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Electroporation: high frequency of occurrence of a transient high-permeability state in erythrocytes and intact yeast

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We present the first determinations of population distributions of macromolecule uptake due to electroporation, the percentage of cells which participate and, for the yeast, the subpopulation of cells whose membranes exhibit significant recovery following macromolecule uptake. Flow cytometry is used to measure the uptake of a first test molecule (green fluorescence, FITC-dextran; 70 kDa) and also, for the yeast, the subsequent uptake of a second, much smaller, test molecule (red fluorescence, propidium iodide; 660 Da), which provides a measure of membrane recovery. A dramatic 20% (erythrocytes) to 75% (intact *Schizosaccharomyces pombe*) of cells can take up the first test molecule within 5 min of a pulse.

Electroporation; Membrane pore; Permeability; Erythrocyte; Flow cytometry; (Yeast)

1. INTRODUCTION

Electroporation [1,2] is emerging as a general method for introduction of molecules into cells [1,3–14]. Although microinjection is broadly applicable to mammalian cells and to protoplasts [15], it is tedious, and generally inapplicable to intact microorganisms with cell walls. In contrast, a large number of cells can be simultaneously exposed to strong electric fields, which under proper conditions leads to electroporation, the occurrence of pores or membrane openings through which both ions and molecules can pass [16]. Electroporated cells experience a very rapid (microseconds) membrane discharge (reversible electrical breakdown) [16–19], followed by a longer-lived high-permeability state that allows significant uptake or release of molecules [1,3–11]. Thus, electroporation is increasingly used to cause cellular uptake of macromolecules, e.g. enzymes and DNA [1,4–14]. However, previous experiments do not determine the number of cells experiencing electroporation.

For example, successful introduction of DNA by electroporation not only requires uptake, but also non-degradative intracellular transport, correct incorporation and expression, and such experiments score only the small number of eventual transformants. Other studies are based on the average molecular uptake (or release) by an entire cell population [1,4,5] and give the average response of the cell population, while still other studies monitor the uptake or release of fluorescence by a single cell [20,21]. In contrast, we determine how many cells experience electroporation by measuring uptake of fluorescent test molecules by each cell within a large, statistically significant population. Flow cytometry provides individual fluorescence measurements of large numbers of cells, followed by computational construction of the population distribution for uptake of two different fluorescence-labeled molecules by each cell. This allows direct computation of the percentage of cells which take up a significant amount of the first test molecule, and such cells are interpreted as having achieved a transient high-permeability

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state. Our approach complements single-cell studies, and can, by computation, provide the average cell response given by the 'total response' studies, while at the same time revealing the significant heterogeneous response of a cell population.

Erythrocytes (red blood cells, RBCs) have been used in many previous electroporation studies [4,5,21,22], and intact yeast are of interest because they are readily tested for viability by plating, and because the creation and use of protoplasts [9,14] can be avoided [8,11] for introduction of macromolecules if electroporative uptake occurs with high frequency in intact cells. The morphology of RBCs and the two yeasts differs significantly: (i) an RBC is a 7 μm biconcave disc, (ii) *Saccharomyces cerevisiae* is an approx. 7 μm spheroid, and (iii) *Schizosaccharomyces pombe* is a rod about 3–5 μm in diameter and 5–15 μm in length [23]. In addition to studying cells with different properties, we used the test macromolecule, FITC-dextran, which has relatively small electrical charge, and which is therefore not strongly excluded from pores because of a Born energy interaction [21,24,25]. For this reason, FITC-dextran is a good probe for openings or pores in cell membranes.

2. MATERIALS AND METHODS

Yeast were grown in YPD medium, harvested while in exponential phase and resuspended in a minimal medium (50 mM Na + 50 mM K phosphate buffer, pH 6.8, 1 mM glucose, 5 mM NH_4Cl) and 0.1 mM FITC-dextran (green fluorescence, GF; 0.01 mol FITC per mol glucose; 70 kDa, Sigma; effective radius about 5.8 nm) [21]. Control aliquots were removed before and after the electrical pulse experiments, corresponding to exposure of 5 and 40 min to the FITC-dextran. These initial and final controls are used to determine the spontaneous uptake and adsorption, and also loss of viability which is unrelated to electroporation. For each electric field strength, E, a single aliquot (14–25 μl; 6×10^7 cells/ml) of cell suspension was used to fill a parallel plane (stainless-steel electrodes) electroporation chamber at room temperature (23 ± 3°C). Because large potential drops occur at the electrode, the current associated with each pulse was measured and used with the medium conductivity and chamber geometry to calculate E. Cells were then placed in a micro-centrifuge tube, held for a 5 min uptake/recovery period, centrifuged at 5000–7500 × *g* for 3 min, and washed twice with minimal medium. The much smaller test molecule propidium iodide (PI; red fluorescence, RF; 660 Da; effective molecular radius about 0.8 nm) is supplied 25 min after pulsing at a concentration of 5.1×10^{-6} M, which stains cellular RNA and DNA with RF if a cell's membrane is compromised [26–28], and provides a measure of membrane recovery. Because RBCs contain negligible RNA and DNA, and therefore do not stain strongly with PI, this membrane recovery test was not used for RBCs. Yeast cells were analyzed with an Ortho Cytofluorograf IIs using an argon laser 488 nm 'blue' line at 35 mW for fluorescence excitation, with 90° light scatter (forward blue scatter, FBS; 488 nm) used as a trigger event, and with both GF and RF measured for each of 15000 individual cells. Similar analysis was used for RBCs, but with the difference that FBS was both a trigger event and a measured parameter, and GF, but not RF, was measured [29].

Whole heparinized human blood was obtained from healthy volunteers. RBCs were washed 4 × with PBS, pH 7.2, a suspension of 10^7 cells/ml electrically pulsed in the PBS with 10^{-4} M FITC-dextran, and then washed 2 × with pure PBS. Yeast viability was determined by direct plating, for which a cell concentration of 5×10^4 cells/ml was used, so that a 5 μl aliquot could be directly placed on a YPD plate containing 0.1 ml sterile 50 mM Na + 50 mM K PO_4 at pH 6.8, and spread. Plates were incubated at 26 ± 2°C for 2 days (*S. cerevisiae*) or 5 days (*Sch. pombe*). Percent viable cells was determined by direct plating of yeast onto YPD plates (mean of four plates) relative to the control (fig.3). For RBCs and both yeasts a high percentage of the cells can achieve the transient high-permeability state, taking up significant GF after application of a 50 μs pulse. Fluorescence microscopy showed that cells with GF were uniform in fluorescence emission, in contrast to a bright fluorescent rim due to surface binding. This demonstrated that macromolecule uptake rather than some type of electrically assisted surface binding occurred.

3. RESULTS AND DISCUSSION

In spite of large differences in cell morphology and membrane recovery behavior, all three cell types can achieve a transient high-permeability state. The high permeability is indicated by uptake of a significant amount of FITC-dextran, is measured by flow cytometry and computational analysis, and is found to be orders of magnitude larger than transformation frequencies. Of particular interest is the comparison between the transfection frequency (0.00006%) for intact *S. cerevisiae* KK4 [8], and the 35% uptake of 70 kDa FITC-dextran found here for intact *S. cerevisiae*. Further, as revealed by exclusion of PI 25 min after the pulse, significant membrane recovery often occurred for yeast cells with significant GF uptake, confirming the transient nature of the high-permeability state (figs 1,2).

Interestingly, pulsed cells can have very different fates (fig.3). RBCs are terminally differentiated, cannot be tested for growth, do not have DNA and RNA for PI staining, but are observed by us using microscopy to form spherical 'ghosts' [21] if there is significant FITC-dextran uptake. For the yeasts

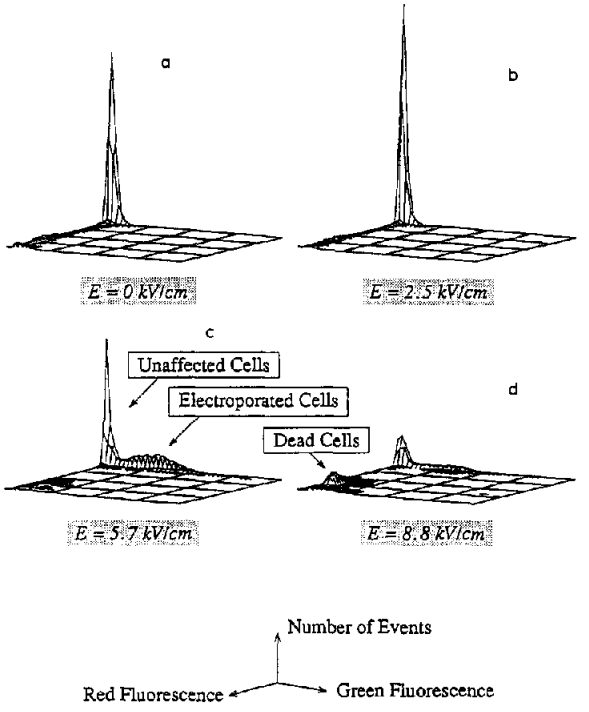

Fig.1. Typical two-dimensional histograms (cytograms) for flow cytometer two-color fluorescence measurements of 15000 individual yeast (*S. cerevisiae*; ATCC 2341) cells showing red fluorescence (RF) (vertical) vs green fluorescence (GF) (horizontal) for a single 50 μs pulse at several field strengths, E. The percentage of cells interpreted as having achieved a transient high-permeability state is determined by computing the number of cells within a rectangular box. This percentage is denoted %EP ('percent electroporated'), as indicated by exhibiting significant GF uptake. (a) E = 0, control with FITC-dextran present; %EP = 2.2%, due to surface adsorption or spontaneous uptake, (b) E = 2.5 kV/cm; sub-threshold with %EP = 2.0%, (c) E = 5.7 kV/cm, optimal with %EP = 38%, (d) E = 8.8 kV/cm, excessive with %EP = 17%.

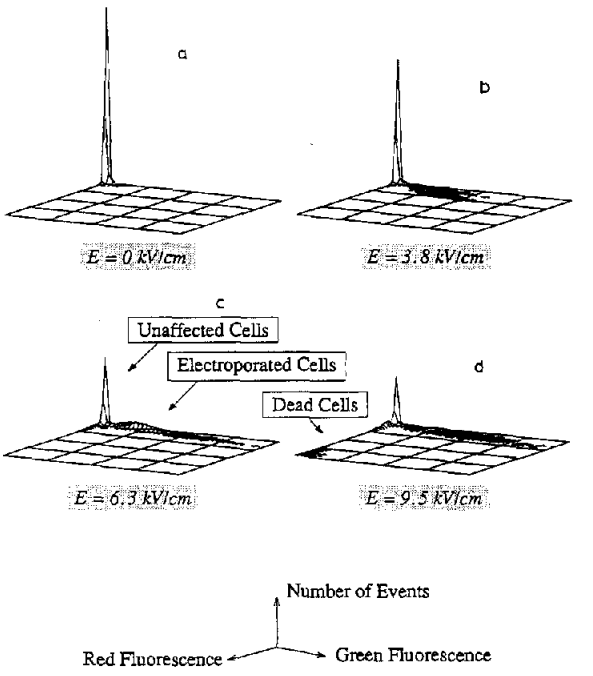

Fig.2. *Sch. pombe* (ATCC 26189) two-color bivariate histograms at four field strengths for a single 50 μs square wave pulse, using the methodology and terminology of fig.1: (a) E = 0, control with FITC-dextran present; %EP = 1.7%, due to surface adsorption or spontaneous uptake, (b) E = 3.8 kV/cm; sub-threshold with %EP = 25%, (c) E = 6.3 kV/cm, optimal with %EP = 51%, (d) E = 9.5 kV/cm, excessive with %EP = 41%.

average viability was determined by direct plating. Pulsed *S. cerevisiae* exhibits high survival rates at the electroporative uptake optimum (table 1), whereas *Sch. pombe* behaves quite differently: cell death commences at about 2 kV/cm, and then increases dramatically for large electric fields (at 10 kV/cm an impressive 75% take up FITC-dextran, but only 15 ± 5% of the cells are viable). Further, *Sch. pombe* with significant GF (FITC-dextran uptake) have negligible RF following exposure to PI 25 min after the pulse, indicating significant resealing of pores through which FITC-dextran earlier entered. However, this degree of membrane recovery does not rule out the presence of still smaller pores, which can lead to chemical imbalances and eventual cell death [30]. Here, for reasons not presently understood, significant delayed cell death occurs for *Sch. pombe* but not *S. cerevisiae*.

The main finding of this study is that a large percentage (20–75%) of cells can take up FITC-dextran following a single 50 μs 'square wave' pulse of suitable amplitude (table 1). The view that electroporation is a highly probable event in artificial bilayer and cell membranes is supported by this result, as significant uptake occurs even though both the morphology and the eventual fates of the three cell types are quite different. A closely related finding is that cell participation is, however, not 100%. Instead there is a significant distribution of electroporation behavior within a cell population, of which some is probably due to

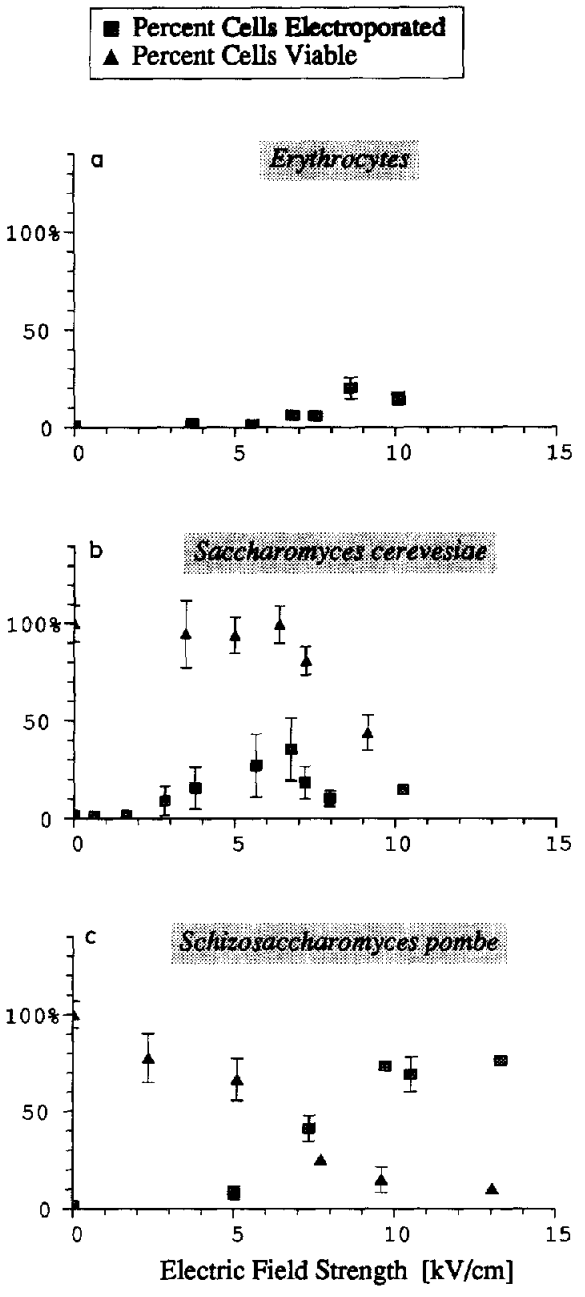

Fig.3. Average percent of electroporated cells from several experiments (open squares), as determined by analysis of flow cytometer cytograms (e.g. figs 1,2), at different electric fields, *E*, for: (a) human red blood cells, (b) *S. cerevisiae*, and (c) *Sch. pombe*. For the yeasts the percent viable cells (solid triangles) was determined by plating.

Table 1

Comparison of electroporative uptake of a test macromolecule (70 kDa FITC-dextran) following application of a single 50 μs pulse

	Maximum percent participation	Percent survival by plating	Optimal field strength (kV/cm) for electroporative uptake
S. cerevisiae	35	75	5– 6
Sch. pombe	75	15	8–12
Erythrocytes	20	N.A.	8

These maximum percentages are orders of magnitude larger than the reported values of electroporative transformation [7–14], and support the view that electroporation can occur with high frequency in any bilayer or cell membrane

variations in cell orientation with respect to the applied electric field. The existence of major subpopulations with different electroporation behavior strongly suggests that the heterogeneity of response must be understood if full insight into electroporation phenomena is to be gained. These experiments demonstrate that fluorescent test molecules, fluorescence microscopy and flow cytometry can be used to determine rapidly conditions for creating, and following the recovery from, the transient high-permeability state caused by electroporation.

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REFERENCES

[1] Neumann, E. and Rosenheck, K. (1972) J. Membrane Biol. 10, 279–290.
[2] Neumann, E., Sowers, A. and Jordan, C. (1988) Electroporation and Electrofusion in Cell Biology, Plenum, New York, in press.
[3] Zimmermann, U., Pilwat, G. and Riemann, F. (1975) Biochim. Biophys. Acta 375, 209–219.
[4] Zimmermann, U., Riemann, F. and Pilwat, G. (1976) Biochim. Biophys. Acta 436, 460–474.
[5] Kinosita, K. jr and Tsong, T.Y. (1977) Nature 268, 438–441.
[6] Neumann, E., Schaefer-Ridder, M., Wang, Y. and Hofschneider, P.H. (1982) EMBO J. 1, 841–845.

[7] Potter, H., Weir, L. and Leder, P. (1984) Proc. Natl. Acad. Sci. USA 78, 7161–7165.
[8] Hashimoto, H., Morikawa, H., Yamada, H. and Kimura, A. (1985) Appl. Microbiol. Biotechnol. 21, 336–339.
[9] Fromm, M.E., Taylor, L.P. and Walbot, V. (1986) Nature 319, 791–793.
[10] Toneguzzo, F., Hayday, A.C. and Keating, A. (1986) Mol. Cell. Biol. 6, 703–706.
[11] Morikawa, H., Iida, A., Matsui, C., Ikegami, M. and Yamada, Y. (1986) Gene 41, 121–124.
[12] Chu, G., Hayakawa, H. and Berg, P. (1987) Nucleic Acids Res. 15, 1311–1326.
[13] Stopper, H., Jones, H. and Zimmermann, U. (1987) Biochim. Biophys. Acta 900, 38–44.
[14] MacNeil, D.J. (1987) FEMS Microbiol. Lett. 42, 239–244.
[15] Pasti, G., Lacal, J.-C., Warren, B.S., Aaronson, S.A. and Blumberg, P.M. (1986) Nature 324, 375–377.
[16] Weaver, J.C. and Powell, K.T. (1988) in: Electroporation and Electrofusion in Cell Biology (Neumann, E. et al. eds) Plenum, New York, in press.
[17] Benz, R., Beckers, F. and Zimmermann, U. (1979) J. Membrane Biol. 48, 181–204.
[18] Benz, R. and Zimmermann, U. (1980) Bioelectrochem. Bioenerg. 7, 723–739.
[19] Powell, K.T., Derrick, E.G. and Weaver, J.C. (1986) Bioelectrochem. Bioenerg. 15, 243–255.
[20] Mehrle, W., Zimmermann, U. and Hampp, R. (1985) FEBS Lett. 185, 89–94.
[21] Sowers, A.E. and Lieber, M.R. (1986) FEBS Lett. 205, 179–184.
[22] Zimmermann, U., Schultz, J. and Pilwat, G. (1973) Biophys. J. 13, 1005–1013.
[23] Russell, P. and Nurse, P. (1986) Cell 45, 781–782.
[24] Parsegian, V.A. (1969) Nature 221, 844–846.
[25] Weaver, J.C., Mintzer, R.A., Ling, H. and Sloan, S.R. (1986) Bioelectrochem. Bioenerg. 15, 229–241.
[26] Crissman, H.A. and Steinkamp, J.A. (1973) J. Cell Biol. 59, 766–771.
[27] Krishan, A. (1975) J. Biol. Chem. 66, 188–193.
[28] Lemasters, J.J., DiGiuseppi, J., Nieminen, A.-L. and Herman, B. (1987) Nature 325, 78–81.
[29] Shapiro, H.M. (1985) Practical Flow Cytometry, Liss, New York.
[30] Kinosita, K. jr and Tsong, T.Y. (1977) Proc. Natl. Acad. Sci. USA 74, 1923–1927.