Electroporation in Biological Cell and Tissue: An Overview

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Abstract In this chapter, basics and mechanisms of electroporation are presented. Most important electric pulse parameters for electroporation efficiency for different applications that involve introduction of small molecules and macromolecules into the cell or cell membrane electrofusion are described. In all these applications, cell viability has to be preserved. However, in some biotechnological applications, such as liquid food sterilization or water treatment, electroporation is used as a method for efficient cell killing. For all the applications mentioned above, besides electric pulse parameters, other factors, such as electroporation medium composition and osmotic pressure, play significant roles in electroporation effectiveness. For controlled use of the method in all applications, the basic mechanisms of electroporation need to be known. The phenomenon was studied from the single-cell level and dense cell suspension that represents a simplified homogenous tissue model, to complex biological tissues. In the latter, different cell types and electric conductivity that change during the course of electric pulse application can significantly affect the effectiveness of the treatment. For such a complex situation, the design and use of suitable electrodes and theoretical modeling of electric field distribution within the tissue are essential. Electroporation as a universal method applicable to different cell types is used for different purposes. In medicine it is used for electrochemotherapy and genetherapy. In biotechnology it is used for water and liquid food sterilization and for transfection of bacteria, yeast, plant protoplast, and intact plant tissue. Understanding the phenomenon of electroporation, its mechanisms and optimization of all the parameters that affect electroporation is a prerequisite for successful treatment. In addition to the parameters mentioned above, different biological characteristics of treated cell affect the outcome of the treatment. Electroporation, gene electrotransfer and electrofusion are affected by cell membrane fluidity, cytoskeleton, and the presence of the cell wall in bacteria yeast and plant cells. Thus, electroporation parameters need to be specifically optimized for different cell types.

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1 Basics and Mechanisms

Electroporation is a method of cell membrane permeabilization that is today widely used in biotechnology and medicine for delivery of drugs and genes into living cells (Neumann et al. 1982; Fromm et al. 1985; Teissié 1988; Ferber 2001; Prud'Homme et al. 2006). It is alternative method for water sterilization and food preservation (Teissié et al. 2002), and it is a prerequisite for cell electrofusion (Zimmermann 1982; Teissié and Rols 1986; Ramos and Teissié 2000a).

The phenomenon of electroporation can be described as a dramatic increase in membrane permeability caused by externally applied short and intense electric pulses. Various theoretical models were developed to describe electroporation, among which the transient aqueous pore model is the most widely accepted. According to this model, hydrophilic pores are formed in the lipid bilayer of a cell membrane when it is exposed to external electric pulses. In the cell membrane, hydrophobic pores are formed by spontaneous thermal fluctuations of membrane lipids. In a cell exposed to an external electric field, the presence of an induced transmembrane potential provides the free energy necessary for structural rearrangements of membrane phospholipids and thus enables hydrophilic pore formation (Neumann et al. 1989; Tsong 1991; Chang et al. 1992; Weaver and Chizmadzev 1996). Hydrophilic pores form only in a small fraction of the membrane exposed to electric field. Even though some attempts to visualize the changes in the membrane structure caused by electric pulse application were made (Stenger and Hui 1986; Chang and Rees 1990), the structural reorganization and creation of hydrophilic pores has so far not been directly observed (Rols 2006). All the data available until now have been obtained as an indirect evidence of membrane permeabilization, such as measurements of conductivity changes caused by electric pulse application and observations of molecular transport through the cell membrane (Neumann et al. 1989; Weaver and Chizmadzev 1996).

Cell membrane electroporation takes place because the cell membrane amplifies the applied external electric field, as its conductivity is several orders of magnitude lower than the conductivities of extra cellular medium and cell cytoplasm. The theoretical description of the transmembrane potential induced on a spherical cell exposed to electric field is known as Schwan's equation (Neumann et al. 1989; Marszalek et al. 1990; Kotnik et al. 1997). The induced transmembrane potential for a spherical cell can be calculated as:

$$U_{TI} = -1.5 r E \cos\varphi \tag{1}$$

where r is the radius of the cell, E is the strength of applied electric field, and φ is the angle between the direction of the electric field and the selected point on the cell surface. The induced transmembrane potential and therefore maximum electroporation occur at the poles of the cell exposed to the electric field facing the electrodes (Fig. 1).

Electroporation can be either reversible or irreversible, depending on parameters of the electric pulses. It is a threshold phenomenon: the induced transmembrane

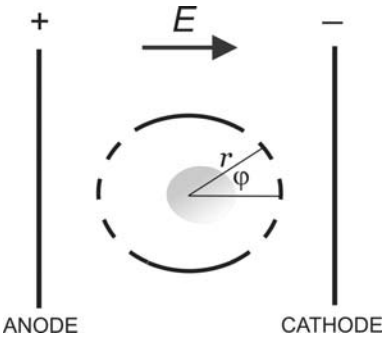

Fig. 1 Cell in an electric field. The induced transmembrane potential is maximal at the poles of the cell in accordance with Equation (1). Electroporated area is presented with dashed line

voltage imposed by external electric field should reach a critical value to trigger formation of transient aqueous pores in the cell membrane (Kinosita and Tsong 1979; Abidor et al. 1979; Neumann and Rosenheck 1972; Kinosita and Tsong 1977). The threshold membrane potential that needs to be reached in the cell membrane is between 200 mV and 1 V (Zimmermann 1982; Tsong 1991; Teissié and Rols 1993). For reversibility of electroporation, the membrane potential has to be kept below the critical value. In such conditions, the cell membrane recovers after electric pulse application (Neumann et al. 1989). On the contrary, when the critical value is exceeded, irreversible electroporation takes place, resulting in cell membrane disintegration and loss of cell viability (Hamilton and Sale 1967; Meaking et al. 1995; Danfelter et al. 1998).

The electroporation process consists of different phases. The first of them is pore formation, which is the cell membrane's response to the induced threshold membrane potential, and lasts a few microseconds. The second phase is a time-dependent expansion of the pore size taking place in a time range of hundreds of microseconds to milliseconds, and lasts throughout the duration of pulses. The last phase is membrane recovery, which takes place after electric pulse application and consists of pore resealing, and lasts several minutes (Kinosita and Tsong 1977; Hibino et al. 1993; Neumann et al. 1999; Leontiadou et al. 2004). This resealing phase is strongly affected by temperature (Kinosita and Tsong 1977) and cytoskeleton integrity (Rols and Teissié 1992a; Teissié and Rols 1994). The first phase of electroporation can be measured by changes in membrane conductivity and is related to short-lived transient pore formation, which does not contribute to molecular transport (Pavlin et al. 2007). Molecular transport across the permeabilized cell membrane associated with electroporation is observed from the pore formation phase until membrane resealing is completed (Gabriel and Teissié 1997, 1999; Prausnitz et al. 1995; Puc et al. 2003; Pavlin et al. 2007).

Electroporated membranes are also a prerequisite for associated membrane phenomena termed electrofusion. During electric pulse application and immediately after it, the cell membrane is capable of fusion: it is in a so-called fusogenic state (Teissié et al. 1982; Zimmermann 1982).

In brief, electroporation is a useful technique in biotechnology and medicine for introduction of different molecules into the cell, electrofusion, or water sterilization

and food preservation. Among different theoretical models that describe electroporation, the transient aqueous pore model is most widely accepted. This model predicts hydrophilic pore formation as a response to induced external electric field on the cell membrane. Electroporation can be reversible or irreversible, depending on the electric pulse parameters used.

2 Influential Parameters

Electroporation is affected on the one hand by parameters of electric pulses and chemical composition of the media used and on the other by the characteristics of the cell that is exposed to the electric field. The effect of the electric pulse parameters and electroporation media are described in this section.

2.1 Parameters of Electric Field

The parameters of electric pulses were extensively investigated. The most important electric pulse parameters are amplitude, duration, number, and repetition frequency (Rols and Teissié 1990a; Wolf et al. 1994; Gabriel and Teissié 1995a; Vernhes et al. 1999; Maček-Lebar et al. 1998; Maček-Lebar and Miklavčič 2001; Bilska et al. 2000; Canatella et al. 2001). If those parameters exceed the optimal values, irreversible electroporation takes place due to cell membrane disintegration (Hamilton and Sale 1967; Danfelter et al. 1998) and DNA damage (Meaking et al. 1995), resulting in cell lysis. The choice of electric pulse parameters thus depends on the desired application. Some applications require reversible, while others require irreversible electroporation. For loading of foreign molecules into the cell, reversible electroporation is required. The choice of electric pulse parameters depends on the type of the foreign molecule that is being introduced. For small molecules, such as different drugs or fluorescence dyes, a train of relatively short pulses (time duration in range of microseconds to milliseconds) is sufficient. For large molecules, such as DNA, longer pulses (range of few milliseconds) or a combination of high-voltage short-duration pulses and low-voltage long-duration pulses is used (Wolf et al. 1994; Klenchin et al. 1991; Sukharev et al. 1992; Šatkauskas et al. 2002).

Besides the before mentioned parameters of electric pulses, different pulse shapes can also be used. The most frequently used are exponential and square wave pulses. One should be careful when comparing results obtained by different pulse shapes, as the membrane polarization process that takes place during the pulse application is different (Neumann 1992).

Electric pulses can be applied in one direction or their orientations can be changed during the pulse application. Such protocols were successfully used for electrochemotherapy and gene electrotransfer (Rols, Teissié 1990a; Tekle et al. 1991; Serša et al. 1996; Vernhes et al. 1999; Kotnik et al. 2001a; Kotnik et al. 2001b; Golzio et al. 2002; Faurie et al. 2004; Faurie et al. 2005; Reberšek et al. 2007).

2.1.1 Introduction of Small Molecules

For introduction of small molecules, short electric pulses in a range of tens to hundreds of microseconds are generally used. The most important parameter is pulse amplitude. It should reach a threshold value at which the electroporation of cell membrane is triggered. Above the threshold value the increase in electroporation is obtained with increase of pulse duration and number of pulses (Fig. 2). The increase in pulse duration increases the electroporation of cells until a plateau is reached and further increase in number of pulses or its duration does not affect cell electroporation (Rols and Teissié 1990a; et al. 1993; Maček-Lebar and Miklavčič 2001). At the same time the increase in pulse number and pulse duration affects cell viability (Gabriel and Teissié 1995b; Maček-Lebar and Miklavčič 2001). The following explanation for the relationship between the pulse amplitude and the pulse number or duration was proposed: increasing the pulse amplitude results in larger area of membrane electroporation with smaller extent of electroporation, while increase in pulse number or duration does not affect the electroporated membrane area but increases the extent of electroporation (Fig. 3) (Rols 2006). Nevertheless, when increasing the duration of the pulse, one should also consider that longer pulses cause significant Joule heating of the sample (Pliquett et al. 1996).

Systematic study of electric pulse parameters revealed that electroporation and cell viability are not related to the total electrical energy delivered (Maček-Lebar et al. 1998; Vernhes et al. 1999). Further examinations of different parameters of electric pulses indicate complex dependence between electric pulse parameters and degree of electroporated cell membrane (Canatella et al. 2001).

Another electric pulse parameter affecting electroporation of the cell membrane is pulse repetition frequency. When pulses are applied with high repetition frequency, above 1 kHz, the pause between two consecutive pulses is too short and does not allow cell membrane to return to pre-pulse state. From the experimental results it can be concluded that cell viability and cell membrane electroporation is optimal in the frequency range from 0.5 to 10 Hz and decreases at higher frequencies (Vernhes et al. 1999; Pucihar et al. 2002; Pavlin et al. 2005).

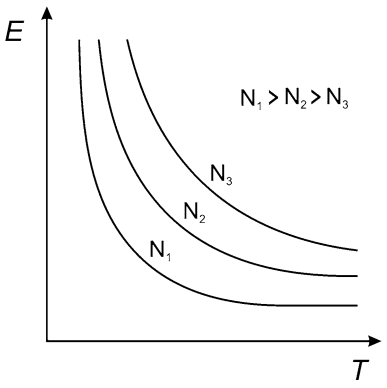

Fig. 2 Fraction of electroporated cells is increasing with increasing number of applied pulses. E, electric field strength, T, pulse duration, N, number of applied pulses

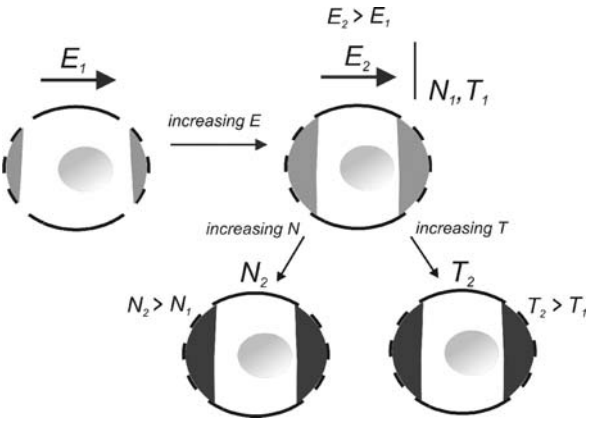

Fig. 3 Increasing the pulse amplitude results in larger area of membrane with smaller extent of electroporation, while increase in pulse number or duration does not affect the membrane area but increases the extent of electroporation

For reversibility of electroporation, the membrane potential has to be kept below the critical value. In such conditions, the cell membrane recovers after the electric pulse application (Neumann et al. 1989). On the contrary, when critical value is exceeded, irreversible electroporation takes place, resulting in cell membrane disintegration and loss of cell viability (Hamilton and Sale 1967; Meaking et al. 1995; Danfelter et al. 1998).

2.1.2 Introduction of Macromolecules

The optimal conditions for introduction of macromolecules are different from optimal conditions for introduction of small molecules (Wolf et al. 1994). Most experiments were performed with long, 5 to 10 ms pulses with relatively low pulse amplitude. When those results were compared with results obtained with higher-voltage microsecond pulses, typically used for introduction of small molecules, it was established that many different pulse parameters are capable of delivering plasmid DNA into the cell. Protocols employing millisecond pulses are more efficient than microsecond pulses for long-term gene expression in vivo (Lucas and Heller 2001).

The efficiency of gene electrotransfer into mammalian cells was first related to the pulse shape used, and exponentially decaying pulses were reported as more effective that the square wave pulses (Andreson and Evans 1989). Later, the use of combination of high-voltage and low-voltage pulses was suggested. High-voltage pulse causes electroporation of cell membrane, while the low-voltage pulse helps highly charged DNA entrance into the cell interior. A low-voltage pulse thus provides electrophoretic movement of DNA into the cell in in vitro conditions, or it can be a powerful driving force for improving interstitial transport of DNA during gene delivery in vivo (Klenchin et al. 1991, Sukharev et al. 1992; Zaharoff

et al. 2002; Zaharoff and Yuan 2004). The effect of electrophoretic pulses was successfully used and demonstrated in in vivo experiments in mammalian tissues (Bureau et al. 2000; Somiari et al. 2000; Šatkauskas et al. 2002; Šatkauskas et al. 2005; Andre and Mir 2004; Zampaglione et al. 2005; Pavšelj and Preat 2005a). Nevertheless, the role of electrophoretic force in DNA movement across permeabilized membrane is questioned for in vitro gene electrotransfer as no contribution of electrophoretic force could be detected (Wolf et al. 1994). Lately the effect of electrophoretic movement of DNA by low-voltage pulse has also been questioned for in vivo applications (Liu et al. 2006).

The effect of low-voltage electric pulse on the highly charged DNA is alternatively attributed to electrophoretic accumulation of DNA on the cell membrane (Wolf et al. 1994). It has also been demonstrated by visualization of DNA interaction with the cell membrane that the electric field orientation plays an important role in gene electrotransfer (Golzio et al. 2002; Faurie et al. 2004; Faurie et al. 2005; Reberšek et al. 2007). Similar to small molecules, asymmetric DNA uptake is observed during electroporation (Mehrle et al. 1985; Tekle et al. 1991). Nevertheless, DNA, unlike small molecules that enter cell cytoplasm on the membrane-facing cathode, enters the cell on the surface-facing anode (Golzio et al. 2002; Faurie et al. 2004; Faurie et al. 2005). Another main difference between introduction of small molecules and DNA is that for successful gene electrotransfer, DNA has to be present in the medium before electric pulses are applied (Fig. 4) and the transport of the DNA through cell membrane takes place minutes after the pulse

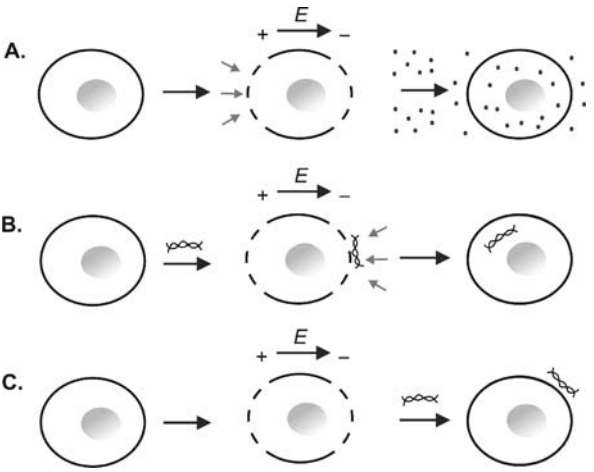

Fig. 4 Introduction of small and large molecules by electroporation. (**A**) Introduction of small molecules takes place during and predominantly after the pulse. Electroporation of the cell membrane is asymmetrical and occurs first at the anode side (small *grey* arrows). (**B**) Introduction of DNA into the cell. DNA must be present before electric pulses are applied. The initial step is DNA adsorbtion to the cell membrane, which takes place in the cell membrane facing cathode (small *grey* arrows). (**C**) When DNA is added after the pulse application it cannot be introduced into the cell

application (Golzio et al. 2002). No spontaneous interaction of DNA with the cell membrane was detected. The complex between the DNA and the membrane forms only when the membrane is electroporated. If DNA is added after pulse application, no transfection can be observed. It was, however, demonstrated that transfection is successful if the DNA is added after the high-voltage pulse and before low-voltage pulse, but the level of DNA expression is lower (Šatkauskas et al. 2002).

2.1.3 Electrofusion

The electrofusion is a two-step process; it involves cell membrane electroporation and a close physical contact of two electroporated membranes in fusogenic state (Zimmermann 1982; Saunders et al. 1986). The electric field parameters needed for introduction of small molecules and for electrofusion are similar. The main difference between two processes is the critical voltage required for electrofusion, which is higher than for electroporation (Teissié and Rols 1993; Abidor et al. 1994; Teissié and Ramos 1998), and the duration of fusogenic state, which is shorter than cell membrane resealing process. The resealing of the cell membrane after electroporation can take up to tens of minutes, while membrane fusion is only possible if the contact of permeabilized membranes is achieved within few minutes after pulse application (Teissié and Rols 1986; Sowers 1986; Ramos and Teissié 2000a). The contact needed for electrofusion can be obtained before or immediately after the electroporation pulse. When the cell contact is obtained before electroporation, most often dielectrophoresis is used (Zimmermann 1982), while the contact of cells after electroporation is obtained by centrifugation of fusogenic cells (Teissié and Rols 1986; Sowers 1986).

The close physical contact obtained by dielectrophoresis results in pearl chain formation (Zimmermann 1982). For this application, an alternating electric field of low amplitude on the order of few hundred volts per centimeter and frequencies in the range of 10 kHz to 6 MHz is used (Zimmermann 1982; Vienken and Zimmermann 1985; Saunders et al. 1986). During electrophoresis the polarized cells are attracted to the areas of high field strength (Oblak et al. 2007). Cells migrate toward each other and form pearl chains. The procedure is rapid and has negligible effect on cell viability (Saunders et al. 1986). The alternating electric field is then switched off and an electroporation pulse is applied. To maintain cells in the close contact after electroporation, the alternating electric field is applied again for a short duration (Vienken, Zimmermann 1985).

When the contact of electroporated cells is obtained after the pulse (Teissié and Rols 1986), better fusion yield is obtained, if a larger membrane area is in fusogenic state. This can be obtained by proper selection of electric pulse parameters, such as number of pulses and their duration (Ramos and Teissié 2000a). When the electric field orientation is changed during the pulse application, it results in increase of electroporated area of cell membrane (Valič et al. 2003). The efficiency of electrofusion was reported to be slightly lower when the contact is obtained after the pulse than with the pre-pulse contact (Wu et al. 1992). Therefore, it is possible that

the membrane merging already starts during the electric pulse application and is concluded after the pulse (Dimitrov and Sowers 1990).

Besides electric field parameters, mechanical forces can increase the fusion yield as they enable good contact of cells (Jaroszeski et al. 1994; Ramos and Teissié 2000b).

2.1.4 Irreversible Electroporation

Irreversible electroporation is in some applications the undesired, while in others it is the desired outcome of the electric pulse application. It is a consequence of membrane rupture that is a directly caused by electric pulse application (Weaver and Chizmadzev 1996). Irreversible electroporation and Joule heating are an integral part of electrical injury, which affects especially nerve and muscle cells due to their size. Release of intracellular components from affected cells cause acute renal failure due to deposition of iron-containing molecules such as myoglobin (Lee and Dougherty 2003). Successful treatment of electroporated membranes with nontoxic polymers can reduce tissue injury produced by irreversible electroporation due to sealing of electroporated cell membranes (Lee et al. 1992; Lee and Dougherty 2003).

Irreversible electroporation is the desired result when it is used for microbial deactivation in water and food treatment. The applied electric pulses should cause irreversible damage of treated cells (Teissié et al. 2002). For effective treatment, critical electric field parameters should be chosen properly. Typical pulse amplitude for microbial deactivation in water and liquid food is between 20 and 35 kV/cm, pulse duration, from micro- to milliseconds, and pulse number varies from ten to hundred pulses (Zhang et al. 1995; Angersbach et al. 2000; Beveridge et al. 2002). For food preservation, amplitudes used are lower than for microbial inactivation in freshwater and liquid food. The main problem is the choice of optimal treatment parameters that would require minimal power consumption and would effectively disintegrate treated cells (Lebovka et al. 2000, 2002).

Recently, irreversible electroporation was reported as an alternative minimally invasive surgical technique in medicine for tissue ablation. The train of ten electrical pulses in the range of 1.5 kV/cm and duration 300 ms was applied three times for effective tissue ablation. The method was also tested in vivo. For in vivo applications, mathematical models provided a valuable tool for proper electrode positioning and optimal pulse parameter determination for effective treatment (Davalos et al. 2005; Miller et al. 2005; Edd et al. 2006; Rubinsky et al. 2007).

2.2 Electroporation Medium Composition

Conflicting reports are found on the effect of medium composition on electroporation. In some reports, increasing the ionic strength of the medium resulted in cell membrane electroporation at lower electric field intensities. The nature of monovalent ions such as sodium, potassium, or lithium (Na, K, Li) does not affect the

electroporation. On the other hand, presence of bivalent calcium ion in the medium resulted in cell lysis and death (Rols and Teissié 1989). Nevertheless, toxicity of calcium ions was reported independently of electroporation, as they are involved in different physiological processes in the living cell. Because of sudden and uncontrolled increase of calcium in the cytoplasm, the cell cytoskeleton is disrupted and uncontrolled activation of calcium-dependent catabolic enzymes takes place (Orrenius et al. 1989).

In some studies, when the medium conductivity was maintained unchanged, the effect of ionic composition and strength of the media on electroporation was almost negligible. Yet, when medium conductivity was decreased, electroporation efficiency increased drastically. In contrast, the resealing of the membrane was independent on medium ionic composition or conductivity (Djuzenova et al. 1996; Barrau et al. 2004). In our study performed in the wide range of medium conductivities it was observed that cell membrane electroporation as such was not affected by medium conductivity, while it had significant effect on cell survival (Pucihar et al. 2001). Medium composition affects heating of the sample during electroporation. When short electric pulses are used (in range of microseconds), Joule heating in high-conductivity media is negligible. On the other hand, when long pulse duration (milliseconds) and high amplitudes are used, Joule heating takes place during electroporation and is more pronounced in high-conductivity than in low-conductivity media (Pliquett et al. 1996; Pavlin et al. 2005).

Medium composition plays an even more important role in gene electrotransfer of bacteria, yeast, plant, and animal cells. Monovalent alkali ions were found to be involved in gene electrotransfer of the plant protoplasts. It was proposed that they increase membrane fluidity or enhance membrane electrical potential, making the protoplast more susceptible to an applied electric pulse (Saunders et al. 1989). In contradiction to the previously reported role of calcium on cell viability, the presence of bivalent cations such as calcium and magnesium (Ca^{2+} Mg^{2+}) was found to improve transfection efficiency of bacteria and yeast (Xie et al. 1990; Neumann et al. 1996). The role of bivalent cations in gene electrotransfer is attributed to improved DNA adsorbtion to the cell membrane.

Electrofusion yield is also improved by the presence of bivalent cations in the medium (Ohno-Shosaku and Okada 1985; Vienken and Zimmermann 1985), while the presence of monovalent ions decreased the fusion yield (Rols and Teissié 1989). Nevertheless, cell electrofusion is a complex process and several biologically active substances affect its yield (Grobner, Velizarov, Berg 1996; Velizarov and Berg 1998a; Velizarov et al. 1998b; Liu et al. 2000).

2.3 Osmotic Pressure

Electroporation is further affected by electroporation buffer osmolarity. When it is carried out in a hypertonic media, cells are permeabilized at a lower voltage than cells maintained in isotonic media and exposed to the same electric pulse parameters. On the other hand increasing the osmotic pressure of the post-electroporation

media (hypertonic media) facilitates the resealing of electroporated cells (Rols and Teissié 1990b).

Osmolarity of the electroporation media affects the cell size and shape changes caused by electroporation. The electroporation of cells in suspension results in an increase in cell diameter up to 30%, which corresponds to 100% of volume increase, in isotonic medium, while the increase is significantly lower in hypertonic medium. In addition, the osmolarity of the medium plays an important role in post-pulse incubation (Golzio et al. 1998; Barrau et al. 2004).

As electrostatic and electrorepulsive forces play an important role in an initial step of gene electrotransfer process, when a highly charged DNA molecule adsorbs to cell membrane, the medium osmolarity is an important factor in this process. Hypotonic media facilitate the gene electrotransfer in mammalian cell because of the decrease in repulsion between DNA and cell membrane. The initial step of successful DNA–membrane interaction is a key step for successful gene transfer (Wolf et al. 1994; Golzio et al. 1998). On the other hand, a hypertonic medium improves gene electrotransfer of gram-positive bacteria because of improved cell survival. Higher electric pulse amplitudes can be used, which result in better electroporation of the cell membrane and DNA loading into the cell (Xue et al. 1999). Also in plant cells, a hypertonic medium is used for improved gene electrotransfer. Osmotic treatment of an intact plant cell causes plazmolysis, which is a consequence of water loss from the vacuole. The plant cells vacuoles maintain high turgor pressure, which enables cell membrane to attach closely to the cell wall. When a cell is placed in hypertonic solution, the membrane is pulled away from the cell wall because of water loss from the cytoplasm, and the cell shrinks. These partial detachments of the cell wall from the membrane cause a void space between the rigid cell wall and the cell membrane and enables the required contact between the cell membrane and the macromolecule that is being introduced into the cytoplasm (Fig. 5) (D'Halluin et al. 1992; Ganeva et al. 1995; Sabri et al. 1996a; Eynard et al. 1997; Wu and Feng 1999).

As in gene electrotransfer, the medium osmolarity also plays an important role in cell electrofusion. The electrofusion efficiency is increased in hypotonic medium due to increased osmotic pressure in the cell (Rols and Teissié 1990b, Barrau et al. 2004). When the distance between adjusted cells is reduced, repulsive forces between neighboring cells become significant; however, those forces are balanced by osmotic pressure. In a hypertonic electroporation medium, electrofusion yield is reduced (Abidor et al. 1994).

In brief, in this section the effects of electric pulse parameters, electroporation medium composition, and osmotic pressure are described. Among electric pulse parameters, pulse amplitude, duration, number, and repetition frequency significantly affect electroporation. When these parameters exceed their optimal values, cell viability is affected and irreversible electroporation takes place. For introduction of small and large molecules, different electric pulse parameters need to be used. Small molecules are efficiently introduced into the cell by application of short electric pulses in range of tens to hundreds of microseconds. The transport of small molecules takes place predominately after the pulse by diffusion. On the

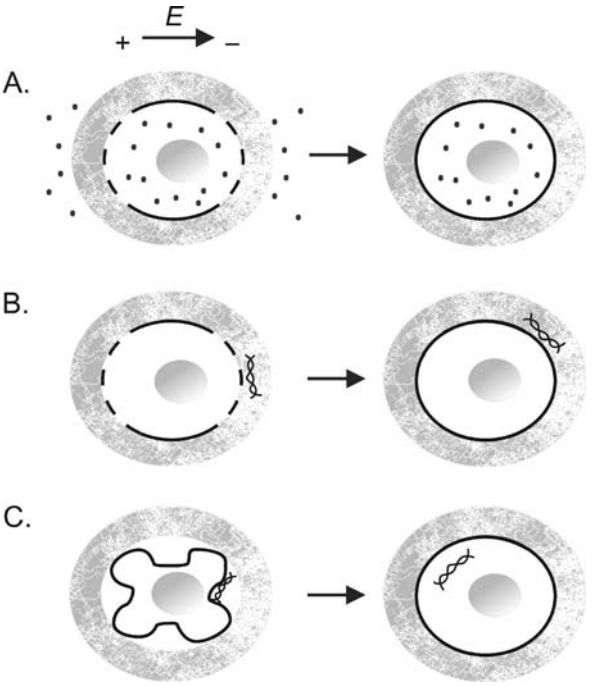

Fig. 5 Electroporation of a cell with cell wall. (**A**) Introduction of small molecules is not affected by cell wall. (**B**) DNA molecule is trapped in the cell wall. (**C**) Plasmolysis improves DNA transport into the cell

other hand, for macromolecules, long 5 to 10 μs pulses with relatively low pulse amplitude are used. Besides, for successful gene electrotransfer, DNA has to be present in the medium before electric pulses are applied, while small molecules can enter the cell even if added after the pulse. Electric pulse parameters for cell electrofusion are similar to those used for introduction of small molecules, but the critical voltage required is higher. For irreversible electroporation that is used for inactivation of microorganisms, the electric pulse parameters should exceed critical value, as cell death is the desired result of such application. In addition to the electric pulse parameters, electroporation medium composition and its osmolarity strongly affect electroporation as well as related gene electrotransfer and electrofusion.

3 From Single-Cell to Tissue

Single-cell electroporation is a suitable tool for the study of basic electroporation mechanisms. A few attempts were made to observe ultra-structural changes related to electroporation (Stenger and Hui 1986; Escande-Geraud et al. 1988); however, the process is too fast. Besides, chemical composition and fluid characteristics of the thin cell membrane make direct observation of primary membrane changes

related to electroporation very difficult (Weaver and Chizmadzev 1996). The attempt was made to use rapid freezing scanning microscopy to determine the changes in membrane structure (Chang and Rees 1990); however, the size of the pores observed was 20 nm up to 120 nm, too large compared to theoretically estimated 1 nm (Weaver and Chizmadzev 1996), and the observed pores were most probably secondary structures (Rols 2006).

At the cell membrane level, the induced transmembrane potential was imaged by fluorescence probes sensitive to transmembrane potential changes induced by an external electric field. Temporal and spatial induction of transmembrane potential on the cell membrane that responds to externally applied electric field was observed with potentiometric dyes (Gross et al. 1986; Kinosita et al. 1988; Tekle et al. 1990; Tekle et al. 1991; Hibino et al. 1991). The results obtained in those experiments on a single spherical cells are in good agreement with the theoretically calculated values obtained by Schwan's equation (Loew 1992). The value of induced transmembrane potential sustainable for living cell electroporation was determined to be 1 V (Zimmermann 1982; Tsong 1991). Later the value of the induced transmembrane potential that triggers electroporation was determined to be in the range of 200–500 mV (Marszalek et al. 1990; Grosse and Schwan 1992; Teissié et al. 1993). These values obtained by fluorescence imaging and calculations were further confirmed by direct measurement at the single-cell level using patch clamp technique (Ryttsen et al. 2000).

The value of induced transmembrane voltage depends on the cell size, shape, and the position of the cell with respect to the direction of applied electric field (Sale and Hamilton 1967; Zimmermann 1982; Graškova et al. 1996; Teissié et al. 1999; Kotnik and Miklavčič 2000; Valič et al. 2003; Valič et al. 2004). For a spheroidal cell, the maximum induced transmembrane potential strongly depends on its orientation with the respect to the electric field (Fig. 6). It is maximum when the spheroidal cell is parallel to the applied electric field (Valič et al. 2003).

The distribution of induced transmembrane potential is asymmetric due to native transmembrane potential that is present in live cells. As the induced transmembrane potential caused by externally applied electric pulses is superimposed to the resting membrane potential of the cell, the side of the cell facing the anode is hyperpolarized while the side facing the cathode is depolarized (Mehrle et al. 1985; Gabriel and Teissié 1997, 1999; Pucihar et al. 2006). The membrane labeling with fluorescent probes allows imaging of the membrane area affected by applied electric pulse (Gabriel and Teissié 1997). It was found that the membrane resting potential has a significant effect on asymmetric electroporation, especially when the induced transmembrane potential is close to the threshold voltage that triggers electroporation. This, however, is the case in majority of the applications in which cell viability needs to be preserved (Valič et al. 2004).

The cell shape affects the site of cell membrane electroporation, and it is especially important in attached cells, as they are not at regular shape. The calculation of induced transmembrane potential on single cells, therefore, depends on the realistic cell shape that needs to be taken into account as it affects the calculated distribution of the induced transmembrane potential (Pucihar et al. 2006).

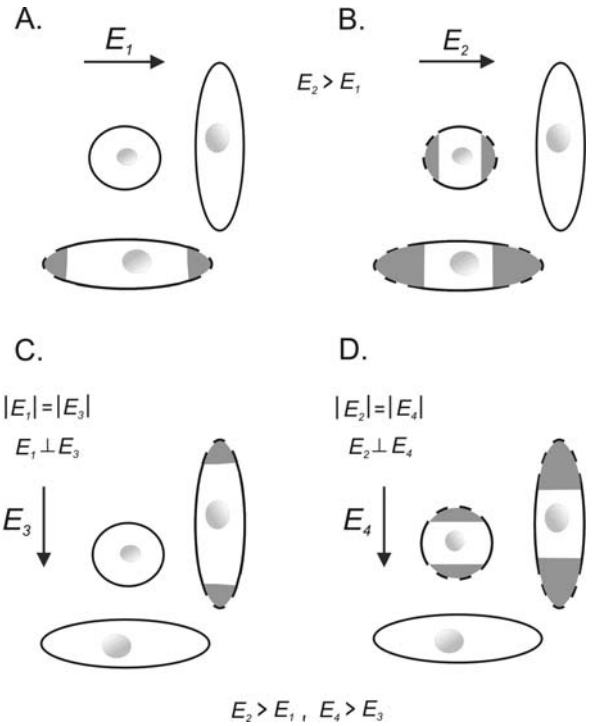

Fig. 6 Effect of electric field orientation on electroporation of different cell sizes and shapes. (**A**) Electric field parallel to elongated cell. (**B**) Electric pulse amplitude is increased. (**C**) Orientation of electric field is changed. (**D**) Electric pulse amplitude is increased

Although a single-cell model is a valuable tool for the study of basic mechanisms of electropotaion, it is not the best method to predict electroporation behavior in a tissue. As a tissue is composed of cells that are close to each other, dense cell suspensions represent an intermediate level between the single-cell level and the tissue (Abidor et al. 1994). Neighboring cells, even if they are not in direct contact, affect each other due to mutual electrical shading (Susil et al. 1998; Pavlin et al. 2002; Pucihar et al. 2006). For electroporation of cell suspensions, the proportion of the cells in the total volume is important. When they represent less than 1% of the volume fraction they behave as single cell, while for volume fraction greater than 10% or for clusters of cells, the induced transmembrane potential is affected by the suspension density (Susil et al. 1998, Pavlin et al. 2002; Pavlin et al. 2007). The fraction of electroporated cells decreases with increase in cell density and the resealing of cells in dense cell suspensions is slower. In dense cell suspensions, cell clusters, and multicellular spheroids it was found that the molecular transport is slower due to slower diffusion of molecules into the interior of such cluster or spheroid (Abidor et al. 1994; Canatella et al. 2004; Pucihar et al. 2007).

Dense cell suspensions can serve as a model for tissues with homogeneous structure composed of similar cells in close contact; nevertheless, most tissues are

not homogeneous. Tissues are composed of different cell types that are irregularly shaped, are vascularized, and present different electrical properties. All the mentioned factors affect the distribution of electric field within the tissue and consequently its electroporation efficiency (Miklavčič et al. 1998; Šemrov and Miklavčič 1998; Pucihar et al. 2006). Furthermore, cells in tissue are connected by gap junctions for intracellular communications and transport, which change the electroporation behavior of such cells, and they behave as a single larger cell (Fear and Stuchly 1998a, 1998b). For efficient tissue electroporation *in vivo,* the electric field distribution, which depends on electrode geometry, position, and electrical properties of the sample, is crucial (Šemrov and Miklavčič 2000). The electrical properties of biological tissue such as conductivity and permitivity change once the tissue is permeabilized and the electric field distribution is changed. The largest part of these changes is attributed to increased membrane conductivity due to electroporation (Pavšelj et al. 2005b; Šel et al. 2005). Changes in membrane conductivity need to be taken into account when performing electroporation with multiple needle electrodes and can be used for detection of cell membrane electroporation and for pulse delivery control. Recently these changes were used for regulating the output voltage for *in vivo* gene transfection (Cukjati et al. 2007). One of the major problems with respect to conductivity measurements *in vivo* is the inhomogeneous distribution of current density and electric field due to inhomogeneous and anisotropic properties of the tissue. For successful tissue electroporation, anatomically based mathematical models are important tools for prediction of the outcome of the treatment (Miklavčič et al. 1998; Šemrov and Miklavčič 1998; Brandinsky and Daskalov 1999; Miklavčič et al. 2000; Šel et al. 2007; Miklavčič et al. 2006a).

In brief, in this section the differences between single-cell and tissue electroporation are described. Single-cell electroporation is a suitable tool for study of basic electroporation mechanisms. The situation is more complex in tissues as they are composed of cells that are in close contact with each other and their proximity affect electroporation. Besides, most tissues are not homogenous structures, they are composed of different cell types that are irregularly shaped, are vascularized, and have different electrical properties that affect current density and electric field distribution, all of these affecting electroporation effectiveness. Mathematical models are thus a valuable tool for predicting electroporation behavior of the tissue.

4 Electrodes/Shaping the Electric Field

Electroporation is used for different purposes and depending on the application one should chose the right electrodes to obtain the desired result.

For different applications, different types of electrodes are available and can be classified according to their geometry into different groups: plate, needle, wire, and tweezers electrodes (Miklavčič and Puc 2006b). In certain cases, special electrodes are needed; for example, for individual-cell electroporation, specially designed microelectrodes are required (Lundqvist et al. 1998; Ryttsen et al. 2000; Olofsson et al. 2003). For treatment of large volumes of sample and for flow electroporation,

electroporation chambers that allow efficient treatment were designed and successfully tested. They were successfully used for gene transfection or water treatment (Stopper et al. 1987; Teissié and Conte 1988a; Teissié and Rols 1988b; Rols et al. 1992b; Li et al. 2002; Teissié et al. 2002). The choice of most suitable electrodes for a given application depends also on the characteristics of the treated sample (Miklavčič et al. 2006b).

For reversible electroporation used in medicine, electrode design have to allow efficient electroporation and at the same time cause as little cell damage of the surrounding tissue as possible. In *in vivo* electroporation, electrical properties of the treated tissue have to be taken into account, as they vary significantly among different tissues. In electroporation, mathematical models taking into account the tissue conductivity changes can be very useful for proper electrode selection and their positioning with respect to the tissue that needs to be electroporated (Pavšelj et al. 2005b; Šel et al. 2005, 2007), since the electric field distribution can be efficiently modified by electrode geometry and their position during the pulse application (Šel et al. 2005, 2007).

Irreversible electroporation, used in water sterilization and food preservation, where large volumes need to be treated and high electric fields need to be applied, requires different methodologies (Teissié et al. 2002). For flow electroporation, it is crucial that the pulse delivery frequency is linked to the flow rate in such a way that each cell that passes electroporation chamber receives electric pulse treatment. Liquid flow during electroporation affects causes cell elongation therefore electric field orientation with respect to cell is important (Fig. 6) (Teissié et al. 2002).

In brief, in this section, electrode type and effects of electrode shape and positioning on electroporation effectiveness are described. The choice of proper electrode shape and their position during the pulse application is crucial for successful treatment, as they affect the electric field distribution. The most appropriate electrode type and positioning depends on the application.

5 Different Applications

Various applications of electroporation have already been proposed, ranging from gene electrotransfer in biotechnology, biology, and medicine to cell killing in water sterilization, food preservation, and tissue ablation (Fig. 7) (Miklavčič et al. 2006b). These electroporation-based technologies and treatments require proper selection and choice of pulse parameters, electrodes, and pulse generators (Puc et al. 2004). In this section different applications in biology, biotechnology, and medicine are briefly reviewed.

5.1 Use in Medicine

In medicine, electroporation is used with the method called electrochemotherapy in clinical practice for improved drug delivery for cancer treatment, and in preclinical

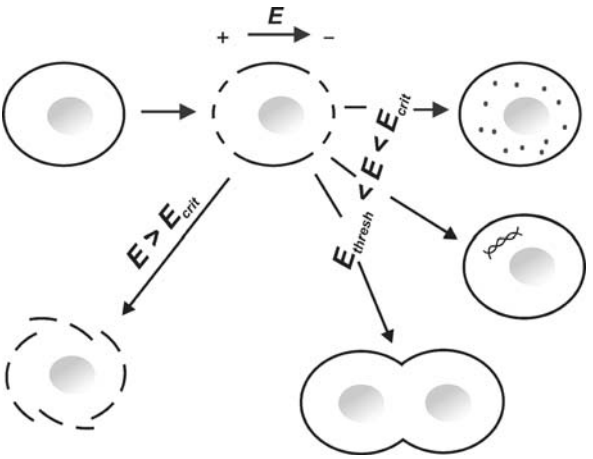

Fig. 7 Different application of electroporation. When external electric field reaches threshold value (E_{thresh}) cell membrane is electroporated. Small and large molecules can be introduced into the cell or when two cells are in close contact their membranes can fuse. When external electric field exceeds certain critical value (E_{crit}) irreversible electroporation occurs resulting in cell membrane disintegration and cell death

trails for gene electrotransfer (Serša et al. 1995; Serša et al. 1998; Serša et al. 2003; Heller et al. 1999; Mir and Orlowski 1999; Mir 2000). From the point of view of medical applications, it is more convenient to use a high-repetition pulse frequency rather than 1 Hz pulse repetition, which is currently used in clinical trials. This is important when larger tumor nodules need to be treated and when multiple needle electrodes are used. In that case, a large number of pulses need to be delivered to each of the pairs of the electrodes, which would represent an unpleasant and a relatively long treatment time, if pulses were delivered at 1 Hz repetition frequency. The application of pulses with higher repetition frequency does not significantly affect the electrochemotherapy efficiency and the treatment is less unpleasant than application of pulses with standard 1 Hz repetition frequency (Miklavčič et al. 2005; Županič et al. 2007).

At the in vivo level, tissue vascular lock is observed due to disruption of blood vessel network after the application of high-voltage pulses. Consequently, the tissue oxygenation level is reduced by electroporation resulting in enhanced tumor cell death (Serša et al. 1999; Čemaar et al. 2001; Serša et al. 2002; Gehl et al. 2002; Kanthou et al. 2006).

5.2 Water Sterilization and Food Preservation

Irreversible electroporation is used in food technology for liquid food sterilization, food preservation, and water treatment as a tool for efficient cell killing (Graškova et al. 1996; Danfelter et al. 1998; Lebovka and Vorobiev 2004), which

is important for nonthermal food preservation and for freshwater treatment (Gould 1995; Lebovka et al. 2002; Lebovka and Vorobiev 2004; Teissié et al. 2002).

The benefit of nonthermal food preservation is the maintenance of food quality (Zhang et al. 1995; Ade-Omowaye et al. 2001). The design of static and flow chambers for liquid food pasteurization by electroporation has to take into account sufficient electric field strength and treatment times (Zhang et al. 1995). For efficient use of irreversible electroporation in food industry, identification of optimal parameters is crucial (Angersbach et al. 2000, Lebovka et al. 2000, 2002). In some cases, irreversible electroporation is combined with other treatments for superior results. For example, inactivation of *Escherichia coli* was obtained by combination of electroporation and high-temperature treatment. For efficient liquid food sterilization, a apparatus was developed, which combines thermal, high pressure, and electric pulse treatment. The main advantage of the system is that it is not only effective for inactivation of vegetative cells but it efficiently eradicates even spores (Uemura and Isobe 2002). As yet, irreversible electroporation treatment alone is effective for inactivation of vegetative microorganisms; its effectiveness is not sufficient for the inactivation of their spores (Gould 1995).

Similarly, as for food preservation, the combination of irreversible electroporation and other established methods is used for freshwater treatment. Such a combination was applied for electroporation-assisted water chlorination, which was efficient for elimination of *Giardia muris* (Haas and Atrualiye 1999). Further, synergistic effect of electroporation and photodynamic treatment was reported. Such combined treatment reduced the time needed for efficient cell elimination as compared with photodynamic treatment alone (Wang et al. 1998; Zhou et al. 2000).

5.3 Electroporation of Bacteria and Yeast

Gene electrotransfer of bacteria provides an important methodology for the improvement of microorganisms used in food and pharmaceutical industry. Electroporation is used as an efficient transformation technique for gram-positive and gram-negative bacteria (Chassy et al. 1988; Dower et al. 1988; Fiedler and Wirth 1988; Tryfona and Bustard 2005). Mechanisms of gene electrotransfer were studied extensively, among which surface binding and diffusion through electropores, effective electric pulse parameters, and the effect of DNA topology on transformation efficiency were investigated (Xie et al. 1990; Xie and Tsong 1992; Xie et al. 1992).

The optimal temperature for bacterial gene electrotransfer depends on the strain used. For slow-growing mycobacteria, elevated temperatures markedly increases electrotransformation efficiency. On the contrary, for fast-growing strains the highest transformation is achieved at low temperatures (Wards and Collins 1996). Furthermore, different bacterial culture conditions were reported for optimal electrotransformation of *Corynobacterium*. In some species of Corynobacterium, cultivation at suboptimal temperature conditions and heat shock following electric pulse application significantly increased gene electrotransfer. The heat shock effect contributed to the inactivation of the restriction system present in bacteria, as it was observed only

with xenogenic DNA, where the restriction system inhibits DNA expression (Van der Rest et al. 1999). Optimization of technical conditions for gene electrotransfer in bacteria is crucial for successful use in industry (Kim et al. 2005; Mason et al. 2005). Among the most important factors for improved gene electrotransfer of bacteria is the disruption of the cell wall, which presents an obstacle for macromolecular uptake by the cell. Optimization of electric pulse parameters and the choice of the compatibility of foreign and endogenous plasmids is also required (Kim et al. 2005). Optimization of conditions for gene electrotransfer is not only species and strain specific, it also depends on the environmental conditions, from which bacteria was isolated (Mason et al. 2005).

The complexity of cell wall and cell shape of given bacterial strain determines the optimal parameters for efficient gene electrotransfer to bacteria. The optimal field strength is usually lower for gram-positive bacteria, rod-like bacilli, and cocci, and higher for gram-negative bacteria (Dower et al. 1992). Rod-like cells orient with the long axis in the direction of the electric field (Neumann 1992). Electroporation of rod-like bacteria was thus described as a multistep process in which orientation of the rod in the electric field plays an important role. When the rod is parallel to the electric field, the effective electroporation takes place at lower pulse amplitudes as compared to non-oriented one. The pulse duration must thus be sufficient for effective orientation and successful electroporation (Eynard et al. 1997; Eynard et al. 1998).

Similar to bacteria, the yeast species have a cell wall that interferes with the transport of molecules to the cell. Macromolecules are trapped in the yeast cell wall (Ganeva et al. 1995). At the same time the cell wall also presents a barrier for macromolecule release from the cell. Different yeasts species belonging to *Saccharocmyce* taceae family are used in biotechnology as a cell factory due to their ability to produce desired proteins (Meilhoc et al. 1990). When electroporation is used for macromolecular release, besides cell membrane alteration produced by electric pulses, the cell wall alterations were proposed as mechanism responsible for macromolecule release from the cell interior (Ganeva et al. 2003, 2004; Suga et al. 2007). This statement, however, is not in agreement with other authors who assume that the cell wall, at least in plant species, is not altered by electric pulse application (Joersbo and Brunstedt 1991).

5.4 Plant Protoplast Electroporation

Electroporation can be used as an efficient method for transfer of foreign genes into plant protoplasts of monocotyledons and dicotyledons (Fromm et al. 1985). In case of gene electrotransfer, the range of plants is not limited by pathogen host specificity as in the case of gene transfer by *Agrobacterium tumefaciens*. Besides, large amounts of protoplasts can be transformed at the same time (Saunders et al. 1989).

Gene electrotransfer of plant protoplasts was successfully applied for transformation of several crop species such as maize, rice, wheat, sorghum, soybean, and rye (Fromm et al. 1986; Lee et al. 1986; Christou et al. 1987; Pitt et al. 1997;

Quecini et al. 2002). Cell viability preservation is crucial for production of transgenic plants, as transformed protoplast should maintain the ability of normal organogenesis. Conflicting reports about electrotransfected plant protoplast regeneration ability are found in the literature. Some authors reported increased cell division, plant regeneration, and DNA synthesis in protoplast transformed by electroporation (Rech et al. 1987; Rech et al. 1988; Chand et al. 1988; Ochatt et al. 1988; Joersbo et al. 1991), while others found slower plant regeneration of electrotransfected protoplasts (Quecini et al. 2002). In some studies, increasing electric filed strength and the number of pulses decreased plant protoplast viability and plating efficiency. Nevertheless, the regeneration of plantlets was stimulated (Mordhorst and Lorz 1992).

Electroporation can be successfully used for production and extraction of plant metabolites from cell culture. Plany cell suspension cultures can be used for large-scale production of many plant secondary metabolites, such as different alkaloids (Kutney 1982; Yang and Bayraktar 2003, Ladygin 2004; Vanisree et al. 2004). One of the advantages of such production of secondary metabolites is that they are extractable form the cell culture. When plant cell culture is combined with efficient cell transfection methods, it can provide constant levels of desired metabolite production and therefore an important source for plany secondary metabolites (Vanisree et al. 2004). Electroporation is a suitable technique for such applications as it is applicable to different species and suitable for continuous production of desired product. It is important to note that cell viability and cell biosynthetic capabilities are not affected by the treatment when electroporation parameters are chosen properly (Yang et al. 2003).

Another important application of electroporation in plant protoplast is electrofusion that allows production of hybrid plant cells. As an effective field strength for cell fusion depends on the cell diameter, the amplitude needed for protoplast fusion is much lower than for animal or bacterial cells, as protoplast diameter is much larger than that of animal or bacterial cells. However, the method presents its limitations as hybrid cells obtained from electrofusion are mainly genetically instable and present multiple ploidity levels (Saunders et al. 1989).

5.5 Transfection of Intact Plant Tissue

The limitations related to electrotransfected protoplast regeneration are overcome by gene electrotransfer in the intact plant cells. Even though the cell wall represents a barrier, osmotic shock pretreatment that provokes plasmolysis can be used to create a passage of molecules through the cell wall (D'Halluin et al. 1992; Ganeva et al. 1995; Sabri et al. 1996a; Eynard et al. 1997; Wu and Feng 1999).

Reactive oxidative species are produced in response to oxidative stress in mammalian and plant cells exposed to electric pulses (Biedinger et al. 1990; Gabriel and Teissié 1995a, Maccarrone et al. 1995; Sabri et al. 1996b, 1998). Even if the cell viability is not directly correlated with reactive oxidative species production, gene

electrotransfer efficiency is improved by post-pulse treatment with antioxidants, which protect the cell from reactive oxidative species (Sabri et al. 1996; Sabri et al. 1998).

Electroporation is an alternative method for plant transformation. It is, however, still not widely used due to its low efficiency. Although it was effective in some species, such as maize (D'Halluin et al. 1992), a much lower efficiency was obtained in other species, such as wheat (Walden and Wingender 1995; Rakoczy-Trojanowska 2002). In some cases, gene electrotransfer in wheat was successful and electrotransfected explants were able to regenerate plants via somatic embryogenesis; however, the transformation was transient (He and Lazzeri 1998). The production of fertile transgenic wheat plants via tissue electroporation still depends on the quality of plant material used (Sorokin et al. 2000). The stable electrotransformation procedure as an alternative method for Triticae family crop species (wheat) transformation is still in development. Fully fertile plants that expressed transgenes and transmitted them to progenity were obtained from tritordeum, fertile amphiploid derived from durum wheat and wild barley, by tissue electroporation (He et al. 2001). Barley transfected by tissue electroporation resulted in stable genetic transformation (Gurel and Gozukirmizi 2000).

In brief, in this section different applications of electroporation were described. The method is successfully used in medicine in clinical practice as electrochemotherapy. Preclinical trials for gene electrotransfer are progressing and irreversible electroporation has a potential as a new surgical method for tissue ablation. Besides, irreversible electroporation is used for water sterilization and food preservation. In biotechnology gene electrotransfer is successfully used for improvement of microorganisms used in food and pharmaceutical industry. Gene electrotransfer is also used as efficient tool for manipulation of yeast cells and their ability to produce desired proteins. On the other hand, plant protoplast gene electrotransfer and electrofusion is used to obtain transgenic plants while plant cell cultures serve as bioreactors to produce desired secondary metabolites of economical interest. For production of transgenic plants, limitations associated to electroporated/fused protoplast regeneration are overcome by gene electrotransfer into intact plant tissue. The method has already been used successfully for some economically important species while for others the transfection efficiency and transformation stability is still not sufficient for wider use and needs further improvements.

6 Understanding Electroporation of Different Cell Types

Electroporation can be successfully used for different cell types although they differ in their electroporation behavior. While part of the differences can be attributed to the differences in cell size and shape, already mentioned before, some differences are related to biological characteristics of the treated cell (O'Hare et al. 1989; Rols and Teissié 1992a; Rouan et al. 1991; Čemaar et al. 1998; Čegovnik and Novaković 2004; Kandušer et al. 2006). Among such biological factors that affect

cell membrane electroporation are membrane fluidity, cell cytoskeleton, and cell wall in bacteria, yeast, and plant cells.

6.1 Influence of Cell Membrane Fluidity

Cell membrane fluidity is a physical characteristic of biological membrane that changes with membrane composition and temperature. The content of cholesterol and the ratio between saturated and unsaturated fatty acids that are part of the membrane lipids determine cell membrane fluidity. It can be altered by chemical compounds that integrate into the membrane bilayer or by rapid temperature changes. On the other hand, slow environmental temperature changes cause changes in membrane composition in bacteria, yeast, and plant cells, as these organisms regulate their membrane fluidity in response to environmental factors.

It was reported that membrane fluidity affects the electroporation response of a cell exposed to electric pulses. Two conflicting findings on membrane fluidity effect on electroporation were reported. On the one hand, at physiological temperature less fluid membranes are permeabilized at lower voltages than the more fluid ones (Rols et al. 1990c; Kandušer et al. 2006). On the other hand, the effect of cell membrane fluidity on electroporation is just the opposite when membrane fluidity is altered by chilling. Different responses are found in different cell types. Low temperature had almost no effect on erythrocyte electroporation (Kinosita and Tsong 1979). In alga *Valonia*, rye leaf protoplast, porcine stratum corneum, and in our recent study on mammalian cell lines, exposure of cells to low temperature has as a consequent increase in a voltage required for successful electroporation (Coster and Zimmermann 1975; Pitt et al. 1997; Gallo et al. 2002; Kandušer, Šentjurc, Miklavčič, 2008). These temperature effects on electroporation were attributed to the lipid fluidity change produced by lower temperature (Gallo et al. 2002). Probably more than overall lipid fluidity changes, the membrane domain structure is responsible for the observed differences in electroporation behavior. Besides, the temperature probably affects electroporation by other means not only by cell membrane fluidity alterations.

Membrane fluidity is probably also involved in cell membrane electrofusion. It was reported that the membrane fluidity could be an important factor affecting molecular rearrangements in the electroporated cell membrane responsible for the cell fusion (Dimitrov and Sowers 1990). Moreover, in biological membrane fusion, the process depends on properties of the membrane lipid bilayer. It was shown that biological fusion is altered by changes in membrane lipid composition (Chernomordik et al. 1995). In addition, in electrofusion the presence of anesthetic agents or polylysine, substances that affect cell membrane fluidity, also affect cell fusion (Grobner et al. 1996; Velizarov et al. 1998b). It was also reported that in bacteria different temperature and culture conditions that affect membrane lipid composition and fluidity affect efficiency of gene electrotransfer (Wards and Collins 1996; Van der Rest et al. 1999). The effect of membrane fluidity on efficiency of gene electrotransfer was also observed in plant cells (Wu and Feng 1999).

6.2 Influence of Cell Cytoskeleton

The cell cytoskeleton is a very dynamic structure, which is composed of actin filaments, microtubules, and intermediate filaments. It is responsible for cell shape maintenance and mobility (Janmey 1995). As the cell cytoskeleton interacts with cell membranes, it is expected that it also affects cell membrane electroporation.

Tubulin, which is a main component of microtubules, was found to play an important role in electroporation and electrofusion (Blangero et al. 1989; Rols and Teissié 1992a; Teissié et al. 1994; Kanthou et al. 2006). The experiments in which cell cytoskeleton was disrupted by chemical agents showed that the first two phases of electroporation, pore formation and expansion, are not affected by cytoskeleton integrity. On the contrary, the third phase of the electroporation process, cell membrane resealing is dramatically affected. In cells with disrupted cytoskeleton, cell membrane resealing is significantly faster than in intact cells. Similar results were obtained when erythrocytes cytoskeleton was disrupted by heat treatment or when cells in the phase of mitosis, when tubulin cytoskeleton is rearranged in mitotic spindle were electroporated (Rols and Teissié. 1992a; Teissié et al. 1994).

The effect of electroporation on cell cytoskeleton was studied in different cell types, and its disorganization was observed during cell electrofusion (Blangero et al. 1989; Wu and Feng 1999; Rols and Teissié 1992a; Harkin and Hay 1996; Teissié et al. 1998; Kanthou et al. 2006). In some cases, tubulin and vimentin intermediate filaments disruption was dependent on the composition of electroporation media. In media with similar ionic composition as cytoplasm, the cell cytoskeleton disruption was prevented (Harkin and Hay 1996). Disruption of cell cytoskeleton is observed immediately after electroporation and recovery took place in 1 hour after the pulse application. Although electroporation interferes with the organization cytoskeleton filaments, it does not result in degradation of cytoskeletal proteins (Kanthou et al. 2006).

6.3 Influence of Cell Wall in Bacteria, Yeast, and Plants

The cell wall chemical composition varies from bacteria, to yeast, to plant cells. The bacterial cell wall is composed of cross-lined peptidoglycans and polysaccharides; nevertheless, its composition varies in different types of bacteria. Cell walls of bacteria present additional surface structures such as capsules, slimes, S layers, and sheals (Beveridge and Graham 1991; Schaffer and Messner 2005). In yeast species, the chemical composition of cell walls varies with species and is composed, in case of *Saccharomyces cerevisiae*, of glucan, manoprotein, and chitin. The main component is glucan that forms a microfibrilar matrix to which other components are bound (Mazan et al. 2006). In plant species the primary cell wall is composed of cross-linked pectines and hemicellulose molecules. The free spaces among molecules that constitute the cell wall are species and tissue specific, ranging from 3.5 to 5.2 nm (Carpita et al. 1979). The cell wall structure is permeable to

small molecules but represents a barrier to large molecules such as DNA or proteins (Wu and Feng 1999).

Regardless of the cell wall chemical composition, it presents a barrier to electrofusion and is a limiting factor for gene electrotransfer. Nevertheless, the cell wall does not affect transport of small molecules. The cell wall does not interfere with electric pulses, which cause electroporation of cell membrane, as only slight differences were obtained when the electroporation of plant protoplast and intact plant cells was compared (Saunders et al. 1995). Small molecules can freely diffuse through the cell wall; therefore, their loading into cytoplasm was not affected significantly by the presence of the cell wall in bacteria, yeasts, or plants. From those results, it was concluded that electroporation of cell membrane on itself is not affected by the presence of the cell wall (Ganeva et al. 1995; Aouida et al. 2003; Sauders et al. 1995).

On the other hand, when large molecules need to be introduced into the cell, such as DNA, for gene electrotransfer of bacteria, transfection efficiency is improved when the cell wall is partially disrupted by chemical agents (Ganeva et al. 1995). It was also reported that electrotransformation of the gram-positive bacteria is less effective than in gram-negative bacteria due to the thicker and denser cell walls in gram-positive species (Dower et al. 1992; Trevors et al. 1992, Kim et al. 2005). A similar situation occurs in yeast species, where a cell wall represents a barrier for introduction of macromolecules into the cell. Observation of fluorescent 70 kDa dextranes during electroporation of yeast revealed that those macromolecules are trapped in the wall. The presence of macromolecules at the cell membrane level was thus reduced and consequently their loading into the cytoplasm was smaller than it would be in a cell without a cell wall (Ganeva et al. 1995). To improve the transport of macromolecules through the cell wall of bacteria, yeast, and plant cells, different pretreatments were suggested. Before electroporation, partial disruption of the cell wall was effective for bacteria and yeast, while for plant cells, pre-pulse plasmolysis was successfully applied (D'Halluin et al. 1992; Ganeva et al. 1995; Sabri et al. 1996a; Eynard et al. 1997; Wu and Feng 1999).

In brief, in this section characteristics of different cell types on electroporation effectiveness are described. Biological characteristics of treated cells such as membrane fluidity, integrity of cytoskeleton, and presence of cell wall in bacteria, yeast, and plant cells affect electroporation. These characteristics need to be taken into account when optimizing electroporation parameters. Besides, for improved loading of macromolecules into the cells with the cell wall, pretreatments that partly disrupt cell wall or cause plasmolysis can be successfully used.

7 Conclusions

Electroporation is a useful technique in biotechnology and medicine for introduction of different molecules, electrofusion, or water sterilization and food preservation. Among the different theoretical models that describe electroporation, the transient aqueous pore model is the most widely accepted. This model predicts hydrophilic

pore formation that takes place in a cell membrane as a response to an induced electric field. Electroporation can be reversible or irreversible, depending on the electric pulse parameters used. The effectiveness of electroporation is determined by electric pulse parameters, electroporation medium composition, and its osmotic pressure. Among the electric pulse parameters, pulse amplitude, duration, number, and repetition frequency are most important. Pulse amplitude is a critical parameter as, when it reaches threshold value, it triggers the electroporation process. When electric pulse parameters exceed their optimal values, cell viability is affected and irreversible electroporation takes place. For introduction of small and large molecules, different electric pulse parameters need to be used. Small molecules are efficiently introduced into the cell by application of short electric pulses in range of tens to hundreds of microseconds. The transport of small molecules takes place predominately after the pulse by diffusion. On the other hand, for macromolecules, long 5 to 10 μs pulses with relatively low pulse amplitudes are used. In addition, for successful gene electrotransfer, DNA has to be present in the medium before electric pulses are applied, while small molecules can enter the cell even if added after the pulse is applied. Electric pulse parameters for cell electrofusion are similar to those used for introduction of small molecules, but the threshold voltage required is higher. For irreversible electroporation that is used for inactivation of microorganisms, the electric pulse parameters should exceed critical value as cell death is the desired result of such applications. Electroporation medium composition and its osmolarity affect electroporation and related gene electrotransfer and electrofusion.

The basic mechanisms of electroporation were mainly studied at the single-cell level, although the situation is more complex in a tissue. The tissue is composed of cells that are in close contact with each other and their proximity affects electroporation. In addition, most tissues are not homogenous structures. They are composed of different cell types that are irregularly shaped and have different electrical properties that affect current density and electric field distribution and consequently also electroporation effectiveness. The mathematical models are thus a valuable tool for prediction of electroporation behavior of the tissue. In addition, the electrode type, shape, and positioning affect electroporation effectiveness. The choice of proper electrode type, shape, and positioning is crucial for successful treatment, as it affects the electric field distribution and depends on the application.

Electroporation has many different applications; the method is successfully used in medicine in clinical practice as electrochemotherapy. Preclinical trials for gene electrotransfer are in progress, and recently irreversible electroporation was suggested as a new surgical method for tissue ablation. In addition, irreversible electroporation is used for water sterilization and food preservation. In biotechnology, gene electrotransfer is successfully used for improvement of microorganisms used in food and pharmaceutical industry and for plant cell cultures that produce secondary metabolites. On the other hand, gene electrotransfer in plant protoplast or protoplast electrofusion is used to obtain transgenic plants. Limitations found in protoplast regeneration are overcome by gene electrotransfer into intact plant tissue. The method is successfully used for some important crop species, while for others the

transfection efficiency and transformation stability is still not sufficient for wider use and needs further improvements.

Although electroporation is used in a wide range of different cell types, biological characteristics of the treated cell, such as membrane fluidity, integrity of cytoskeleton, and presence of cell wall in bacteria, yeast, and plant cells, affect its efficiency. Specific characteristics of different cells need to be taken into account when optimizing electroporation parameters. The cell wall that presents a barrier to large molecules loading into the cell can be partly disrupt or cell can be plasmolysed. Such pretreatment improves electroporation effectiveness.

It can be concluded that electroporation can be efficiently used for different applications in biotechnology and medicine if proper conditions are chosen and characteristics of the treated sample are taken into account.

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