Introduction of Macromolecules into Living Dictyostelium Cells by Electroporation

Shigehiko Yumura†, Rika Matsuzaki, and Toshiko Kitanishi-Yumura
Department of Biology, Faculty of Science, Yamaguchi University, Yamaguchi 753, Japan

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ABSTRACT. An attempt was made to optimize conditions for introduction of macromolecules into Dictyostelium cells by electroporation. The amount of fluorescein-labeled bovine serum albumin (FITC-BSA) introduced into cells was measured by fluorometry after extraction of FITC-BSA from cells with detergent. The amount increased as the applied voltage and capacitance of the discharger were increased. However, the survival of cells decreased at higher voltages and elevated capacitance. FITC-BSA was introduced into 80-90% of treated cells. FITC-BSA at 0.25 mg/ml was introduced into cells under optimum conditions when the concentration of the extracellular protein was 2.5 mg/ml. Several discharges in sequence improved the extent of introduction of FITC-BSA although viability decreased. There was a linear correlation between final intracellular concentration and the initial extracellular concentration of FITC-BSA, suggesting the possible quantitative introduction of the protein into cells. The membrane pores that opened during electroporation closed within 2.5 sec after the discharge. FITC-labeled dextran with molecular weights of less than 5 x 10^5 were able to pass through these pores. Our results show that electroporation provides a quantitative and reproducible method for introduction of macromolecules into living Dictyostelium cells.

Microinjection via a microcapillary is the major method used for introduction of macromolecules into living cells. However, the disadvantages of this method are as follows. 1) It cannot be easily applied to large numbers of cells, for example, 1,000 cells. 2) The cells must be large enough to accommodate a microcapillary. 3) The method is very time-consuming. Other methods, such as the scrape-loading method, the sonication loading method, the method using glass beads, and the red blood cell ghost-mediated method have been developed (8, 4, 3, 5, 9, 13). It is difficult to microinject material into Dictyostelium via a microcapillary since the diameter of each cell is about 7.5 μm, although such experiments have been attempted (1). Recently, an indicator of calcium ion and antibodies were introduced into Dictyostelium by the scrape loading method and the sonication loading method, respectively (12, 6). In the scrape-loading method, macromolecules were introduced by scraping cells with a rubber policeman in the presence of macromolecules after the cells had been tightly attached to coverslips. The macromolecules were introduced when cell membranes were torn by scraping, but the cell membranes were immediately repaired. From 5 to 10% of cells could be loaded with macromolecules by this method. The sonication-loading method depends on a principle similar to that of the scrape-loading method but cell membranes are torn by sonication. None of the methods mentioned so far allows satisfactory quantitative introduction of macromolecules into living cells.

In this study, we examined conditions for the introduction of macromolecules into living Dictyostelium cells by electroporation. We knew already that large amounts of small molecules or small amounts of DNA and plasmids could be introduced into mammalian cells by electroporation (11). In addition, we were also aware of a report that proteins, such as antibodies, could be introduced (2). We attempted to identify conditions for the introduction of large amounts of large macromolecules into living cells. We succeeded in the highly efficient introduction of proteins, namely, 80-90% of treated cells were loaded and 10-20% of the extracellular protein was introduced into cells. This method provided quantitative and reproducible results. Furthermore, our method required only 30 μl of a solution of protein in a small chamber, and less than 10 min were required for treatment of more than 10^6 cells. This method should serve as a powerful tool for studies of the cell biology of small living cells.

MATERIALS AND METHODS

Cell culture. Cells of Dictyostelium discoideum strain NC4 were cultured on nutrient agar with Escherichia coli for

† To whom correspondence should be addressed.
40 hours as previously described (16). The cells were then washed three times in cold 15 mM sodium and potassium (Na/K) phosphate buffer (pH 6.4) by centrifugation and spread on non-nutrient agar at 21 °C for 3–6 hrs.

**Fluorescent macromolecules.** Fifty mg of bovine serum albumin (fraction V; Sigma) dissolved in 0.25 M carbonic acid buffer (pH 9.0) were mixed with 3.4 mg of fluorescein isothiocyanate (Sigma) that had been dissolved in dimethyl sulfoxide and the mixture was incubated at 4°C for 1 hr to allow the coupling reaction to proceed. The solution was dialyzed for two days against 15 mM Na/K phosphate buffer, and then residual free fluorescein was removed by gel filtration on a column of Biogel P-6 that had been equilibrated with phosphate buffer. The molar ratio of bound fluorescein to protein was 1.2. Dextran was also coupled to fluorescein by the same method. Some fluorescein-labeled dextrans (M.W. 4,400 and 9,400) were also purchased from Sigma.

**Electroporation.** For typical electroporation experiments, cells were harvested from an agar plate, washed, and then suspended in chilled 15 mM phosphate buffer at 5 x 10^7 cells/ml. Then 30 μl of the suspension of cells were mixed with 30 μl of a solution of fluorescein-labeled bovine serum albumin (FITC-BSA; 5 mg/ml), and the mixture was placed in a precooled electroporation chamber with stainless-steel electrodes. The distance between electrodes was 1.4 mm. An electrical discharge was generated by a 3.3 μF condenser that had been charged by a power supply for electrophoresis. The diagram of the circuit used for electroporation is shown in Fig. 1. The applied voltage was typically 240 V. The time constant (τ) of the pulse current was estimated to be 150 msec. For determination of viability after discharges, 940 μl of a 5 mM solution of MgCl₂ were added to the contents of chamber and living cells were counted under a phase-contrast microscope. Alternatively, 5 min after discharges, cells were suspended in cold 5 mM MgCl₂ and then washed three times by centrifugation at 1,500 rpm.

**Quantitative analysis of incorporated macromolecules.** The electroporated cells were washed in 5 mM MgCl₂ and suspended in 0.5 ml of phosphate buffer. Ten μl of the suspension were used to determine the number of cells with introduced FITC-BSA by fluorescence microscopy. The incorporation rate was calculated by dividing number of cells with introduced label by the total number of cells. Five min after 0.5 ml of lysis buffer (1% Triton X-100, 100 mM KCl, 1 mM DTT, 0.1 mM PMSF, 5 mM benzamidine, 0.05% sodium azide, 20 mM PIPES; pH 7.5) had been added to the rest of the suspension of cells, insoluble materials were removed by centrifugation at 10,000 rpm for 10 min. The fluorescence of the supernatant was determined with a fluorometer (RF-500; Shimazu) and the amount of protein was estimated from a standard curve for FITC-BSA. The amount of FITC-BSA introduced into 1.5 x 10⁶ cells was calculated by subtracting the amount of non-specifically bound FITC-BSA and multiplying by the incorporation rate. The amount of non-specifically bound FITC was determined from the averages of cells that had been incubated with FITC-BSA without electroporation. The average concentration of introduced FITC-BSA in each cell was calculated by dividing the amount of introduced FITC-BSA by the volume of 1.5 x 10⁶ cells (3.3 x 10⁻⁴ cm³; calculated for an average cell diameter of 7.5 μm).

**Miscellaneous methods.** Protein was quantified by the method of Lowry et al. (7). Cells that had been loaded with FITC-BSA were photographed under an epifluorescence microscope on TMAX 3200 film (Kodak).

**RESULTS**

Dictyostelium cells were subjected to an electropulse by discharge of condenser in the presence of 2.5 mg/ml BSA in chamber in which the distance between electrodes was 1.4 mm. Ruptured cells began to appear when a condenser of 3.3 μF was charged at 220 V; at higher voltages, the viability decreased still further (Fig. 2). The voltage at which ruptured cells began to appear...
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Fig. 3. The effect of the external concentration of protein on the viability of *Dictyostelium* cells after electroporation. The viability curve was shifted to higher voltages when the concentration of extracellular BSA was increased.

Determined with higher capacitance of the condenser (4.7 μF or 6.6 μF). When the concentration of protein mixed with cells increased, the viability curve shifted to higher voltages (Fig. 3). This result suggests that the conductance of the solution in the chamber affects the voltage at which cells begin to rupture. Indeed, the voltage increased when the ionic strength was increased by addition of KCl (data not shown). The density of cells also influenced the viability (data not shown). In the present study, the density of cells in the electroporation chamber was fixed at 2.5 x 10^7 cells/ml.

At higher voltages, when ruptured cells appeared, FITC-BSA was incorporated into 80-90% cells, as determined by fluorescence microscopy (Fig. 4). However, the percentage of incorporated cells decreased at higher voltages, under conditions where more than 80% cells were disrupted. Fluorescence microscopy revealed that an almost equal amount of FITC-BSA was incorporated into each cell (Fig. 5). FITC-BSA was located diffusely within cells and was not sequestered in specific vacuoles or organelles, as indicated by observations at higher magnification. The amount of FITC-BSA incorporated into cells was calculated by measuring fluorescence of extracted FITC-BSA after cells had been treated with a lysis buffer that contained 0.5% Triton X-100. The optimum capacitance of the condenser was 3.3 μF for maximum incorporation of FITC-BSA (Fig. 6). The concentration of FITC-BSA in cells was calculated to be 0.25 mg/ml under optimum conditions when

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![Fig. 3](image1.png)

**Fig. 3.** The effect of the external concentration of protein on the viability of *Dictyostelium* cells after electroporation. The viability curve was shifted to higher voltages when the concentration of extracellular BSA was increased.

![Fig. 4](image2.png)

**Fig. 4.** The percentage of cells into which FITC-BSA was incorporated by electroporation. The number of cells that incorporated FITC-BSA was counted by fluorescence microscopy. Each point represents the mean±S.E. of results from three separate experiments.

![Fig. 5](image3.png)

**Fig. 5.** A fluorescence micrograph (right panel) and a phase-contrast micrograph (left panel) of cells into which FITC-BSA had been introduced by electroporation. The fluorescence was evenly distributed in the cytoplasm in most cells. Bar, 10 μm.

![Fig. 6](image4.png)

**Fig. 6.** Optimum capacitance of the condenser used for introduction of FITC-BSA. Incorporated FITC-BSA was extracted from cells by treatment with lysis buffer. The intracellular concentration of FITC-BSA was calculated as described in the text. The optimum capacitance was 3.3 μF. Each point represents the mean±S.E. of results from three separate experiments.
the extracellular concentration of FITC-BSA was 2.5 mg/ml and the diameter of cells was taken as 7.5 μm. There was a linear correlation between the concentration of incorporated FITC-BSA and the extracellular concentration of FITC-BSA (Fig. 7). The intracellular concentration was about 10% of the extracellular concentration under optimum conditions. Next, the effect of the number of electric pulses was examined (Fig. 8). When the number of pulses was increased, the amount of incorporated protein also increased but viability decreased simultaneously.

Next, we attempted to characterize the opening of pores in cell membranes that was caused by electroporation. We examined how long pores remained open after the electric pulse. Cells were incubated in the chamber for indicated times after an electric pulse in the presence of 2.5 mg/ml FITC-BSA, and then they were washed free of extracellular FITC-BSA. Even after a 10-sec incubation, the concentration of incorporated protein decreased.

**Fig. 7.** The correlation between the initial extracellular concentration of FITC-BSA and the concentration of incorporated FITC-BSA. When the initial extracellular concentration of FITC-BSA was increased, the concentration of incorporated FITC-BSA increased. To equalize concentrations of BSA in the chamber, non-labeled BSA was added to bring the final concentration of BSA to 2.5 mg/ml in each sample. Each point represents the mean±S.E. of results from five separate experiments.

**Fig. 8.** The effect of the number of pulses on the incorporation of FITC-BSA. The extracellular concentration of FITC-BSA was 1 mg/ml. When the number of electric pulses was increased, the concentration of incorporated FITC-BSA (open circles) increased, but the viability (open triangles) decreased. Each point represents the mean±S.E. of results from three separate experiments.

**Fig. 9.** Optimum incubation time after electroporation. Cells were electroporated in the presence of 2.5 mg/ml FITC-BSA and washed after indicated times. The concentration of incorporated FITC-BSA was calculated as described in the text. No further FITC-BSA was incorporated into cells after a 10-sec incubation. Each point represents the mean±S.E. of results from three separate experiments.

**Fig. 10.** Duration of the opening of pores in the cell membrane through which FITC-BSA could pass. At indicated times after electroporation, FITC-BSA at 2.5 mg/ml was added to the suspension of cells. For zero time, cells were electroporated in the presence of FITC-BSA. The abscissa shows the time at which FITC-BSA was added after electroporation. Only when FITC-BSA was added at zero time or 2.5 sec after electroporation was any FITC-BSA incorporated into cells. Each point represents the mean±S.E. of results of three separate experiments.
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Molecular weight

Fig. 11. Incorporation of FITC-dextrans of various molecular weights. FITC-dextrans of various molecular weights (4.4 x 10^3, 9.4 x 10^4, 1.56 x 10^5, and 5 x 10^6) were incorporated into cells by electroporation. The ordinate shows the concentration in cells of incorporated FITC-dextran. Smaller dextrans were incorporated more effectively into cells. Each plot represents the mean ± S.E. of results of four separate experiments.

incubation, the amount of incorporated protein reached a maximum level (Fig. 9). Then FITC-BSA was added for indicated times after a pulse in the presence of 2.5 mg /ml non-labeled BSA. FITC-BSA was incorporated only at a low rate for 2.5 sec after a pulse (Fig. 10). These results indicate that the pores through which FITC-BSA could pass closed several seconds after the pulse. Next, the size of pores was examined. FITC-dextrans of different molecular weights (4.4 x 10^3, 9.4 x 10^4, 1.56 x 10^5, and 5 x 10^6) were incorporated into cells. Smaller dextrans were incorporated more effectively but the largest FITC-dextran conjugate was also incorporated into cells (Fig. 11). It was obvious that there were differences in rates of incorporation between different molecular species. FITC-BSA (M.W. 6.8 x 10^4) was incorporated more easily than FITC-dextran (M.W. 4.4 x 10^3).

Incorporated FITC-BSA was retained within cells even after 12 hrs. This method might be useful for labeling of specific cells for tracing of cell lineages during development.

DISCUSSION

In this study, we succeeded in the introduction of macromolecules, namely, a protein and dextrans into living Dictyostelium cells. Macromolecules with molecular weights of less than 5 x 10^6 (higher molecular weights were not examined) were incorporated into cells. The maximum concentration of incorporated FITC-BSA was about 10% of the extracellular concentration after a single electric pulse. Furthermore, 80-90% of cells incorporated macromolecules during a pulse. The scrape-loading method was described in the recent literature as suitable for application to Dictyostelium but only 5-10% of cells incorporated material. The electroporation method enabled us to incorporate macromolecules quantitatively and into a large number of cells. Furthermore, only small volume of protein solution (30 μl for one pulse) was necessary.

The new method does have some defects. It is not suitable for observations of cells immediately after incorporation of macromolecules. For example, it is difficult to observe the way in which fluorescent actin is incorporated into the preexisting cytoskeleton in a cell immediately after a pulse. It is necessary to determine optimum conditions for specific macromolecules because the ionic conditions of the solution in which macromolecules are suspended have a considerable effect on the incorporation. It is also necessary to consider differences among molecular species to be introduced. There were differences in rates of incorporation between FITC-BSA and FITC-dextrans. The three-dimensional conformation or hydrophobicity of macromolecules might be important here. It is known that linear DNA is introduced into cells more easily than circular DNA (11).

It is important to evaluate the cytoplasmic concentration of incorporated material in cells. When the extracellular concentration of FITC-BSA was 2.5 mg/ml, FITC-BSA was incorporated at 0.25 mg/ml if we assume that the average diameter of a Dictyostelium cell is 7.5 μm. This latter concentration corresponds to 3.7 μM but it is an underestimate because no correction was made for the volume of intracellular vacuoles that contained no FITC-BSA. The value is, however, higher than the concentration of most cellular proteins. It might be possible to incorporate antibodies so that they saturate the binding sites of antigen to inhibit the function of the antigen. Actin and myosin reorganize during the active amoeboid movement of Dictyostelium cells (14, 15). The method described in this report should enable us to observe the dynamic organization of cytoskeletal proteins in living Dictyostelium cells after incorporation of fluorescent cytoskeletal proteins. This possibility has previously been limited to large cells that were subjected to microinjection. Thus, the electroporation method should serve as a powerful tool for many investigations of small cells.

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REFERENCES


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