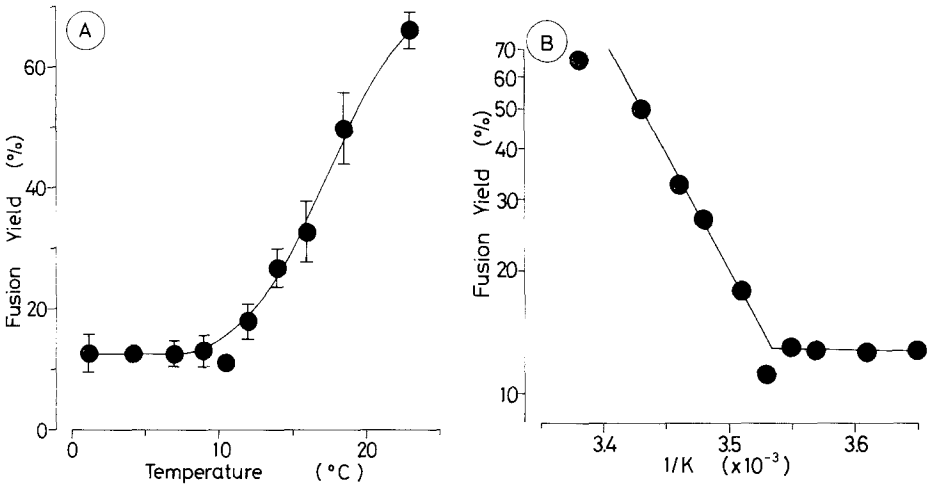

Fig. 5. Effects of temperature on the fusion yield. The stored cells were exposed to electric fields in the control fusion medium containing 10 μg/ml dispase. Cell viability was about 95% regardless of temperature except for 23°C at which it was 81%. From the slope in the Arrhenius plot (*B*) over 11°C, the activation energy is estimated as about 24 kcal/mol, providing that the fusion yield is, to the first approximation, proportional to the rate of chemical reactions responsible for cell fusion. Each point represents the mean value of 4 to 22 observations. Vertical bars, the standard errors of the mean

(Zimmermann & Scheurich, 1981). Similarly, in our preliminary observations, the fusion yield of L5178Y was not sizably affected by temperature changes between 30 and 40°C. However, the fusion yield was strongly dependent on temperatures below 25°C (Fig. 5*A*). At higher than 11°C, cell fusion was enhanced with increasing temperature. Below 11°C, electrofusion was consistently suppressed. Cell viability was not affected by temperature changes in a range from 0 to 20°C (*see* legend for Fig. 5). In the Arrhenius plot, the fusion efficiencies obtained between 0 and 20°C could be described by two straight lines with an intersection near 11°C (Fig. 5*B*). A similar temperature dependency with a transition temperature was also found in Sendai virus-induced fusion of liposomes (Haywood & Boyer, 1982) and in histamine exocytosis by mast cells (Lagunoff & Wan, 1974). However, a transition temperature found in the present study (11°C) is lower than that observed in liposome fusion (18°C) or mast cell exocytosis (16°C). There is a possibility that the local temperature between platinum electrodes was somewhat higher than the temperature measured in the bulk solution because of heat production (Joule heating) by the electric fields. At any rate, a distinct discontinuity in the

Table 1. Effects of phospholipases on the fusion yield and viability of lymphoma cells exposed to electric pulses in the control fusion medium without proteases[a]

Phospholipase	Concentration (μg/ml)	Fusion yield (%)	Cell viability (%)	*n*
Control	—	21.7 ± 1.4	79.6 ± 2.7	23
Phospholipase-A_2	0.01–0.03	18.8 ± 3.0	71.9 ± 5.9	6
	0.1–1	5.1 ± 2.6*	38.3 ± 19.6	4
Phospholipase-C	0.01–0.5	15.6 ± 3.4	84.9 ± 3.4	3
	1 –10	5.6 ± 1.8*	45.2 ± 14.6	6
Phospholipase-D	500	18.1 ± 4.6	75.9 ± 6.3	6

[a] The cells were treated with phospholipases for 2 to 4 min prior to the pulse application.
* Significantly different from the control with $P < 0.05$.

Table 2. Effects of proteases on the fusion yield and viability of lymphoma cells exposed to electric pulses in the control fusion medium

Protease	Concentration (mg/ml)	Treatment time (min)	Fusion yield (%)	Cell viability (%)	*n*
Control	—	—	23.2 ± 1.4	72.4 ± 2.8	36
Pronase E	0.05	3–4	73.2 ± 4.6*	84.5 ± 4.3	4
Dispase	0.01	26 – 110	58.2 ± 3.3*	83.9 ± 2.5	12
Trypsin	0.3–3	4–5	53.9 ± 3.5*	82.9 ± 4.9	8
Chymotrypsin	0.3–1	3–4	50.1 ± 2.2*	62.5 ± 5.5	5
Papain	0.01	5–29	37.7 ± 1.1*	82.2 ± 2.1	6
Protease Type-I	0.05	2–5	51.8 ± 3.6*	86.0 ± 1.0	12
Protease Type-IV	1	3–4	39.7 ± 5.0*	83.5 ± 2.1	3
Protease Type-XI	0.05–0.5	4–5	56.4 ± 3.7*	72.0 ± 3.7	4

* Significantly different from the control with $P < 0.05$.

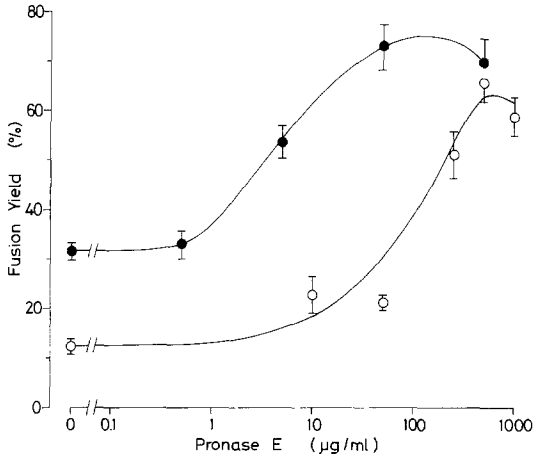

Fig. 6. Effects of the concentrations of pronase E on the fusion yield. The cells were incubated in the control fusion medium containing 0.1 mM Ca^{2+} (closed circles) or the nominally Ca^{2+}-free fusion medium (open circles), and treated with pronase E for 2 to 5 min prior to the pulse application. Each point represents the mean value of four observations. Vertical bars, the standard errors of the mean

Arrhenius plot strongly suggests that the electrofusion of L5178Y cells depends on the physical state of membrane lipids.

Effects of Phospholipases and Proteases

To examine whether the hydrolysis of membrane phospholipids affects the electrofusion, three types of phospholipases were exogenously applied during the field application. Without affecting dielectrophoresis, phospholipase-A_2 and -C inhibited electrofusion at doses of 0.1 to 1 and 1 to 10 μg/ml, respectively (Table 1). They suppressed both the fusion yield and cell viability after exposure to electric pulses, though the latter effect was not statistically significant. Neither dielectrophoresis nor electrofusion was affected by phospholipase-D even with a much higher dose (Table 1). These results suggest that both the fatty acids and phosphoryl groups of phospholipids are important sites for electrofusion.

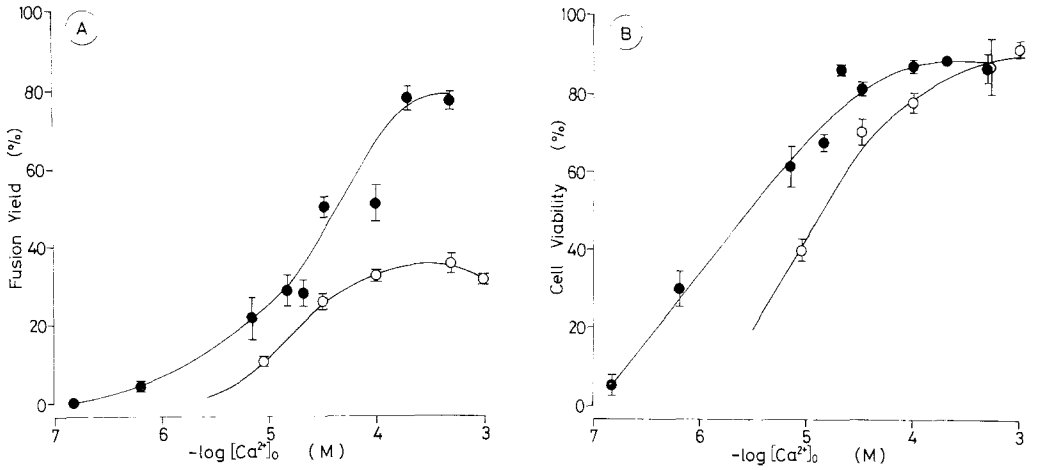

Fig. 7. Effects of the extracellular free Ca^{2+} concentrations ($[Ca^{2+}]_o$) on the fusion yield (*A*) and cell viability (*B*) after the pulse application in the presence (closed circles) or absence (open circles) of 10 μg/ml dispase. Each point represents the mean value of four to 30 observations. Vertical bars, the standard errors of the mean

In contrast, treatment of the cells with proteases (all eight types employed) enhanced the fusion efficiency without affecting cell-to-cell contact induced by dielectrophoresis (Table 2). Most of the proteases tested improved slightly the viability of the cells exposed to electric pulses (Table 2). The following results indicate that proteolytic activities of proteases are responsible for their facilitating effects on electrofusion: First, heat-inactivated pronase E (90°C, 3 min) was no longer effective. Second, protease inhibitors aprotinin (125 μg/ml) and TLCK (5 mM) suppressed the effect of trypsin. Third, pretreatment of the cells with dispase or pronase E was as effective as exposure of the cells to the enzyme during the field application. Thus, it is obvious that limited lysis of the membrane proteins offers favorable conditions for electrofusion. This is consistent with the observations that pretreatment of hen erythrocytes with proteinase enhanced the polyethylene glycol-induced fusion (Hartmann et al., 1976) and that the virus-associated protease is involved in the Sendai virus-induced fusion of human erythrocytes (Israel et al., 1983).

The dose-response curves for pronase E effects are illustrated in Fig. 6. In the nominally Ca-free fusion medium, more than 300 μg/ml of pronase E were necessary to facilitate the electrofusion. This is consistent with the previous observations that the mg/ml order of concentrations is needed to stimulate electrofusion of a variety of animal cells (Pilwat et al., 1981; Scheurich & Zimmermann, 1981; Zimmermann et al., 1981*a*; Zimmermann et al., 1982; Vienken et al., 1983). However, in the presence of 0.1 mM Ca^{2+}, much lower doses of pronase E facilitated the electrofusion. The fusion efficiency increased with an increase in the pronase concentration, reaching a maximum plateau at 50 μg/ml. The data can be explained in two ways: the proteolytic activity of pronase E may be enhanced by the coexistence of Ca^{2+}, and alternatively, the pronase E preparation might have been contaminated by a small dose of Ca ions which may independently facilitate the electrofusion. The former possibility can be ruled out, because pretreatment of the cells suspended in the nominally Ca-free fusion medium with 50 μg/ml pronase E underwent fusion with high probabilities after applying electric pulses in the presence of 0.1 mM Ca^{2+}. In addition, 1 mg/ml pronase E was found to contain free Ca ions of about 0.1 mM with a Ca^{2+}-selective electrode. Therefore, the latter explanation is highly probable.

Effects of Calcium Ions and Other Electrolytes

The possibility that calcium ions may facilitate electrofusion was tested by adding EGTA or $CaCl_2$ to change the Ca^{2+} concentration over a range of 10^{-7} to 10^{-3} M in the presence of 1 mM Mg^{2+}. The free Ca^{2+} concentration in the fusion medium was directly measured with Ca^{2+}-selective electrodes in these experiments. Dielectrophoretic cell adhesion was independent of the $[Ca^{2+}]_o$ values, but the fusion efficiency (Fig. 7*A*) and cell viability (Fig. 7*B*) were sharply dependent on the $[Ca^{2+}]_o$. Viability of the cells exposed to electrical fields increased with an increase in the $[Ca^{2+}]_o$, exhibiting a saturable relation to the $[Ca^{2+}]_o$ values. The half-maximal effect was observed at about 10 μM in the absence of

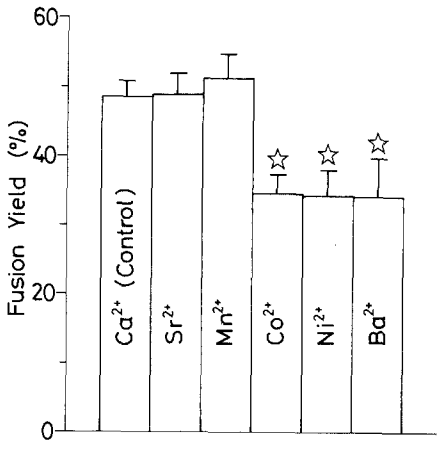

Fig. 8. Effects of various divalent cations on the fusion yield. The cells were incubated with 1 mM Mg^{2+}, 0.1 mM divalent cations other than Mg^{2+} and 10 μg/ml dispase. The free Ca^{2+} concentrations were found to be about 30 μM in the fusion media other than the control Ca^{2+} medium, mainly due to the Ca^{2+} contamination in the dispase preparation. Note that the substitution of Ca^{2+} by Co^{2+}, Ni^{2+} or Ba^{2+} reduced the fusion yield down to about 35%, which was comparable to the yield obtained at about 30 μM of Ca^{2+} (Fig. 7*A*). Cell viability was 80 to 85%. Each represents the mean value of six to 12 observations. Vertical bars, the standard errors of the mean. Stars indicate that the values are significantly different from the control at $P < 0.05$

exogenous proteases. In the presence of dispase, cell viability similarly depended upon the $[Ca^{2+}]_o$ values. However, exogenous dispase decreased the half-maximal $[Ca^{2+}]_o$ down to 2 μM. These data suggest that resealing of the membrane suffered from electrical breakdown depends on extracellular Ca ions. The yield of cell fusion was also intensified by an increase in the extracellular Ca ions. The fusion yield showed a sigmoid relation to $\log[Ca^{2+}]_o$, and the half-maximal effect was observed at 20 to 25 μM $[Ca^{2+}]_o$. The addition of dispase pronouncedly enhanced the fusion yield but did not change the half-maximal $[Ca^{2+}]_o$ values. It is noteworthy that almost identical sigmoidal dependence on the extracellular Ca^{2+} concentration has been found in the fusion of various secretory vesicles (Gratzl et al., 1977; Gratzl & Dahl, 1978; De Lorenzo et al., 1979; Ekerdt et al., 1981; Knight & Baker, 1982).

At the Ca^{2+} concentrations lower than 10^{-5}M, almost all the cells could not fuse, while a considerable number of the cells were still viable over 1 hr after the pulse application (Fig. 7). The $[Ca^{2+}]_o$ value for half-maximal cell fusion is different from that for half-maximal cell viability. Thus, it appears that the Ca-dependency of fusion yield is independent of the Ca-dependency of cell viability. At the submicromolar Ca^{2+} concentrations, cell fusion was abolished and cell viability was greatly reduced (Fig. 7). This phenomenon could be due to some intracellular side effects of EGTA, if EGTA enters the cells through the pores transiently brought about by electrical membrane breakdown. However, when the $[Ca^{2+}]_o$ was reduced to 0.74 μM by adding immobilized EDTA coated on Chelex beads (77.5 mg/ml) to the nominally Ca-free fusion medium containing 1 mM Mg^{2+} and 10 μg/ml dispase, both viability and fusion of the cells exposed to electric pulses were very much suppressed. Therefore, it can be concluded that Ca ions over micromolar concentrations in the extracellular solution are indispensable for electrofusion of L5178Y cells. This conclusion is in sharp contrast to the reports that reduction of extracellular Ca^{2+} concentrations down to 10^{-6} M slowed down the electrofusion kinetics but did not suppress the fusion *per se* of plant protoplasts adhered under dielectrophoresis (Zimmermann & Scheurich, 1981; Zimmermann, 1982), but is consistent with the observations that millimolar Ca ions facilitated electrofusion of protoplasts adhered mechanically (Senda et al., 1982).

The application of Ca^{2+} ionophore A23187 (0.5 to 1 μg/ml) to the fusion medium containing 0.1 mM Ca^{2+} enhanced the efficiency of electrofusion of L5178Y cells. In the absence of proteolytic enzymes, A23187 significantly increased the fusion yield from 24.2 ± 3.5 to 40.2 ± 4.1% ($n = 6$, $P < 0.05$). In the presence of pronase E, a similar facilitating effect of A23187 on electrofusion was also observed, though less remarkable. Therefore, it is likely that Ca^{2+} plays a part in electrofusion by interacting with the cytoplasmic side of the plasma membrane.

The data shown in Fig. 7 also indicate that the effects of Ca^{2+} cannot be substituted by Mg^{2+}. Effects of other divalent cations on electrofusion of the stored cells were examined by replacing Ca^{2+} in the fusion medium with a variety of divalent cations in the presence of 1 mM Mg^{2+}. Sr^{2+} and Mn^{2+} of 0.1 mM were as effective as 0.1 mM Ca^{2+}, whereas Co^{2+}, Ni^{2+} and Ba^{2+} were ineffective at 0.1 mM (Fig. 8).

The addition of 1 mM Mg^{2+} is known to improve the degree of electrofusion of monolayer fibroblasts and of plant protoplasts in a nominally Ca-free solution (Teissie et al., 1982; Chapel et al., 1984). In the case of stored L5178Y cells, in fact, the yield of viable fused cells was very low under Mg-free conditions (Table 3). Although cell fusion proceeded normally in the Mg-free fusion medium containing 0.1 mM Ca^{2+}, most fused cells became irreversibly damaged. Thus, incubation of the cells in the Mg-free fusion medium containing 0.1 mM Ca^{2+} for more than 3 hr (at 0°C) seemed not to interfere with the membrane fusion but to render the cells very

Table 3. Effects of 1 mM Mg^{2+} (or Ca^{2+}) on the fusion yield and viability of "stored" or "fresh" lymphoma cells exposed to electric pulses in the fusion medium containing 0.1 mM Ca^{2+} and 100 μg/ml pronase E

Cells	Conditions	Fusion yield (%)	Cell viability (%)	n
"Stored" cells (3 to 5 hr at 0°C)	Control (1 mM Mg^{2+})	61.4 ± 5.0	76.3 ± 9.0	4
	Mg-free	18.1 ± 4.5*	32.1 ± 2.4*	4
	Mg-free + 1 mM Mg^{2+}**	63.0 ± 3.5	79.5 ± 6.8	4
	Mg-free + 1 mM Ca^{2+}**	56.6 ± 3.2	92.4 ± 1.2	4
"Fresh" cells (30 to 50 min at 22 to 27°C)	Control (1 mM Mg^{2+})	50.0 ± 2.1	88.9 ± 0.7	3
	Mg-free	52.7 ± 2.0	85.6 ± 2.9	3

* Significantly different from the control with $P < 0.05$.
** Immediately before pulse applications, 1 mM Mg^{2+} or Ca^{2+} was added to the Mg-free fusion medium containing 0.1 mM Ca^{2+}.

sensitive to pulse application (at room temperature). The addition of 1 mM Mg^{2+} or Ca^{2+} at the moment of exposure to electric pulses prevented the cell damage and thus recovered the fusion yield (Table 3). Therefore, it can be concluded that the existence of millimolar concentrations of Mg (or Ca) ions, in addition to micromolar concentrations of Ca^{2+} (Fig. 7), is prerequisite to the resealing of membrane suffered from electrical breakdown, probably due to the so-called screening effect (McLaughlin et al., 1971). On the other hand, in the case of fresh cells, removal of Mg^{2+} did not impair fusion nor viability of cells, if the incubation time of the cells in the Mg-free fusion medium (containing 0.1 mM Ca^{2+}) was shorter than 1 hr (Table 3). This fact strongly suggests that the Mg-free effect on the stored cells is due to the reduction of Mg ions within the cells.

The process of rounding up of fused cells was also dependent on the millimolar divalent cations. The fresh cells, which had been fused in the Mg-free fusion medium containing 10 μM Ca^{2+} and 10 μg/ml dispase, could not proceed to round up in the Mg- and Ca-free fusion medium, even though this medium was found to be contaminated with 2 to 3 μM Ca^{2+}. The addition of 1 mM Mg^{2+} or Ca^{2+} restored this process, as reported by Zimmermann and Vienken (1984).

When Tris-HCl buffers in the control fusion medium were replaced with Tris-H_2SO_4 or phosphate buffers, keeping the pH at 7.2, neither the fusion yield nor viability of the cells exposed to electrical pulses was influenced. Thus, electrofusion of lymphoma cells appears to be independent of species of anions employed.

The addition of NaCl or KCl (7.5 to 15 mM) to the control fusion medium slightly enhanced the fusion efficiency. At the same time, the temperature of the fusion medium containing these concentrations of NaCl or KCl was found to increase by about 2 to 4°C during the field application. Thus, it is likely that the effects of NaCl or KCl are the result of the heat development (Joule heating) due to the presence of a considerable amount of electrolytes.

Effects of Drugs which Affect Cytoskeletons and Calmodulin

Since the selectivity for divalent cations (Fig. 8) suggests the possible involvement of some Ca^{2+}-binding proteins in electrofusion, effects of putative blockers for Ca^{2+}-binding cytoskeletal proteins and calmodulin were examined. The external application of 10 μM cytochalasin B, which impairs actin gelation and microfilament function, did not affect the fusion efficiency, the cell viability after exposure to pulses (Table 4) and the subsequent rounding up process. This is consistent with the report that cytochalasin B or D was without effect on exocytosis of catecholamine by adrenal medullar cells exposed to electric fields (Knight & Baker, 1982), but in contrast with the report that cytochalasins suppressed virus-induced fusion of Ehrlich ascites tumor cells (Asano et al., 1974). Also, no significant inhibition was observed following the application of 10 μM colchicine which depolymerizes microtubules (Table 4). Thus, the present data provide no support for a role of microfilaments or microtubules in electrofusion. The fusion efficiency was significantly suppressed by 25 to 50 μM CPZ (Table 4). In contrast, CPZ-sulfoxide, which is a much weaker calmodulin inhibitor although its hydrophobicity is comparable to CPZ (Douglas & Nemeth, 1982), did not significantly affect the fusion yield TFP (25 μM) also somewhat suppressed the fusion yield (Table

Table 4. Effects of putative antagonists for cytoskeletons and calmodulin on the fusion yield and viability of lymphoma cells exposed to electric pulses in the control fusion medium containing 50 to 500 μg/ml pronase E (upper group) or 10 μg/ml dispase (lower group)

Drug	Concentration (μM)	Treatment time (min)	Fusion yield (%)	Cell viability (%)	*n*
Control (pronase E)			53.7 ± 2.9	86.8 ± 2.1	14
Cytochalasin B	10	4–5	56.1 ± 2.5	82.5 ± 4.9	4
Colchicine	10	15–27	53.5 ± 4.7	87.2 ± 1.1	6
TFP	25	10–32	47.2 ± 2.8	81.3 ± 4.2	6
Control (dispase)			48.4 ± 2.3	78.2 ± 3.4	12
CPZ-sulfoxide	25–50	3–4	50.7 ± 2.2	81.3 ± 1.9	4
CPZ	25–50	3–4	35.3 ± 4.8*	55.7 ± 7.6*	4

* Significantly different from the control with $P < 0.05$.

4), but not the rounding up of fused cells (*data not shown*). Cell viability was, to some extent, suppressed by CPZ or TFP (Table 4). Since CPZ-sulfoxide did not significantly diminish cell viability, it is unlikely that decreased cell viability resulted from nonspecific effects of these hydrophobic agents. Thus, a possibility remains that roles of Ca^{2+} in membrane fusion and resealing upon exposure to electric pulses are mediated by activation of calmodulin. A role of calmodulin has been suggested in the secretory granule-plasma membrane fusion during exocytosis (De Lorenzo et al., 1979; Burgoyne et al., 1982; Douglas & Nemeth, 1982) and in the spontaneous fusion of myoblasts (Bar-Sagi & Prives, 1983).

Discussion

Although detailed mechanisms involved in the membrane fusion are not as yet known, all fusion events presumably share several basic processes in common; that is, a close contact of two membranes, emergence of protein-free areas of phospholipid bilayer, and intermingling of lipids of two membranes. The data obtained in the present experiments on electrofusion of lymphoma cells are compatible with this notion. From the temperature dependence (Fig. 5), it is strongly suggested that electrofusion is also crucially dependent on the physical state of membrane lipids. The experiments with phospholipases (Table 1) suggest that fatty acids and phosphoryl groups constituting membrane phospholipids are important sites for electrofusion. Exogenous proteases markedly facilitated, through their proteolytic activities, the electrofusion (Table 2, Figs. 6 & 7*A*). Since treatment of the cells with proteases prior to the pulse application was also effective, the facilitating effect was not due to degradation of some intracellular proteins by the proteases which entered upon pulse application through the pores produced by electrical membrane breakdown. The simplest explanation for the protease effects would be that removal of cell coats composed of glycoprotein matrixes enables the two membranes to take in more juxtaapposition and/or that limited degradation of integral membrane proteins creates protein-free, fusion-potent lipid moieties.

Calcium ions are widely believed to play a causal role in cell fusion (Okada, 1969; Lucy, 1978; Poste & Pasternak, 1978; David & Higginbotham, 1981). In this respect, electrofusion was considered to be unique because removal of extracellular Ca^{2+} failed to abolish the fusion (Zimmermann, 1982). However, in the experiments without using chelating agents, it is feasible that a small amount of Ca ions might be contaminated in the chemicals employed (especially pronase). In fact, the present study with Ca^{2+}-selective electrodes indicated that this is the case in some protease preparations, and the effect of pronase E could be explained partly by the contaminated Ca^{2+} (Fig. 6). Conversely, quantitative reassessment in the present study gives evidence that Ca^{2+} is involved in electrofusion of lymphoma cells. First, cell damage upon pulse application due to irreversible electrical breakdown was greatly reduced in the presence of extracellular Ca^{2+} over micromolar concentrations (Fig. 7*B*). Exogenous protease reduced the Ca sensitivity down to submicromolar ranges (Fig. 7*B*). Second, fusion of viable cells was also highly dependent on extracellular Ca^{2+} concentrations. Ca^{2+} ions of over micromolar concentrations were prerequisite to the fusion in the presence or absence of exogenous proteases (Fig. 7*A*). The fusion efficiency increased with an increase in the $[Ca^{2+}]_o$ until the maximal effect was attained at 0.1 to 0.2 mM (Fig. 7*A*). Sr^{2+} and Mn^{2+} were able to substitute for Ca^{2+} in this effect, but Mg^{2+}, Co^{2+}, Ni^{2+} and Ba^{2+} were not (Fig.

8). This selectivity for divalent cations cannot be explained in terms of the so-called screening effect but might suggest the possible involvement of some Ca^{2+}-binding site(s) or protein(s) in cell fusion. Since calmodulin inhibitors partially inhibited electrofusion (Table 4), this Ca^{2+}-binding protein remains as one of the candidates. On the other hand, the experiments with cytochalasin B and colchicine did not provide any evidence for the involvement of microfilaments or microtubules in the electrofusion (Table 4). Further investigations are needed to determine the precise binding site of Ca^{2+} responsible for electrofusion.

In addition to the above-mentioned Ca^{2+}-specific effects, the presence of millimolar concentrations of divalent cations (Ca^{2+} or Mg^{2+}) was to be essential for the cell fusion processes. First, the resealing of membranes suffered from electrical breakdown was dependent on the millimolar divalent cations in the extracellular or intracellular milieu. The fresh cells, in which millimolar Mg ions probably exist, were resistant to the pulse application even without extracellular divalent cations of millimolar concentrations. However, the stored cells, which had been maintained in Mg-free fusion medium for over 3 hr at 0°C, were sensitive to electric pulses, resulting in irreversible cell damage, and addition of the millimolar divalent cations upon the field application prevented cell damage. Second, the rounding up process was also dependent on the presence of millimolar divalent cations in the fusion medium.

An electrofusion technique has a number of advantages compared to conventional fusion techniques by means of virus or chemical agents. Cell fusion can take place synchronously and immediately after exposure to electric pulses. All the processes can be readily monitored by light microscopy. Fusion can be induced under physiological conditions without adding any exogenous fusogens, and even with adding a variety of electrolytes, if an appropriate electronics instrument is employed to overcome the limitations due to the high conductivity of cell suspensions. Furthermore, the yield of cell fusion is remarkably high. Under optimum conditions, almost all (70 to 90% of) the cells were found to be viable and partake in cell fusion after exposure to electric pulses. Cytoplasmic fusion was confirmed by three criteria: 1) fused cells became gradually spherical after switching off the dielectrophoretic field (Fig. 3); 2) vitally staining dyes within one cell could move to the partner cells several minutes after the exposure to electric pulses; 3) fused cells behaved as one volume conductor in simultaneous intracellular recordings (Fig. 4). A number of previous studies have shown that the hybrids obtained by electrofusion can continue to grow and reproduce (Richter et al., 1981; Bischoff et al., 1982; Bates et al., 1983; Halfmann et al., 1983; Finaz et al., 1984). Heterokaryons of mammalian cells have also been obtained in high yields with the electrofusion technique (Bischoff et al., 1982; Vienken & Zimmermann, 1982; Finaz et al., 1984; Ohno-Shosaku et al., 1984). Recently, using this technique, monoclonal antibody production by hybridomas has succeeded (Lo et al., 1984). Therefore, it appears that electrofusion technique is a useful tool in studies on gene mapping, genetic complementation and genetic engineering.

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