ELECTROPORATION OF BIOLOGICAL MEMBRANES

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Summary. In recent years, manipulation of biological cells and cell tissue by external electric fields gains increasing importance for biophysics and cell biology in general and in biotechnology and medicine in particular. Especially, the method of electroporation has become a powerfool tool for cell manipulations. In electroporation cells are subjected to a pulsed high-voltage electric field, resulting in a temporary increase of cell membrane conductivity and permeability. This increase of permeability is large enough to allow both small molecules and macromolecules to enter or leave the cell. The process can be fully reversible and after resealing of the membrane, the cell regains its original state.

Although the actual molecular mechanism underlying this process is not yet fully understood, most investigators agree that transient hydrophilic pores are responsible for a membrane's behaviour at elevated membrane voltages.

Here, a short historical survey on the investigations of the influence of strong electric fileds on biological membranes has been presented. Main features of cell electroporation phenomenon and underlying mechanism have also been discussed.

Key words: Cell electroporation, electrofusion, electroinsertion, electrotransformation, transdermal drug delivery.

BIOLOGINIŲ MEMBRANŲ ELEKTROPORACIJA

Santrauka. Pastaraisiais metais biofizikai, biologai, biotechnologai ir medikai domisi galimybe keisti biologinių membranų ir audinių savybes. Jau keli dešimtmečiai žinoma, kad biomembranos elektrinį laidumą ir pralaidumą įvairioms medžiagoms galima pakeisti biologinę ląstelę paveikus trumpu, tačiau stipriu elektros lauku. Reiškinys buvo pavadintas elektroporacija. Membranos pralaidumas padidėja tiek, jog ne tik mažos, bet ir didelės molekulės gali patekti į ląstelės vidų. Pokyčiai membranoje gali būti pilnai grįžtami. Nors detalus šio reiškinio mechanizmas nėra aiškus, daugelis mokslininkų sutinka, kad veikiant stipriam elektros laukui membranoje susidaro hidrofilinės poros, kurių diametras gali siekti iki kelių nm.

Šiame straipsnyje pateikiama trumpa istorinė biologinių membranų elektroporacijos reiškinio tyrimų istorinė apžvalga, aptariami pasiūlyti elektroporacijos mechanizmai bei savybės.

Raktažodžiai: lastelių elektroporacija, membranų pralaidumas.

Introduction. A multiplicity of phenomena have been associated with the interactions of electric fields with cells. It has been shown that development (Jaffe, 1979), regeneration (Borgens et al., 1981), and repair (Kenner et al., 1975) are affected by electric fields and that many other basic cellular functions including motility (Cooper and Keller, 1984) and receptor regulation (Young and Poo, 1983) are modulated by applied external electric fields. In addition, cell membrane permeabilization and fusion have been effected by applied fields (Neumann et al., 1989). In recent years, manipulation of biological cells and cell tissue by external electric fields gains increasing importance for biophysics and cell biology in general and in biotechnology and medicine in particular (Chang et al., 1992; Neumann et al., 1989). In particular, the method of electroporation has become a powerful tool for cell manipulations. In electroporation, cells are subjected to a pulsed high-voltage electric field, resulting in a temporary breakdown of the cell membrane and the formation of pores that are large enough to allow both small molecules and macromolecules to enter or leave the cell. The process is reversible and after resealing of the membrane, the cell regains its original state.

Electroporation is now widely used for the introduction into or getting out of living cells of membrane-impermeable substances (Auer et al., 1976; Berglund et al., 1989; Hughes and Crawford, 1989; Kilbane and Bielaga, 1991; Knight and Baker, 1982; Laban and Wirth, 1989; Lambert et al., 1990; Neumann et al., 1982; Winegar et al., 1989; Zimmermann et al., 1980), electrofusion (Zimmermann, 1982), introduction of foreign DNA into cells (Chu et al., 1987; Mann and King, 1989; Potter, 1988; Shillito et al., 1985; Satoh et al., 1990), the electro-insertion of foreign glycoproteins into the membrane of blood organelles (Mouneimne et al., 1989), electrochemotherapy (Mir et al., 1991), and electrically-enhanced transdermal delivery of drugs (Prausnitz et al., 1993).

Regardless the numerous applications of electroporation and the fact that both theoretical and experimental investigations of the electroporation process have been carried out over a period of more than three decades (Abidor et al., 1979; Crowley, 1973; Glaser et al., 1988; Kinosita and Tsong, 1977a,b; Neumann and Rosenheck, 1972; Sale and Hamilton, 1967, 1968; Schwister and Deuticke, 1985; Sowers and Lieber, 1986; Zimmermann et al., 1974), the detailed molecular membrane processes of electroporation are not yet well understood. Due to this, data analysis and technical optimization strategies are still generally empirical. No doubt, further progress in goal-directed applications of electroporation methods in cell biology, biotechnology and medicine will greatly benefit from understanding of the molecular mechanism of membrane electroporation.

In this paper, we will discuss main features of cell electroporation phenomenon. Its applications will be discussed in a subsequent paper.

A short historical survey. Under normal physiological conditions, the cell plasma membrane is a highly impermeable barrier for ions and hydrophilic molecules. This permeation barrier can be modified by imposing a transmembrane electric potential (Chang *et al.*, 1992; Neumann *et al.*, 1989). When a strong electric field is applied, the membrane conductivity increases dramatically in microseconds (Hibino *et al.*, 1993; Kinosita and Tsong, 1979).

The phenomenon of the electric modification of cell membrane conductivity has been known since the 1940s (Cole, 1972). Goldman (1943) measured the voltage-current (V-I) characteristics of the membrane of *Chara australia* and found a phenomenon similar to the dielectric breakdown of cell membrane i.e., an abrupt increase in the membrane conductance when the membrane was hyperpolarized beyond a certain potential (Goldman, 1943). This effect was reversible: repetitive voltage scans did not alter the V-I characteristics of the membrane. Coster called it the *reversible electric punch through* (Coster, 1965). Irreversible electric breakdown of BLM and cell membranes has also been noted and the dielectric strengths determined for BLM of various lipid compositions (Huang *et al.*, 1964; Tien, 1974). It was found that for BLM the breakdown potential is in the range from 150 to 500 mV when the field duration is in microseconds to milliseconds (Cole, 1972). The publications by Sale and Hamilton in 1967 and 1968 (Sale and Hamilton, 1967, 1968) are of particular interest to investigators working in the area of electroporation,. The authors observed that the exposure of suspensions of yeast, bacteria, and erythrocytes to intense electric pulses in the range of kilovolts per centimeter and of microsecond duration causes lysis of cells (Sale and Hamilton, 1967, 1968). The results indicated the electric field effect to be cleary a cell membrane phenomenon; the authors suggested that the increased transmembrane potential may cause conformational changes in the membrane structure resulting in lysis. They estimated this transmembrane potential $\Delta\Phi_m$ from the equation (Cole, 1972) where E_0 is the strength of the external electric field, a is the radius of the cell, and θ is the angle between the direction of the field and the normal to the cell surface. The critical transmembrane potential built up by the external field was found to be about 1 V (for erythrocytes, bacterial protoplasts and spheroplasts). The phenomenon was called *electric breakdown* by Sale and Hamilton (1968).

$$\Delta\Phi_m = 1.5 E_0 a \cos\vartheta$$

It soon became apparent that a field-induced permeability increase could be transient in nature although long-lived compared with the field duration. Neumann and Rosenheck (1972) found that above a threshold value of the initial field strength E_0=18 kV cm^{-1}, the chromaffin granules release some of their content of catecholamines and ATP. They introduced the term "electropermeabilization" to explain the occurrence of permeability changes introduced by electrical impulses in vesicular membranes. It was later shown by Rosenheck *et al.* (1975) that the electric field only led to a transient change of the chromaffin granules. Zimmermann *et al.* (1973) attributed resistance changes of *E. coli* in Coulter counter measurements to dielectric breakdown. Kinosita and Tsong (1977a,b, 1978, 1979) studied effects of intense, pulsed electric fields on human erythrocytes and presented evidence that a primary effect of the electric field was the implantation of aqueous pores of limited size into the cell membranes (Kinosita and Tsong, 1977a,b, 1978, 1979). It was shown that electric field-induced pores could reseal under some conditions and the cytoplasmic macromolecular contents could be retained (Kinosita and Tsong, 1977a,b,1978). Since then, a number of research groups have focused their attention on the study of mechanisms of pore formation and characterization of the electric field modified cell membranes (Abidor *et al.*, 1979; Benz *et al.*, 1979; Chernomordik *et al.*, 1987; Glaser *et al.*, 1988; Powell *et al.*, 1986; Schwister and Deuticke, 1985; Sowers and Lieber, 1986; Sugar, 1983). Main experimental observations and theoretical considerations are discussed below.

A finding which is closely associated with electroporation and has attracted much attention among cell biologists and biophysicists is that high electric pulses can induce fusion of cells. Senda *et al.* (1979) with the aid of micromanipulator brought two electrodes into contact with adjoining *Rauwolfia serpentina* protoplasts and after applying a brief electric pulse, induced fusion. The fused plant protoplasts were viable for at least several hours. The viable giant cells were first obtained by simple electropulsing of a suspension of cells of the eukaryotic microorganism *Dictyostelium discoideum* by Neumann *et al.* (1980). To obtain close contact between cells, it was proposed by Scheurich and Zimmermann (1981) to utilize the phenomenon of dielectrophoresis (Pohl, 1978), when cells aggregate in long chains in an alternating electric field.

In 1982, Neumann *et al.* (1982) described transfection of a foreign gene into eukaryotic cells by the electroporation method. They observed that the transfected gene was expressed in the host cells. In recent years, electroporation has been widely used to introduce exogenous DNA into various cells (Potter, 1988).

It has been shown that all direct factors leading to an increase in the membrane permeability in pathology (lipid peroxidation, phospholipase action, mechanical expansion of membrane, and changes in protein-lipid interaction) decrease the electrical stability of the lipid bilayer. It has therefore been assumed that an electrical breakdown of the membrane by its intrinsic potential may be an important mechanism by which membranes lose their barrier function in pathologic situation (Putvinsky *et al.*, 1979). Electroporation was also postulated to be an important mechanism of tissue damage in electrical trauma (Lee and Kolodney, 1987).

In the experiments carried out on preparations of viable frog skin, Powell *et al.* (1989) have demonstrated that electroporation could be made to occur repeatedly in a tissue without apparent damage. Subsequent studies have shown that the electroporation of skin could be used to enhance transdermal drug delivery (Prausnitz *et al.*, 1993, 1994).

In 1989 it was reported that the application of electric fields pulses on a suspension of cells in the

presence of a selected membrane protein having a membrane spanning sequence resulted in the implantation of the protein in the cell's plasma membrane (Mouneimne *et al.*, 1989). This phenomenon was called *electroinsertion*.

Later, electroporation of exitable membranes was observed (Chen and Lee, 1994; O'Neil and Tung, 1991). O'Neil and Tung (1991) observed electrically induced membrane breakdown of isolated cardiac cells. Chen and Lee (1994) reported the asymmetrical electropermeabilization of frog skeletal muscle fibers with respect to the stimulation pulse polarity

All these goal-directed applications of electroporation methods in cell biology, biotechnology, and medicine are described in more details in the subsequent paper. Now we proceed to analysis of the experimental studies on cell electroporation.

Features of permeabilization. A few characteristics of the cell membrane permeabilization induced by strong electric fields are evident:

1) The critical transmembrane potential $\Delta\Phi_{cr}$ required to induce electroporation was found to be dependent on the duration of the electric field pulse (Kinosita and Tsong, 1977; Sale and Hamilton, 1967). Kinosita and Tsong (1977) and Riemann *et al.* (1975) studied dependence of the critical field intensity $E_{0.5}$ at which 50% of the erythrocytes either hemolyse or release potassium on the pulse duration. They have found the strong increase in $E_{0.5}$ at pulse durations shorter than 5 to 10 ms. Above 5 to 10 ms $E_{0.5}$ was less dependent on pulse duration.

2) The critical transmembrane potential $\Delta\Phi_{cr}$ or the duration of the pulse required to induce electroporation is not dependent on pH, at least in the range of 5 to 9 (Benz *et al.*, 1979; Zimmermann *et al.*, 1977) and the ionic strength of the medium (Kinosita and Tsong, 1977b; Rols and Teissie, 1989), but is influenced by temperature (Kinosita and Tsong, 1979; Benz *et al.*, 1979; Coster and Zimmermann, 1975) and some other membrane and system parameters (Deuticke *et al.*, 1991; Gneno *et al.*, 1986; Lovelace *et al.*, 1985). It has been found that the cell membrane composition (Gneno *et al.*, 1986; Lovelace *et al.*, 1985), the physiological state of the cells (Zimmermann *et al.*, 1974), chemical modifications (Deuticke *et al.*, 1991), the osmotic (Zimmermann *et al.*, 1974) or hydrostatic (Zimmermann *et al.*, 1980) pressure gradient, - all affect the value of $\Delta\Phi_{cr}$.

3) The increase of permeability is large enough to allow ions and small molecules (ATP, mannitol, sucrose, etc.) as well as macromolecules (dextran of molecular weight up to 154 kDa, proteins - up to 5×10^6 daltons, and DNA - up to 240 kb) to enter or leave the cell (Hughes and Crawford, 1989; Lambert *et al.*, 1990; Kinosita and Tsong, 1977b; Liang *et al.*, 1988; Graziadei *et al.*, 1991; Grinstein and Furuya, 1988; Sheng *et al.*, 1995; Swezey and Epel, 1989; Yumura *et al.*, 1995).

4) The pores are greater in the solution of low ionic strength (Kinosita and Tsong, 1977a; Rols and Teissie, 1989; Teissie and Tsong, 1981) and in the presence of PEG (Hood and Stachow, 1992). When *Schizosaccharomyces pompe* cells were electroporated in the presence of large FITC-dextran (150 kDa), no uptake was observed. However, when electroporated cells were incubated in PEG before exposure to the 150-kDa FITC-dextran, uptake was observed (Hood and Stachow, 1992).

5) Permeability is bidirectional i.e., intracellular compounds (e.g., ions, glycine, ATP, proteins, etc.) can leak from pulsed cells (Moser *et al.*, 1995; Neumann and Rosenheck, 1972; Schwister and Deuticke, 1985) as well as foreign substances can enter the cell (Kinosita and Tsong, 1977a,b; Swezey and Epel, 1989; Zimmermann *et al.*, 1980).

6) No apparent molecular binding event, as a transporter, is involved with uptake of external substances. The enhanced permeability shows the characterristics expected of true pores in the plasma membrane, as opposed to those of a saturable carrier as no competition between influx of labeled and unlabeled molecules was found (Swezey and Epel, 1989).

7) Electrically induced leaks exhibit some chemical selectivity (Deuticke and Schwister, 1989; El-Mashak and Tsong, 1985; Schwister and Deuticke, 1985). The pores discriminated among anions but not among cations and distinguished divalent from monovalent ions (Deuticke and Schwister, 1989).

8) Swezey and Epel (1989) showed that both negatively and positively charged molecules could enter electroporated cells, albeit the ease of loading decreased with the charge of the molecule.

9) Evidence suggests that it is most probably the lipid part of the biological membrane which is transiently permeabilized by an electroporation pulse (Chernomordik *et al.*, 1987; Teissie and Tsong, 1981). However some part of permeability may also be associated with perforation of (Na, K) pumps (Teissie and Tsong, 1980). Ouabain, a potent inhibitor of (Na, K) ATPase, partially blocked the membrane current in a manner consistent with the drug's physiological effect (Teissie and Tsong, 1980). At a saturation concentration, ouabain blocked 30% of the total voltage-induced current.

10) The electroporation enhances phospholipid transbilayer mobility (Dressler *et al.*, 1983). This finding is consistent with the formation of hydrophilic pores.

11) Phospholipids in the membrane display major structural changes under electroporation conditions (Neumann *et al.*, 1992). These data support the idea of a rapid transition ($\tau<1\mu s$) from hydrophobic to hydrophilic pores.

12) Electropermeabilization of cells can be asymmetrical: pore populations in two hemispheres may differ in the size and (or) number of pores (Saulis, 1993; Sowers and Lieber, 1986).

13) The change of the membrane permeability can be fully reversible - when the conditions for the pulse characteristics and the medium are properly chosen, electropores have a finite lifetime (Kinosita and Tsong, 1977a,b; Saulis, 1997; Saulis *et al.*, 1991;).

Theoretical considerations. Theoretical models, which have been suggested to explain the experimental data on the electroporation of biological membranes, can be devided in two main groups: (i) electromechanical models (Crowley, 1973; Dimitrov, 1984; Zimmermann *et al.*, 1974) and (ii) statistical models (Abidor *et al.*, 1979;

Glaser *et al.*, 1988; Powell and Weaver, 1986; Sugar, 1983).

The former models assume a membrane compression by the external electrical field, leading to a membrane rupture when the electrical force exceeds the elastic (or viscoelastic) restoration force. It predicts the existence of a critical potential, above which the membrane is unstable. This occurs at a critical transmembrane potential where Y_m is the elastic compressive modulus referring to deformations normal to the surface of the membrane; ε_m is the dielectric constant of the membrane; ε_0 is the dielectric constant of the vacuum, and h_{m0} is the thickness of the membrane in the unstressed state.

$$\Delta\Phi_{cr} = [0.3679 Y_m h_{m0}^2 / \varepsilon_m \varepsilon_0]^{1/2} \quad (2)$$

However, electromechanical models could not explain - at least not quantitatively - neither the pulse length dependence of the breakdown voltage in the milliseconds range or the dependence of the membrane lifetime on its area, which was observed by Arakelyan *et al.* (1983), nor the fact that the lifetime of the membrane varies randomly from experiment to experiment (Abidor *et al.*, 1979; Sukharev *et al.*, 1983).

The second class of electroporation theories is based on transient aqueous pores that are explicitly assumed to be created by the combined effects of thermal fluctuations and the local electric field across the membrane. This concept was first suggested by Chizmadzhev and co-workers (Abidor *et al.*, 1979). Similar pore approaches to rupture were subsequently developed by Weaver and Mintzer (1981) and Sugar (1983).

It has been suggested that a certain number of temporal defects of the type of through-going hydrophobic pores (Fig. 1A) having originated in the membrane due to lateral thermal fluctuations of phospholipid molecules are present in a membrane (Abidor *et al.*, 1979). Another possible type of the defect is a hydrophilic pore (Fig. 1B). As a result of the thermal motion of individual phospholipids molecules, the sizes of the hydrophobic pores will vary randomly. As long as the hydrophobic defect sizes do not exceed the critical value r_*, the restoring force acts on them and the region of small radii is therefore stable. If, however, the size of any defect exceeds the critical value r_*, such a hydrophobic defect may transform into a hydrophilic, because this process is accompanied by a decrease of the free energy of the system.

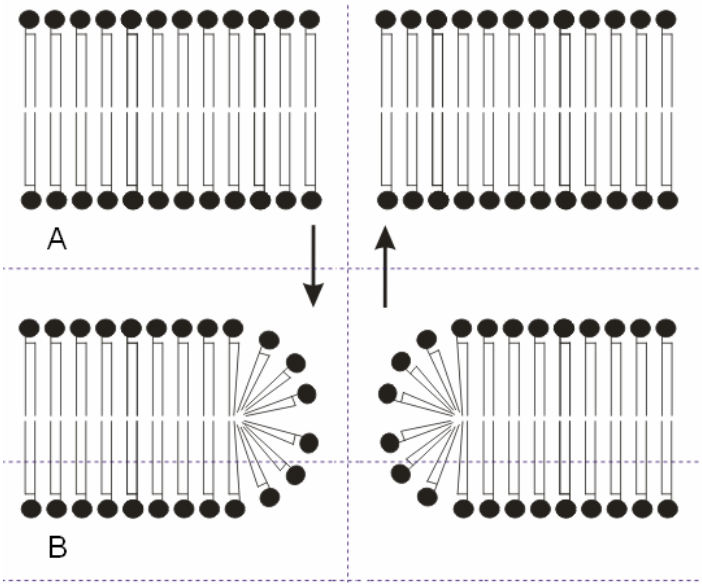

Fig. 1. **Types of pores in lipid membranes: (A) hydrophobic pore, (B) hydrophilic pore.**

The probability of the existence of a hydrophobic pore is determined by the dependence of pore energy on pore radius, and the transformation of a hydrophobic pore into a hydrophilic one requires that some kinetic barrier be surmounted. The comparison of the energies of hydrophobic and hydrophilic pores, W_h (r) and W(r) respectively, shows that the formation of hydrophobic pores in the bilayer is energetically more favourable if the radius is very small (Fig. 2). The life time of these hydrophobic pores is of the order of the lipid fluctuations. They are only intermediate stages in the formation of hydrophilic pores, as when the radius of the hydrophobic pore exceeds a critical value r_*, at which $W_h(r)=W(r)$, a reorientation of the lipid molecules becomes energetically favourable.

An electric field reduces the energy barrier to pore formation (Fig. 2) and, as a result, increases the rate of pore formation which is exponentially dependent on $\Delta\Phi_m^2$. Accumulation of hydrophilic pores in the membrane due to an electric field is considered to be the cause of electroporation (Chernomordik *et al.*, 1983). Even at zero transmembrane potential hydrophilic pores are metastable owing to the existance of an energy barrier to pore resealing $\Delta W_r(\Delta\Phi_m)$, which prevents them from closing (Glaser *et al.*, 1988; Saulis *et al.*, 1991).

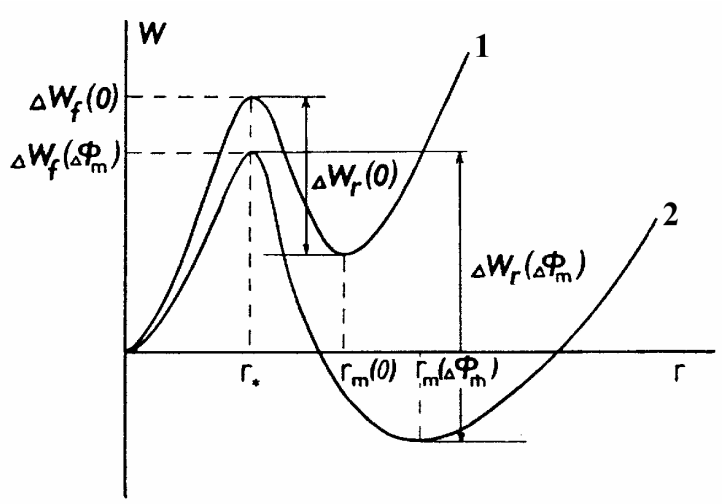

Fig. 2. **Pore energy *W* as a function of pore radius in the absence (upper curve) and presence (lower curve) of a transmembrane potential $\Delta\Phi_m$. Values of pore radius $r < r_*$ correspond to hydrophobic and $r > r_*$ to hydrophilic pores. Hydrophilic pores formed as a result of hydrophilization of hydrophobic pores are metastable owing to the existence of an energy barrier $\Delta W_r(\Delta\Phi_m)$ preventing their closing. The transmembrane potential diminishes the energy barrier to pore formation $\Delta W_f(\Delta\Phi_m)$ and raises the energy barrier to pore resealing $\Delta W_r(\Delta\Phi_m)$. In addition, at higher $\Delta\Phi_m$ the radius corresponding to the local energy minimum $r_m(\Delta\Phi_m)$, is greater.**

From the comparison of the theoretical dependence of the rate of pore formation on the transmembrane potential with experimental data on planar bilayer lipid membranes (BLM) and human erythrocytes, the energy barrier for hydrophilic pore formation, $\Delta W_f(0)$ at zero transmembrane potential, and the critical radius r_*, at which this energy barrier is situated, were estimated. The values for membranes formed from asolectin and modified with uranyl ions and human erythrocytes are: $\Delta W_f(0) \approx$ 40-45 *kT*, $r_* \approx$ 0.3-0.5 nm (Glaser *et al.*, 1988; Leikin *et al.*, 1986; Saulis and Venslauskas, 1993).

Applications of electroporation. Electroporation is now widely used for the introduction into or getting out of living cells of such membrane-impermeable substances as drugs (Zimmermann *et al.*, 1980), proteins (Knight and Baker, 1982; Lambert *et al.*, 1990), enzymes (Winegar *et al.*, 1989), antibodies (Berglund and Starkey, 1989), nucleotides (Hughes and Crawford, 1989), RNA (Auer *et al.*, 1976), DNA (Kilbane and Bielaga, 1991; Neumann *et al.*, 1982) and even small organelles (Zimmermann *et al.*, 1982). Recently, electroporation was postulated to be an important mechanism of tissue damage in electrical trauma (Lee and Kolodney, 1987). Electroporation has become especially popular as an effective technique for introduction of foreign DNA into cells of any origin (Potter, 1988). Various kinds of mammalian cells (Chu *et al.*, 1987), insect (Mann and King, 1989), protozoan (Laban and Wirth, 1989) and plant cells (Shillito *et al.*, 1985) intact bacteria (Satoh *et al.*, 1990), and yeasts (Meilhoc *et al.*, 1990) have been successfully transformed by means of this technique. Further applications of the electroporation technique are electrofusion to produce hybridoma cells (Zimmermann, 1982), the electro-insertion of foreign glycoproteins into the membrane of blood organelles (Mouneimne *et al.*, 1989), electrochemo-therapy (Mir *et al.*, 1991), and electrically-enhanced transdermal delivery of drugs (Prausnitz *et al.*, 1993).

All these applications of cell electroporation phenomenon are discussed in more details in our subsequent paper.

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