Topic Introduction

Electroporation

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Electroporation is a process in which brief electrical pulses create transient pores in the plasma membrane that allow nucleic acids to enter the cellular cytoplasm. Here, we provide information on the history, mechanism, and optimization of electroporation. We also describe nucleofection, an improvement of the electroporation technology that permits the introduction of nucleic acids directly into the nucleus.

INTRODUCTION

Nucleic acids do not enter cells under their own power; they require assistance in crossing physical barriers at the cell boundary and in reaching an intracellular site where they can be expressed and/or replicated. Exposure of many types of cells to an electrical discharge reversibly destabilizes their membranes and transiently induces the formation of aqueous pathways or membrane pores (Neumann and Rosenheck 1972; Neumann et al. 1982; Wong and Neumann 1982; for reviews, see Zimmermann 1982; Andreason and Evans 1988; Tsong 1991; Weaver 1993) that potentiate the entry of DNA molecules (Neumann et al. 1982). This method, which is known as electroporation, has been developed into a rapid, simple, and efficient technique for introducing DNA into a wide variety of cells, including bacteria, yeasts, plant cells, and a large number of cultured mammalian cell lines (see Protocol: **DNA Transfection by Electroporation** [Kumar et al. 2019]). The chief practical advantages of electroporation are that it can be applied to a wide variety of cells, both prokaryotic and eukaryotic, and that it is extremely simple to perform.

THE MECHANISM OF ELECTROPORATION

Because the changes in membrane structure that accompany electroporation cannot be visualized in real time by microscopy, our understanding of the mechanism is based on evidence that is both patchy and circumstantial. The following model (Weaver 1993) has been used for many years to provide a plausible account of the sequence of events that are initiated by increasing the transmembrane voltage from its physiological value of ~ 0.1 to 0.5 – 1.0 V. Figure 1 shows the following sequence of events:

- The onset of electroporation causes a membrane dimple followed by formation of transient hydrophobic pores whose diameter fluctuates from a minimum of 2 nm to a maximum of several nanometers.
- Some of the larger *hydrophobic* pores are converted to *hydrophilic* pores because the energy needed to form an aqueous pore is reduced as the transmembrane voltage is increased and the energy

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FIGURE 1. Hypothetical structures for transient and metastable membrane conformations believed to be relevant to electroporation. (A) Free volume fluctuation; (B) aqueous protrusion or “dimple”; (C,D) hydrophobic pores usually regarded as the “primary pores” through which ions and molecules pass; (E) composite pore with “foot in the door” charged macromolecule inserted into a hydrophilic pore. The transient aqueous pore model assumes that transitions from A → B → C or D occur with increasing frequency as U is increased. Type F may form by entry of a tethered macromolecule, while the transmembrane voltage is significantly elevated, and then persist after U has decayed to a small value through pore conduction. It is emphasized that these hypothetical structures have not been directly observed and that support for them derives from the interpretation of a variety of experiments involving electrical, optical, mechanical, and molecular transport behavior. (Redrawn from Weaver 1993, with permission from John Wiley & Sons, Inc.)

required to maintain the circumference of a large hydrophilic pore is significantly lower than that required to maintain a large hydrophobic pore. For this reason, hydrophilic pores have an extended half-life and may be further stabilized by attachment to underlying cytoskeletal elements. The generation of such long-lived metastable pores allows small ions and molecules to enter and leave the cell long after the transmembrane voltage has returned to low values (Rosenheck et al. 1975; Zimmermann et al. 1976; Lindner et al. 1977). The detailed mechanism by which molecules pass through hydrophilic pores is not known but may include electrophoresis (Chermodnick et al. 1990), electroendo-osmosis, diffusion, and endocytosis (Weaver and Barnett 1992). Reclosing of the pores appears to be a stochastic process that can be delayed by keeping the cells at 0°C. While the pores remain open, up to 0.5 pg of DNA can enter the cell (Bertling et al. 1987). Size seems to be no impediment because DNA molecules up to 150 kb in pass through the pores (Knutson and Yee 1987). Because the DNA enters directly into the cytoplasm, it is not exposed to acid conditions in endosomes and lysosomes. This route may explain why the rate of mutation in DNA introduced to cells by electroporation is apparently very low (Drinkwater and Kleindienst 1986; Bertling et al. 1987) compared with DNA transfected as calcium phosphate coprecipitates (e.g., see Calos et al. 1983). Despite intensive research, nobody has been able to see or provide other evidence of these pores. The alternative term “electropermeabilization” referring to changes that may contribute to make the cell membrane reversibly permeable, without “holes,” has been put forth to explain the mechanism of DNA uptake into cells.

Electropermeabilization might result from the following:

- *The electrocompressive forces generated by an electric field associated with the transmembrane potential difference.* These electrocompressive forces bring the two lipid layers closer together than the distance that allows them to remain parallel to each other, which could disrupt the ordered stacking of the lipids that comprise the plasma membrane.
- *Changes in the lipid polar head orientation,* detected by ^{31}P magnetic resonance changes (Lopez et al. 1988).

- The penetration of water in the lipid layer (hydration of the membrane) resulting from the two aforementioned structural changes of the lipids. This mechanism appears plausible based on simulations of membranes exposed to high transmembrane potential differences, using validated molecular dynamics programs (Tieleman et al. 2003; Tarek 2005; Vernier and Ziegler 2007) that also reveal the influence of the lipid composition on the stability and the electroporation of the membranes.
- Possible changes in the structure of transmembrane proteins (Teissie et al. 2005).

A good discussion of the mechanisms of cell membrane electroporation and electroporation can be found in the review by Teissie et al. (2005).

For *Escherichia coli*, electroporation is currently the most efficient method available for transfection with plasmids. In excess of 80% of the cells in a culture can be transformed to ampicillin resistance by this method, and efficiencies of transfection approaching the theoretical maximum of one transfectant per molecule of plasmid DNA have been reported (Smith et al. 1990). However, the number of transfectants obtained is marker dependent. When pBR322, which carries genes conferring resistance to two antibiotics (ampicillin and tetracycline), is introduced into *E. coli* by electroporation, the number of tetracycline-resistant transfectants is ~100-fold less than the number of ampicillin-resistant transfectants (Steele et al. 1994). This effect is not seen when the plasmid is introduced into the bacteria by the calcium chloride method. One possible explanation is that electroporation damages or changes the bacterial membrane so that it can no longer interact efficiently with the tetracycline-resistance protein.

Typically, between 50% and 70% of cells exposed to high electric field strengths are killed. The lethal effects, which vary in intensity from one cell type to another, are not due to heating or electrolysis and are independent of the current density and energy input. Instead, cell killing is dependent on field strength and the total time of treatment (Sale and Hamilton 1967). The most likely cause of cell killing is the inability to restore membrane structure and barrier function, leading to rupture of cell membranes, a rapid loss of ionic balance, and massive efflux of cellular components. Nevertheless, this process is still termed “reversible electroporation” and should not be confused with “irreversible electroporation,” in which cell death is the intended end point. Irreversible electroporation is used in medical applications like atrial heart ablation (Lavee et al. 2007) or tumor ablation by electrical nonthermal means (Edd et al. 2006; Al-Sakere et al. 2007).

ELECTRICAL CONDITIONS REQUIRED FOR ELECTROPORATION

Electroporation of almost all mammalian cells is induced when the transmembrane voltage, $\Delta U(t)$, is increased to 0.5–1.0 V for durations of microseconds to milliseconds. This translates to an electric field strength of ~7.5–15.0 kV/cm. Because this value is constant and is independent of the biochemical nature of the cell membrane, it seems likely that variations in the efficiency of electroporation from cell line to cell line are due to differences in the rate and efficiency of membrane recovery at the end of the pulse.

The transmembrane voltage, $\Delta U(t)$, induced by electric fields varies in direct proportion to the diameter of the cell that is the target for transfection (Knutson and Yee 1987). Electroporation of mammalian cells, for example, requires smaller electric fields (<10 kV/cm) than does electroporation of yeasts or bacteria (12.5–16.5 kV/cm). Most of the commercial suppliers of electroporation machines provide literature describing the approximate voltages required for transfection of specific types of cells in their particular apparatus.

Three important characteristics of the pulse affect the efficiency of electroporation: the length of the pulse, its field strength, and its shape. Most of the commercial electroporation machines use capacitive discharge to produce controlled pulses whose length is mainly determined by the value of the capacitor and the conductivity of the medium. Thus, the time constant of the pulse can be altered by switching capacitors according to the manufacturer’s instructions or by changing the ionic strength

of the medium. When the charge from the capacitor is directed to a sample placed between two electrodes, the voltage across the electrodes rises rapidly to a peak (V_0) and declines over time (t) according to

$$V_t = V_0[e^{-F(t,T)}],$$

where t is the time constant, which is equal to the time over which the voltage declines to $\sim 37\%$ of peak value. T (measured in seconds) is also equal to the product of the resistance (R , measured in ohms) and the capacitance (C , in farads):

$$T = RC.$$

The field strength (E) of the pulse varies in direct proportion to the applied voltage (V) and in inverse proportion to the distance (d) between the two electrodes, which is usually determined by the size of the cuvette through which the pulse travels:

$$E = F(V, d).$$

Most manufacturers provide cuvettes in three sizes, where the interelectrode distances are, respectively, 0.1, 0.2, and 0.4 cm. When 1000 V is discharged into these cuvettes, E_0 is 10,000 V/cm in the 0.1-cm cuvette, 5000 V/cm in the 0.2-cm cuvette, and 2500 V/cm in the 0.4-cm cuvette. Newer machines like the Gene Pulser MXcell (Bio-Rad) also allow high-throughput electroporation using lower cell numbers in a plate well format (12, 24, or 96 well).

The shape of the pulse is determined by the design of the electroporation device (Fig. 2). The waveform produced by most commercial machines is simply the exponential decay pattern of a discharging capacitor. They are thus defined by only two parameters, the field strength E and the time constant T , as explained above. In some types of electroporation apparatuses, square waves can be generated by rapidly increasing the voltage, maintaining it at the desired level for a specified time (pulse width), and then rapidly reducing the voltage to 0. Thus they can be defined by the voltage delivered, the length of each pulse, the number of pulses, and the length of interval between the pulses. Square pulses can be grouped into two general categories: very high field strength of very short duration (typically 8 kV/cm for 5.4 msec) (Neumann et al. 1982), and low field strength of medium to long duration (e.g., <2 kV/cm for >10 msec) (e.g., see Potter et al. 1984). Although square waves have been reported to offer increased viability and efficiency for particularly sensitive cell lines and for in vivo applications (Hewapathirane and Haas 2008; Jordan et al. 2008), the exponential wave forms produced by most commercial electroporation machines are satisfactory for most applications.

Electroporation of mammalian cells is usually performed in a buffered saline solution or culture medium with pulse lengths of 10–40 msec and strengths of 400–900 V/cm.

OPTIMIZING CONDITIONS FOR ELECTROPORATION

A major advantage of electroporation over other methods of transfection is that it works for a very wide variety of mammalian cells, including those that are difficult to transfect by other means (e.g., see Potter et al. 1984; Tur-Kaspa et al. 1986; Chu et al. 1987). However, despite its advantages, electroporation is not always the most efficient way to introduce DNA into a particular cell line. For example, most cell lines like 293T and Vero are easily transfected by lipofection. To find out whether electroporation is a useful method of transfection for a particular cell line, it is important to use a range of field strengths and pulse lengths/types and thereby to establish conditions that generate the maximum numbers of transfectants. Such conditions have been reported for more than 50 types of mammalian cells, and it is sometimes possible to save a lot of work by simply reading the relevant literature. Most

FIGURE 2. Electrical pulses used for electroporation. Exponential decay pulse: When a capacitor charged to a voltage V_0 is discharged into cells, the voltage applied to the cells reduces exponentially with time. The time required for V_0 to drop to V_0/e is the time constant T , which denotes the pulse length. Square wave pulse: When the pulse from a capacitor is abruptly stopped after discharge, a square wave pulse is generated. The pulse length is the time for which the cells are subjected to the discharge. A slight drop in voltage occurs during the pulse length in all instruments. This is called the “pulse droop” and is measured as a percentage of V_0 .

of the companies that sell electroporation devices produce excellent up-to-date lists of papers in which electroporation has been used for transfection. These bibliographies are often the easiest way to gain access to information about the properties of a particular cell line or its close relatives. However, because of variation in properties between different cultivars of the same cell line, it is important for investigators to confirm that the conditions described in the literature are optimal for cells grown in their laboratory. Newer electroporators also provide both the exponential decay and square wave pulse formats for optimizing the best conditions for DNA delivery into target cells.

Because transfection and cell killing are independently determined by field strength (Chu et al. 1987), it is best to expose aliquots of cells to electric fields of increasing strength with time constants between 50 and 200 msec. For each field strength, measure (1) the number of cells that express a transfected reporter gene (10–40 $\mu\text{g}/\text{mL}$ linearized plasmid DNA in the electroporation buffer) and (2) the proportion of cells that survive exposure to the electric field. Plating efficiency is a more accurate measure of cell survival than staining with vital dyes because, after electroporation, cells can remain permeable to vital dyes such as Trypan Blue for an hour or two. The following are other variables that have been reported to affect the efficiency of electroporation:

- *The temperature of the cells before, during, and after electroporation* (e.g., Potter et al. 1984; Chu et al. 1987). Usually, electroporation is performed on cells that have been prechilled to 0°C . The cells are held at 0°C after electroporation (to maintain the pores in an open position) and are diluted into warm medium for plating (Rabussay et al. 1987).
- *The concentration and conformation of the DNA* (e.g., Neumann et al. 1982; Potter et al. 1984; Toneguzzo and Keating 1986). Linear DNA is preferred for stable transfection; circular DNA is for transient transfection. Preparations containing DNA at a concentration of 1–80 $\mu\text{g}/\text{mL}$ are optimal.
- *The state of the cells.* The best results are obtained with cell cultures in the mid-log phase of growth that are actively dividing.

NUCLEOFECTION

A specialized form of electroporation, under standardized conditions nucleofection permits the introduction of nucleic acids directly into the nucleus as well as into the cytoplasm of the host cell. Although a direct comparison between electroporation and nucleofection has not been made, nucleofection appears to be much more efficient in direct nuclear transfer of the substrate as determined by early expression kinetics of nucleofected genes like GFP (30 min postnucleofection) and detection of

at least three log copies of plasmid DNA in the nucleus by quantitative PCR 4 h after the procedure (Greiner et al. 2004; Johnson et al. 2005). The high operating costs (both the device and specialty reagents) have limited the use of nucleofection to primary cells such as stem cells, neurons, T-cells, macrophages, and other mammalian cells that are refractory to conventional lipid-based transfection methods. However, other commercial vendors (e.g., the Ingenio from Mirus Bio) now market less expensive cell-specific reagents for nucleofection that are compatible with the Amaxa Nucleofector.

As is true for conventional electroporation, nucleofection results in a high rate of cell death and requires optimization for best results.

Optimization of Nucleofection

The optimization process involves subjecting cells to various combinations of electrical parameters (termed “programs” in the nucleofector device) and different nucleofector solutions. The condition that results in the highest transfection efficiencies with the lowest amount of cell death is chosen for the rest of the studies. Important considerations are as follows.

- Excessive cell death during the nucleofection procedure can be minimized by using cells that are maintained under the best possible growth conditions and preferably at a low passage number.
- Cells should be handled gently, and procedures such as centrifugation should be limited and performed at the lowest possible speeds both before and after nucleofection.
- Warm up only the necessary amount of medium (rather than the entire bottle) to obtain consistent growth of cells and reproducible data.
- Cell cultures should be free of mycoplasma contamination because it drastically impairs transfection efficiency.
- DNA used for transfection should be of high purity with an OD₂₆₀ to OD₂₈₀ ratio of ≥ 1.8 and should be freshly prepared to avoid using nicked DNA.

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