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Single-Cell Electroporation of Fluorescent Probes into Sea Urchin Sperm Cells and Subsequent FRAP Analysis

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In sea urchin spermatozoa, the energy required for flagellar motility depends only on the diffusional supply from proximal mitochondria, and thus the diffusion rate inside flagella is one of the most crucial factors limiting the practical size and design of the motile machinery. To determine the diffusion rates of materials inside sperm cells, FRAP (fluorescence recovery after photobleaching) analysis of incorporated fluorescent probes is one of the most powerful approaches. However, the only practically possible method until now was to use the ester forms of fluorescence, and our choice was limited to those of relatively small molecular masses, such as fluorescein derivatives. In this report, we show that a modified single-cell electroporation technique can be applied as a new microinjection method for sperm cells of the sea urchin. The method was applied to FRAP analysis to determine the rate of intraflagellar diffusion.

Key words: single-cell electroporation, sea urchin sperm, FRAP, diffusion, flagella, cilia

INTRODUCTION

Animal spermatozoa are composed of a head and a long tail flagellum. Although new material transportation of synthesized peptides is unlikely after the completion of spermatogenesis, the diffusion of other types of small molecules, such as ATP, ions, and signaling ligands, in sperm cells is crucial to maintain active flagellar motility as well as for regulation during chemotactic swimming and fertilization. In the case of ATP, an appropriate amount produced by the mitochondria must be transported continuously by diffusion from the head to the whole length of the flagellum, although microtubule-based inner structures are apparent obstacles that restrict the free diffusion of materials. Several reports have shown theoretical calculations for ATP diffusion (Brokaw, 1966; Nevo and Rikmenspoel, 1970; Tombes et al., 1987). We directly determined the diffusion coefficients of fluorescent dyes in sea urchin sperm flagella (Takao and Kamimura, 2008) using FRAP analysis. Although our results supported the model by Tombes et al. (1987), more detailed investigation of the diffusion properties of materials inside sperm cells is necessary, by analyzing various other molecules such as fluorescence-labeled ATP or macromolecules.

Various methods, such as the direct microinjection of fluorescent probes or the expression of fluorescent protein genes, have been used to load cells with exogenous molecules for FRAP experiments (Roucou et al., 2005; Eckert, 2006; Guo et al., 2006). However, these techniques are less practical for the sperm cells of most animal species. This is because sperm cells are too small for microinjection, and they are highly differentiated cells in which gene expression is almost completely suppressed. These properties of sperm cells make it difficult to apply the conventional loading techniques for fluorescent probes. On the other hand, electroporation, a method that loads probes after membrane perforation by electric pulses, is useful for various types of cells ranging from bacteria to cultured mammalian cells. It has also been applied to sea urchin eggs (Swezey and Epel, 1989; Laroche and Epel, 1991). However, there have been no reports of its use in sperm cells, and our preliminary trials of conventional electroporation into sperm cells were less successful, obtaining just a few loaded cells among hundreds of cells (unpublished data). Thus, a currently available method was to use the acetoxymethyl (AM) or diacetate (DA) ester forms of fluorescent dyes (Rodriguez and Darszon, 2003; Nakajima et al., 2005; Shiba et al., 2008). This method is easy to use; however, the species of fluorescent probes were limited only to small-sized, commercially available molecules.

A new method called single-cell electroporation was recently established for nerve cells, both in culture and in vivo (Haas et al., 2001; Norkrantz et al., 2001; Bestman et al., 2006; Lang et al., 2006), where electroporation is carried out locally with a microelectrode placed close to the target single cell. We decided to apply this technique to load sea urchin sperm cells with fluorescent probes instead of the conventional electroporation technique, for three reasons. First, the single-cell electroporation has been reported to be...
more efficient than the conventional one (Ionescu-Zanetti et al., 2008). Second, it was efficient when being combined with FRAP experiments. After single-cell electroporation, we could sequentially execute FRAP experiments using the same dye-loaded spermatozoa. Third, we expected to apply the same technique to the analysis of single-molecule diffusion inside cells in the future (Bruckbauer et al., 2007).

With some modifications, we were able to apply the single-cell electroporation technique to sea urchin spermatozoa. Successfully loaded some fluorescent probes, including fluorescein-derivatives and dextran conjugates (MW 3000), into sperm cells and then executed FRAP analysis of these probes in sperm flagella. This is the first report to analyze the diffusion properties of such large molecules inside flagella. We expect that the application of single-cell electroporation to sperm cells will expand the ways in which experimental approaches can investigate mechanisms regulating flagellar movements and other related, issues.

**MATERIAL AND METHODS**

**Materials**

Chemicals were purchased from the following suppliers unless otherwise stated: Wako (Osaka, Japan), Sigma-Aldrich Japan (Tokyo), or Dojin (Kumamoto, Japan). Fluorescent probes (carboxyfluorescein, Oregon Green, calcein, fluorescein-dextran, BODIPY-labeled ATP, and other fluorescence-labeled proteins) were obtained from Invitrogen (Carlsbad, CA, USA). Sea urchin sperm (from *Pseudocentrotus depressus* or *Anthocidaris crassispina*) was obtained by intracoelomic injection of 0.5 M KCl. Spawned sperm was collected and stored without dilution (dry sperm) at 4°C until use. The experimental results obtained with both sea urchin species were almost indistinguishable, and data from both species were treated together and are shown. A solution containing 225 mM K-gluconate, 225 mM mannitol, 20 mM NaCl, 20 mM MgCl₂, 10 mM HEPES (pH 7), and 0.5% (w/v) BSA was used as the experimental medium (modified from the medium used by Swezey and Epel, 1989). Under these conditions, sea urchin spermatozoa were almost all non-motile, and cells adhering to glass surfaces were used for the experiments.

**Setup for single-cell electroporation**

For the experimental setup for the single-cell electroporation, we modified and used the methods previously described by Bestman et al. (2006). Photographs and a schematic drawing of the equipment used are shown in Fig. S1. A micromanipulator (MHW-3; Narishige, Tokyo, Japan) holding a glass microelectrode, a reference electrode, a silicon rubber chamber set on a cover slip, and silicon tubes to perfuse the chamber with experimental solutions were all placed on the specimen stage of an epi-fluorescence microscope (IX-70; Olympus, Tokyo, Japan). Glass micropipettes were made by pulling borosilicate glass capillaries containing filaments (outer diameter 1.5 mm, inner diameter 9 mm, length 90 mm; GD-1.5; Narishige, Tokyo, Japan) with a Flaming/Brown micropipette puller (P-97; Sutter Instrument, Novato, CA, USA). Distilled water containing 200 μM fluorescent probe was backfilled into the pipette by using a 1.5 ml syringe. Platinum wires of 0.25 mm diameter (Nilaco, Tokyo, Japan) were used as electrodes. Electric pulses generated by an electronic stimulator (SEN-2201; Nihon Kohden, Tokyo, Japan) were introduced into the electrodes. The reference electrode placed in the chamber was grounded via a resistor (1–100 kΩ). The resistor was used in order to monitor driving currents during electroporation with a digital oscilloscope (VC-6723; Hitachi, Tokyo, Japan).

Before each experiment, a small amount of dry (i.e., undiluted) sperm was first diluted with the experimental medium without BSA and then transferred into a chamber made of a cover slip and a silicon rubber strip, with the top of the chamber left open. By perfusing the chamber with the experimental medium containing BSA by means of a peristaltic pump (PST-100; Iwaki Glass, Chiba, Japan), free sperm cells were removed. Sperm cells attached to glass surfaces were used for the experiments. BSA was added to the medium in order to avoid sperm adhering to the glass micropipettes. After electroporation, we also perfused the chamber in order to remove excess fluorescent probes released in the chamber.

With a CMOS USB-camera (Qcamm Pro 4000; Logicool, Tokyo, Japan) set on a camera port of the fluorescence microscope, the micropipette and the specimen under LED illumination were observed for micromanipulation. After electroporation and the washing away of excess dyes in the medium by perfusion, the observation light path of the microscope was converted for usual fluorescence observations with an Olympus U-MSWB filter set (for green fluorescence observations). Fluorescence images were observed under continuous blue-light excitation with a high-pressure mercury lamp.

**FRAP**

For FRAP experiments, a fluorescence microscope equipped with laser optics for photo-bleaching was used, as previously described (Takao and Kamimura, 2008). Using this setup, we conducted FRAP experiments on the same sperm cells that were previously loaded with fluorescent dye by single-cell electroporation. FRAP experiments and data analyses were executed similarly, as previously described. All the FRAP and single-cell electroporation experiments were carried out at room temperature (25–28°C).

**RESULTS**

Optimization of experimental conditions for the single-cell electroporation in sea urchin sperm

For the single-cell electroporation of sea urchin sperm, we first determined optimal conditions, including the setting parameters for the electronic stimulator (e.g., pulse duration and frequency, driving voltage, and train length) through trial-and-error, referring to previous methods used for mammalian nerve cells (Bestman et al., 2006; Lang et al., 2006). We chose the conditions where intense enough fluorescence was obtained without apparent damage to the cells. Examples of dye loading into sperm cells by single-cell electroporation are shown in Fig. 1A–C.

The experimental medium was based on those used in the conventional electroporation experiments on sea urchin eggs (Swezey and Epel, 1989). Electroporation trials in usual seawater (artificial sea water) tended to result in failure, or we could not repeat the same results. This might have been due to insufficient driving currents passing across the plasma membrane of the sperm cells. Since seawater contains high concentration of ions (e.g., ~460 mM NaCl) and should have high conductance, there would have been little effective current through the cell membranes during electroporation. By lowering the ionic strength to about one-third that of usual seawater and by adjusting osmosis with mannitol, we obtained a suitable experimental medium (the composition is described in the Material and Methods).

Micropipettes were made similar to those used for conventional patch-clamp experiments. We did not need to carefully choose the shape of the micropipettes, although the inner diameter of the tip opening affected the magnitude of the driving current. We used smoothly tapered micropipettes with an inner tip diameter of ~1 μm.
Electronic pulses for electroporation were generated with an electronic stimulator, and the optimal set of parameters we determined was a driving voltage of 100 V; current monitored by a register connected to a reference electrode of ~1 μA; a pulse frequency and duration of 200 Hz and 3 ms, respectively; and a duration of the pulse trains of 0.5–3 s (100–600 pulses).

In our experiments, the distance between the tip of the microelectrode and the sperm cells was most crucial. The pipette tip should have been as close as possible to the cell surface; however, direct contact of the microelectrode tip with the cell surface seemed to frequently cause some mechanical damage, as shown in Fig. 1D. In such cases, we often observed a rapid reduction in fluorescence, as if the fluorescent dye once incorporated inside the cells then gradually leaked out from the point contacted by the micropipette. The time constant of fluorescence decay in these cases was less than 10 s, while that of photobleaching (with a time constant of ~30 s). The results of these observations were similar to those where spermatozoa were loaded by using the AM or DA ester forms of dyes. This indicates that the present method produced no permanent damage to the sperm membrane, and that any small pores formed during electroporation quickly sealed again.

On the other hand, there were no apparent differences in results depending on the orientation and direction of approach of the microelectrode towards the sperm cell. Whether the electrode tip was placed close to the head or the flagellum of the spermatozoa, the efficiency of fluorescence loading was almost the same (Fig. 1A, B).

Observation of sperm cells during single-cell electroporation

Unless the sperm cells were visibly damaged, fluorescence-loaded spermatozoa were observed with stable brightness for more than one minute under the fluorescence microscope, except for the gradual decay due to photobleaching (with a time constant of ~30 s). The results of these observations were similar to those where spermatozoa were loaded by using the AM or DA ester forms of dyes. This indicates that the present method produced no permanent damage to the sperm membrane, and that any small pores formed during electroporation quickly sealed again.

Although many sperm were immotile under low pH conditions (~pH 7), we sometimes observed that spermatozoa partially attached to glass surfaces were moving, and that they kept beating even after the execution of single-cell electroporation. Thus, single-cell electroporation caused no significant damage to the inner axonemal structures required for motility. In a few cases, we observed that some stimuli given by single-cell electroporation triggered motion of the sperm tail: the sperm tails started a slow and transient bending immediately after the start of the electronic pulses.

Loading sperm cells with fluorescent probes

As shown in Fig. 1, some of the fluorescent probes we tried could be loaded into sea urchin sperm cells by single-cell electroporation using the optimal conditions described above. The results for loading efficiency are summarized in Table 1. For small fluorescein-derivatives (MW 376–623), including carboxyfluorescein, Oregon Green, and calcein, our method was quite effective. Fluorescence intensities in these cases were almost comparable to those of spermatozoa loaded with 20 μM concentrations of AM or DA esters of dyes, indicating that a comparable amount of dye was injected. From the intensity of fluorescence, we estimated the concentration to be 10–100 μM. Fluorescein-dextran (3k-FD), which has a higher molecular weight, could be also incorporated into sperm cells. However, in comparison, the fluorescence intensity of fluorescein-dextran, reflecting the amount of incorporated dye, was relatively lower than for the
smaller fluorescein derivatives. Trials with larger dextran conjugates labeled with fluorescein (MW > 10,000) were not always successful. Even in the successful cases for these larger dextrans, the fluorescence signals obtained were too low for further quantitative FRAP analysis. No protein molecules (BSA or casein) were successful. The success rate in loading BODIPY-labeled ATP was extremely low (this was successful only several times in the entire process of optimizing the single-cell electroporation system), although the brightness of incorporated fluorescence was very high. Therefore, the FRAP experiments using BODIPY-ATP are not shown here.

**FRAP analysis**

For the small fluorescein derivatives (carboxyfluorescein, Oregon Green, and calcein) and 3k-FD, we then conducted FRAP analyses to determine the diffusion coefficients in sperm flagella. The diffusion coefficients obtained are shown in Fig. 2. For the fluorescein derivatives, we compared the diffusion coefficient between probes loaded by two different methods, single-cell electroporation and the use of ester forms of dyes (DA or AM). Carboxyfluorescein, Oregon Green, and calcein showed similar diffusion coefficients (~60 μm²/s) regardless of the method used to load them, while 3k-FD showed a diffusion coefficient of 26 μm²/s.

### DISCUSSION

In this study, we successfully loaded sea urchin sperm cells with fluorescein-derived dyes and 3k-FD by applying the single-cell electroporation technique. We concluded that no significant damage to the sperm cells was caused by the method, from the two points of view. First, unless the sperm cells were obviously damaged, fluorescence-loaded sperm cells were observed with stable brightness, as when cells were loaded by other, conventional methods. This indicates that the present method produced no permanent damage to the sperm membrane and that any small pores formed during electroporation was quickly sealed again. Second, we sometimes observed the movement of spermatozoa partially attached to glass surfaces, even after the execution of single-cell electroporation. Thus, single-cell electroporation caused no significant damage to the inner axonemal structures required for motility. Surprisingly, some stimuli given by single-cell electroporation triggered motion of the sperm tails, as described in the Results section. One possible explanation is that a change in the sperm membrane potential, as well as the influx and/or efflux of ions such as H⁺, Na⁺ or Ca²⁺ through electrically generated pores, stimulated some activating pathways involved in regulating motility (Christen et al., 1983; Lee et al., 1983; Lee, 1984; Darszon et al., 2001). Further investigations under different medium conditions are required to understand the details.

It has been shown that there is no charge-dependent filtering effect of pores for passing materials through pores made by electroporation (Swezey and Epel, 1989). However, the size of the induced pores is highly dependent on cell type, and electropermeability seems to be affected by some differences in the membrane properties of cells. In Chinese hamster ovary cells, for example, the upper size limit of permeable molecules is about MW 1500 for cells that survived after electrical pulsing (Escande-Geraud et al., 1988), whereas larger macromolecules, including DNA and proteins (more than 30,000 Da), pass through the pores in cultured mouse fibroblast cell lines (Stopper et al., 1987; Mir et al., 1988). In the present study using sea urchin sperm, small fluorescein derivatives (MW less than 1000) efficiently penetrated cell membranes via single-cell electroporation. However, the loading of fluorescein-dextran of MW 3000 was less efficient than for smaller fluorescent dyes. In addition, there was almost no incorporation of larger fluorescein-dextran conjugates (MW 10,000–40,000). These findings suggest that the upper limit of molecular size for passing through membrane pores in sea urchin sperm is about MW 3000. The cellular architecture of spermatozoa as well as...
the chemical or physical properties of the membranes (e.g., lipid composition, surface charges, limited intracellular space, or membrane curvature) might be crucial.

The net charge of the molecule seems to be crucial as well. Although BODIPY-labeled ATP has a relatively small molecular mass (MW 933), the efficiency of electroporation was quite low (Table 1). We observed that quite a small amount of BODIPY-ATP fluorescence was released when we gave a train of electrophoretic pulses to the microelectrode, compared with when we were using fluorescein derivatives. Therefore, other molecular properties of the probe species, such as net charge, mobility, and molecular shapes, may be closely related to the efficiency of electroporation. Unlike conventional electroporation methods, where molecules pass through pores largely by passive diffusion, outward electrophoretic streaming or the mobility of molecules from the micropipette tip into the cells would be more crucial in single-cell electroporation. Because of the low electrophoretic mobility, probably due to the net charge, BODIPY-ATP would be less efficient in terms of loading efficiency. On the other hand, 3k-FD can be injected more efficiently despite its relatively large size. Similarly, it has been reported that electrophoresis-assisted single-cell electroporation was more efficient as an intracellular delivery method by one order of magnitude compared to diffusion alone subsequent to electroporation (Ionescu-Zanetti et al., 2008).

So far, injection into sperm cells has been quite difficult due to their small size. We have not been able to inject materials without causing mechanical damage to the cell membranes. Although the efficiency depends on the bathing medium and electric current conditions during electroporation as well as on the charge and molecular size of the materials injected, we expect that the present technique can be applied to loading various types of material into many other types of animal spermatozoa, including mammalian sperm. Applications in biomedical fields, as well as in developmental and reproductive biology, will possible, and the introduction of physiologically or genetically active molecules into sperm cells should allow us to learn more about the mechanisms of sperm motility and fertilization.

In the FRAP analysis, diffusion coefficients we obtained for the small fluorescein-derivatives (carboxyfluorescein, Oregon Green, and calcine) were ~60 μm²/s, regardless of the method used to load them (Fig. 2). This implies that the single-cell electroporation procedures we describe here have few destructive effects on the sperm cell membrane. On the other hand, we obtained a diffusion coefficient of 26 m²/s for 3k-FD, about half the value for smaller fluorescein-derivatives (Fig. 2). If we simply assumed that these molecules are spherical in shape and that their diffusion properties follow the Einstein-Stokes equation, where the diffusion coefficient is inversely proportional to the Stokes diameter, then the ratio of the diffusion coefficient of 3k-FD to that of carboxyfluorescein was consistently estimated to be 0.50 in our observations. The difference in the diffusion coefficients between carboxyfluorescein and 3k-FD is, therefore, as expected from the difference in their molecular masses.

In this study, we showed that with the single-cell electroporation technique, various species of fluorescent probes are useful for FRAP analysis. Along with the analysis of the diffusion coefficients for macromolecules such as GFP or dextran-conjugated dyes larger than MW 3000, if possible, we expect to be able to clarify in more detail the features of molecular mobility inside flagella and, thus, energy and material transportation in flagella. Furthermore, we expect that we can apply the single-cell electroporation technique to many other experiments. For example, single-molecule tracking inside cells may be accomplished when quite a small number of fluorescent molecules are loaded into cells (Bruckbauer et al., 2007). Since practical methods to deliver the external reagents into sperm cells have been limited, as described above, demembranated sperm cells, in which some or almost all of the intracellular soluble components are partially lost, have often been used to investigate the functioning of sperm cells. In principle, by the single-cell electroporation technique, similar experiments to those using demembranated cells can be reproduced using intact cells under more physiological conditions. By loading external components concerned with energy supply or signaling pathways and/or labeling molecules such as tubulin or dynein, we expect that new insights into the regulation of flagellar movement and maintenance of axonemal structures will be obtained.

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