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-A2 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 Mg²⁺ or Ca²⁺) as well as of micromolar concentrations of Ca²⁺ (but not Mg²⁺) was prerequisite to the resealing of membranes suffered from electrical breakdown upon exposure to electric pulses. In addition, extracellular Ca²⁺ (but not Mg²⁺) 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 Ca²⁺, Mg²⁺ 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 x 10⁶/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.
**Experimental Procedures**

Fusion experiments were carried out under a phase-contrast microscope (Tokyo Densi 9M 20A) and monitored on a television screen. 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, \([Ca^{2+}]\), 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 10⁻¹ and 10⁻⁷ M \([Ca^{2+}]\) with a slope of about 25 mV/decade and satisfactory selectivity coefficients against Mg²⁺ (about 10⁻³). 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. Kurithara, 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-A₂, -C and -D (Sigma), chlorpromazine hydrochloride (CPZ, Sigma), CPZ sulfoxide (a gift from Smith-Kline & French Labs.), trifluoperazine dimaleate (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.

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**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 MgCl₂, 0.1 mM CaCl₂ and 5 mM Tris-HCl (pH 7.0 to 7.4). To change the Ca²⁺ concentration, an appropriate amount of CaCl₂, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) or Chelex beads (Bio-rad Labs.), on which ethylenediaminetetraacetic acid (EDTA) is coated, was added to the fusion medium devoid of Ca²⁺. Effects of other divalent cations were studied using a Mg²⁺-beads (Bio-rad Labs.), on which ethylenediaminetetraacetic acid (EDTA) is coated, was added to the fusion medium devoid of Ca²⁺. Intracellular recordings from the fused 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, \([Ca^{2+}]\), 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 10⁻¹ and 10⁻⁷ M \([Ca^{2+}]\) with a slope of about 25 mV/decade and satisfactory selectivity coefficients against Mg²⁺ (about 10⁻³). 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.

**Experimental Setup**

The experimental setup employed is illustrated in Fig. 1A. 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. 1B. Using a switch device installed in the booster amplifier, alternating fields were automatically disconnected during the pulse application.

**CHEMICALS**

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