Eur Biophys J (2006) 35: 196–204
DOI 10.1007/s00249-005-0021-y

ARTICLE

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Cell membrane fluidity related to electroporation and resealing

Received: 20 April 2005 / Revised: 7 July 2005 / Accepted: 10 September 2005 / Published online: 8 October 2005

Abstract In this paper, we report the results of a systematic attempt to relate the intrinsic plasma membrane fluidity of three different cell lines to their electroporation behaviour, which consists of reversible and irreversible electroporation. Apart from electroporation behaviour of given cell lines the time course required for membrane resealing was determined in order to distinguish the effect of resealing time from the cell's ability to survive given electric pulse parameters. Reversible, irreversible electroporation and membrane resealing were then related to cell membrane fluidity as determined by electron paramagnetic resonance spectroscopy and computer characterization of membrane domains. We found that cell membrane fluidity does not have significant effect on reversible electroporation although there is a tendency for the voltage required for reversible electroporation to increase with increased membrane fluidity. Cell membrane fluidity, however, may affect irreversible electroporation. Nevertheless, this effect, if present, is masked with different time courses of membrane resealing found for the different cell lines studied. The time course of cell membrane resealing itself could be related to the cell's ability to survive.

Keywords Electroporation · Fluidity · Order parameter · Membrane domain · In vitro · V-79 · DC-3F · B16-F1

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Introduction

Electroporation is a technique widely used in biotechnology and medicine for the delivery of drugs and genes into living cells (Neumann et al. 1982; Fromm et al. 1986; Mir 2000; Ferber 2001; Gehl 2003; Serša et al. 2003). The reversibility of the process was reported as an important characteristic essential especially in gene delivery. For successful electroporation, the external electric field should induce the electric potential difference across the cell membrane, to reach the critical value in order to permeabilise the membrane. At the same time, electric potential on the membrane should not exceed the value at which membrane rupture takes place which results in cell death (Neumann 1989). Although the exact molecular mechanisms are not clearly elucidated, it is widely accepted that electroporation takes place in the lipid bilayer of the plasma membrane (Chernomordik et al. 1987). The process of electroporation consists of different phases: pore formation as a response to induced super threshold membrane voltage lasting a few microseconds, followed by time dependent expansion of the pore size in time range of 100 μs, and pore resealing in the minute range (Kinoshita and Tsong 1979; Neumann et al. 1999, Leontiadou et al. 2004). Molecular transport takes place in the period after pulse application until the resealing of the cell membrane that is in the millisecond to minute range (Gabriel and Teissié 1997, 1999; Puc et al. 2003).

Reversibility of electroporation is governed by parameters of external electric field magnitude, duration, characteristics of the electroporation medium, and of the cell that is exposed to the electric field. In case of the external electric field it was shown that pulse duration, number, amplitude and repetition frequency should be chosen properly for the reversibility of the process (Rols and Teissié 1990a; Vernhes et al. 1999; Canatella et al. 2001). If those parameters exceed their optimal values, irreversible electroporation takes place. Cell death is caused by severe damage of the DNA

(Meaking et al. 1995) and irreversible loss of membrane function as a semi permeable barrier, that causes cell lyses (Hamilton and Sale 1967; Danfelter et al. 1998).

Concerning the characteristics of the electroporation medium, it was also shown that electroporation depends on the composition of the medium (Rols and Teissié 1989; Djuzenova et al. 1996; Pucihar et al. 2001) and its osmolarity (Rols and Teissié 1990b; Golzio et al. 1998). Conductivity of the electroporation medium, and the electrical and geometrical properties of the cell affect induced electric potential on the cell membrane and therefore electroporation (Kotnik et al. 1997; Valič et al. 2003).

With respect to the biological characteristics of the cell, it was shown that different cell lines differ in their response to the same parameters of the external electric field (O'Hare et al. 1989; Neumann 1992; Čemažar et al. 1998). Part of those differences is due to the cell size and shape and the geometrical properties of the cell, as mentioned before. In a given cell population large cells are more sensitive to given electric field strengths than small ones (Sale and Hamilton 1967; Zimmermann 1982; Gaškova et al. 1996; Teissié et al. 1999). Nevertheless, cell size is not the only characteristic of the cell that determines different response to given parameters of the electric field (O'Hare et al. 1989; Čemažar et al. 1998).

Little is known, however, about the effect of cell membrane properties on electroporation. In the study of Rols et al. it was shown that substances like ethanol and lysolecitin when incorporated into the cell membrane, change its fluidity and affect cell electroporation behaviour (Rols et al. 1990).

As the addition of chemical compounds that incorporate into the membrane could change its characteristics and electroporation behaviour (Kandušer et al. 2003), we decided to use a different approach which avoids addition of chemical compounds. The aim of our study was to investigate the effect of intrinsic cell membrane fluidity of three different cell lines on electroporation behaviour. Electroporation behaviour was defined as the reversible and irreversible electroporation of a given cell line and its cell membrane resealing after the application of the electric pulses.

Materials and methods

Cell culture

Three cell lines, V-79 Chinese hamster lung fibroblasts, DC-3F transformed Chinese hamster lung fibroblasts and B16-F1 murine melanoma, were used in the experiments. All cell lines were grown in an Eagle's minimum essential medium supplemented with 10% foetal bovine serum (Sigma–Aldrich Chemie GmbH, Deisenhofen, Germany) at 37°C in a humidified 5% CO_2 atmosphere in an incubator (WTB Binder, Labortechnik GmbH, Germany). For all experiments cell suspension was prepared from confluent cultures by 0.25% trypsin/EDTA solution (Sigma–Aldrich Chemie GmbH). From the obtained cell suspension, trypsin and growth medium were removed by centrifugation at 1000 *g* (180 *g*) at 4°C for 5 min (Sigma, Germany) and the cell pellet was resuspended in the electroporation medium. As an electroporation medium, Spinner's modification of Eagle's minimum essential medium without calcium was used with pH 7.4, osmolarity 300 mosm/kg and conductivity 1.6 S/m (Life Technologies Ltd., Paisley, UK).

Electroporation

Cell suspension was kept on ice for approximately 5–10 min before the application of electric pulses. A 50- µl drop that contained 10^6 cells and 50 nM bleomycin was placed between two parallel plate stainless steel electrodes spaced 2 mm apart. The amplitude of the applied pulses was from 80 to 400 V in increments of 40 V. For each of the pulse amplitudes, a train of eight rectangular pulses with duration of 100 µs and repetition frequency 1 Hz was applied, and a control consisting of cells that were not exposed to the electric field was prepared. For pulse application, a prototype electric pulse generator described in detail in Puc et al. (2001), developed at the University of Ljubljana, the Faculty of Electrical Engineering, Slovenia were used. After the pulse application all cells were incubated at room temperature (20–25°C) for 30 min to allow for membrane resealing. Eagle's minimum essential medium supplemented with 10% foetal bovine serum was added when the cell membrane was resealed and cells were plated in a concentration of 200 cells per Petri dish for clonogenic assay. Colonies were grown for 5 days in the same conditions described previously for cell cultures. After 5 days, colonies were fixed with methanol (Merck KGaA, Darmstadt, Germany) and stained with crystal violet (Sigma–Aldrich Chemie GmbH). Visible colonies were counted and results normalised to control. The percentage of colonies that survived was subtracted from 100% to obtain the percentage of bleomycin uptake. At least three experiments were pooled together for each data point presented in the Results section.

Reversible electroporation was determined by uptake of a cytostatic drug, bleomycin. When electroporation takes place, bleomycin penetrates into the cell and induces cell death. The method was described in detail by Kotnik et al. (2000), Mir et al. (1996) and Tounekti et al. (2001).

Irreversible electroporation was determined as described above for reversible electroporation. Cells in suspension were exposed to applied electric pulses in the medium without the addition of bleomycin. Results were expressed as a percentage of cell survival.

Differences in electroporation behaviour of three different cell lines were tested by ANOVA test and the differences of the mean values among cell lines were compared with all pair wise multiple comparison procedures (Tukey test).

Membrane resealing was determined for cell lines V-79 and B16-F1 that differed the most in their electroporation behaviour and membrane fluidity by the uptake of a cytostatic drug, bleomycin. In the control treatment bleomycin was added before the pulse application while in the resealing treatments it was added at different time intervals after pulse application. The time intervals after pulse application were as follows: 1–5, 10, 15, 20, 25 and 30 min. The voltage applied was selected for each cell line according to the voltages where we obtained 50% of membranes being permeabilised. This was 147 V for cell line V-79 and 107 V for cell line B16-F1 for the 2 mm distance between the electrodes. The train of eight rectangular pulses with a pulse duration of 100 μs and a frequency of 1 Hz was applied. Cells were incubated for 30 min at room temperature and plated for clonogenic assay. Five independent experiments were performed for each cell line. Data are presented as percentage of cells with the resealed membrane after the pulse application.

The average cell size (cell diameter) of non-electroporated cells in the suspension was determined for each cell line. In three independent experiments 90–130 cells were measured for each experiment and cell line giving a total of 270–390 cells measured per each cell line. Measurements of cell size were performed on living cells in suspension under an inverted microscope (Olympus CK40, Germany), with the objective magnification of 40 times. Images were acquired by digital camera and processed with Olympus DP software. For each cell line, data from three experiments were pooled together and the differences in the mean values among cell lines were compared with all pair wise multiple comparison procedures (Tukey test), as samples followed normal distribution.

Maximal induced transmembrane voltage was calculated for all cell lines based on the equation:

$$U_{TI} = -1.5rE\cos\varphi \quad (1)$$

(Cole 1972; Marszalek et al. 1990; Kotnik et al. 1997), where U_{TI} is the maximal induced transmembrane voltage, r is the radius of the cell, E is the strength of the applied electric field estimated as voltage to distance ratio (U/d) and ϕ is the angle between the direction of the electric field and the selected point on the cell surface.

Cell membrane fluidity

Cell membrane fluidity was measured by an electron paramagnetic resonance (EPR) method, with X-band EPR spectrometer (Bruker ESP 300), using the spin probe methyl ester of 5-doxylpalmitate [MeFASL(10.3)], which is lipophilic and therefore dissolves in the membrane phospholipid bilayer. Since the nitroxide groups of the spin probes inside the cell are reduced by the oxy–redoxy systems to the EPR invisible hydroxyl amines (Chen and Morse 1988), it is believed that the main contribution to the EPR spectrum is from the spin probes in the plasma membrane. Other evidence confirming the proposed location of the spin probes are given in Curtain and Gordon (1984).

Cell suspension (1 ml) in electroporation medium (the same as used and described for electroporation experiments) that contained 20×10^6 cells was incubated for 15 min at room temperature with constant shaking of the spin probe that was prepared as a thin film on the wall of a glass tube (from 60 μl of 0.1 mM ethanol solution). Cell pellets obtained by centrifugation at 1000 g at room temperature was put in glass capillary and was measured with EPR spectrometer at 25°C. Each measurement of the cell pellet was repeated three times and three independent experiments were performed.

From the line shape of the EPR spectra information on the ordering and dynamics of the spin probe, which reflect the motional characteristics of its surroundings, were derived.

As a rough estimation of membrane fluidity we calculated overall empirical correlation time (τ_{emp}) that reflects the average dynamics of motion of the spin probe in the lipid bilayer, using the equation:

$$t_{emp} = K\Delta H_0\left[(h_0/h_{-1})^{1/2} - 1\right], \quad (2)$$

where h_{-1} and h_0 are high and middle field amplitudes of the EPR spectra, ΔH_0 is the line width of the middle field line (Fig. 1) and K is a constant typical for the spin probe used (Marsh 1981). This expression is valid only for fast isotropic motion in solution and can be used in the membrane only to compare relative differences in the spin probe dynamics between different cell lines and different treatments (Marsh 1981). The empirical correlation time (τ_{emp}) is inversely proportional to membrane fluidity.

Average membrane fluidity is described with motional characteristics of the membrane phospholipid alkyl chains and is inversely proportional to average membrane viscosity (Šentjurc et al. 2002). However, since the membrane is heterogeneous, composed of the regions with different motional characteristics, the

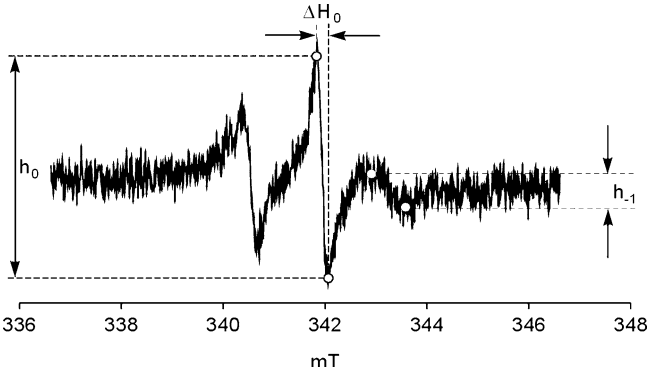

Fig. 1 Typical EPR spectra of MeFASL(10.3) in the membrane of cells at 25°C from which h_0, ΔH_0, and h_{-1} were calculated

fluidity in different regions is different. The regions that have one or more measurable properties that distinguish it from neighbouring regions of the membrane are defined as domains (Bloom and Thewalt 1995).

For a more precise description of complex membrane characteristics, including its lateral heterogeneity, a computer simulation of the EPR spectra line-shape was performed. The model used (Štrancar et al. 2000) takes into account the fact that that the membrane is heterogeneous and composed of domains with different fluidity characteristics. The experimental EPR spectrum is a superposition of spectral components that correspond to the spin probes in the membrane domain types with different fluidity characteristics. Distinct domain types are characterized with sets of spectral parameters, which describe different modes of spin probe motion. The parameters, which describe fluidity characteristics of membrane domains are the order parameter (S) and rotational correlation time (τ_c). The order parameter describes the ordering of lipid hydrocarbon chains, can vary from 1 to 0, and is inversely proportional to the fluidity of certain membrane domain types, while the rotational correlation time describes the dynamics of the spin probe motion in the domain and is proportional to the fluidity. Further, the relative proportion of each spectral component (d) is determined. It describes the relative amount of the spin probe with particular motional characteristics and depends on the distribution of the spin probe between the domain types and on the distribution and position of the spin probe within the domain. It should be stressed that the lateral motion of the spin probe is slow on the time scale of EPR spectra. Therefore, an EPR spectrum describes only the properties of a spin probe's nearest surrounding (i.e. of the range of some nm). The tuning of EPR spectral parameters to obtain the best fit of the calculated spectrum to the experimental spectrum was performed by the program EPRsim 2.6 (Filipić and Štrancar 2001), and is implemented in the software package EPRsim (http://www.ijs.si/ijs/dept/epr/).

From the best fit of the calculated to the experimental spectrum we derived the order parameter, rotational correlation time and the relative proportion of the spin probes in the coexisting domain types. A good fit of the calculated and experimental spectrum was obtained only if three domains were used.

Results

Cell electroporation behaviour

In the present work, we use the term reversible electroporation to refer to the phenomenon where transient cell membrane permeabilization for exogenous molecules is observed. Reversible electroporation with respect to the applied voltage of the electric pulses is presented in Fig. 2a. The dependence of membrane permeabilization on voltages of the applied electric pulses of cell lines

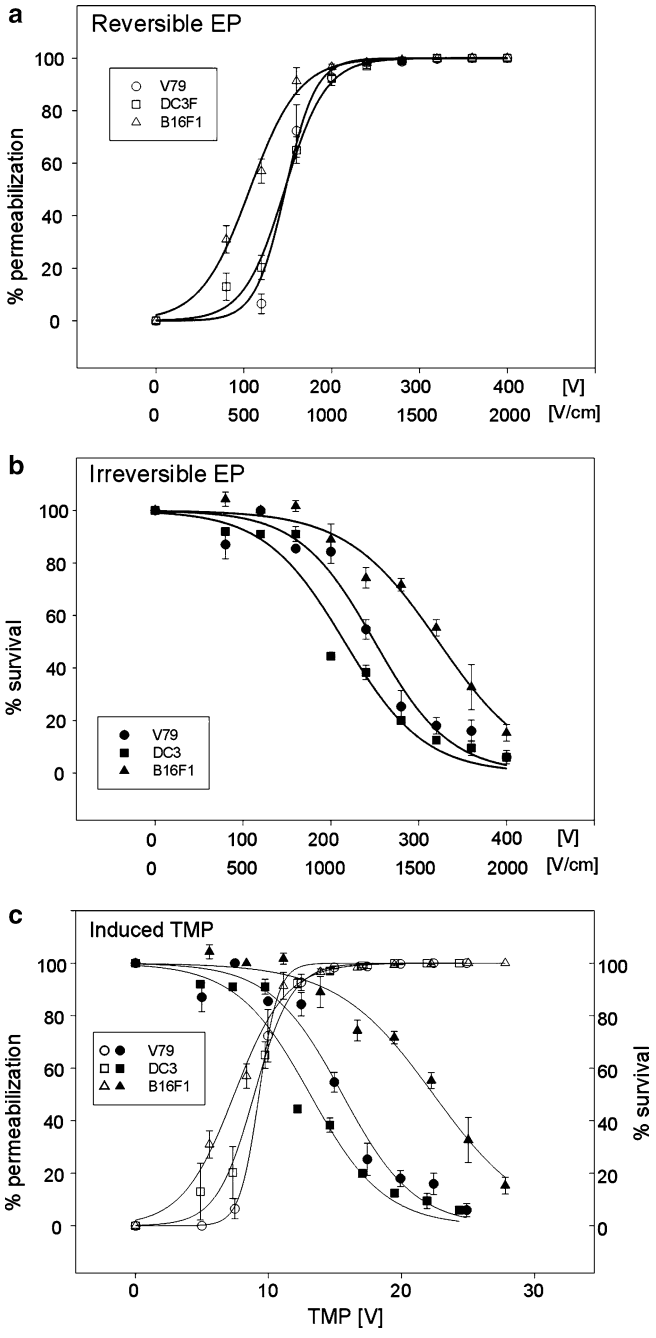

Fig. 2 Reversible electroporation measured by bleomycin uptake (**a**) irreversible electroporation (**b**) and maximal induced transmembrane voltage (**c**) of cell lines V-79, DC-3F and B16-F1. The train of eight pulses, 100 µs and repetition frequency 1 Hz was applied. The voltage applied to 2 mm electrodes increased gradually from 0 to 400 V. Values are means of three independent experiments ± standard error. Two parameters sigmoid curve was fitted to experimental data. *Open symbols* permeabilization, *closed symbols* survival.(*filled circle, open circle*)V-79, (*filled square, open square*)DC-3F, (*filled triangle, open triangle*)B16-F1

DC-3F and V-79 is very similar, while B16-F1 is permeabilised at lower voltage ($P < 0.05$).

The term irreversible electroporation is used for the phenomenon where cell death due to irreversible cell

membrane permeabilization is observed. Irreversible electroporation dependence on the applied voltage of electric pulses is presented in Fig. 2b. The course of cell survival shows that B16-F1 is able to sustain higher values of the applied external electric field than DC-3F and V-79 ($P < 0.01$).

There was no significant difference in cell diameter between V-79 16.5 ± 0.2 μm and DC-3F 16.2 ± 0.2 μm, but both are smaller than B16-F1 18.5 ± 0.16 ($P < 0.001$). Even though the difference in the cell size is only 2 μm it represents a 110–140 mV difference in induced transmembrane voltage. When we calculate the maximal induced transmembrane voltage for our cell lines (Eq. 1), the differences in reversible electroporation among them diminish (Fig. 2c). For irreversible electroporation the calculated maximal induced transmembrane voltage is significantly higher ($P < 0.001$) for the largest cell line (B16-F1) than for the two smaller ones (V-79 and DC-3F) (Fig. 2c).

Membrane resealing was determined for cell lines V-79 and B16-F1 that differed the most in their electroporation behaviour and membrane fluidity by uptake of a cytostatic drug, bleomycin. Cell membrane resealing of cell lines V-79 and B16-F1 is presented in Fig. 3. Cells were permeabilised at voltages where we observe 50% of permeabilization in order to avoid severe membrane damage. Cell line B16-F1 was permeabilised at 107 V, while cell line V-79 was permeabilised at 147 V. In the first 4 min after electric pulse application there is no significant differences in membrane resealing between the two cell lines, nevertheless, the differences appear later on. Membrane resealing in cell line B16-F1 is completed in 50% of the cells in a population 5 min after pulse application, while in cell line V-79 it takes more than 20 min to reach the same value. Even though all the cells in the population reseal within 30 min in both cell lines, the differences between the resealing of the two cell lines are more than 15 min, which can have an important effect on cell survival (Fig. 2b).

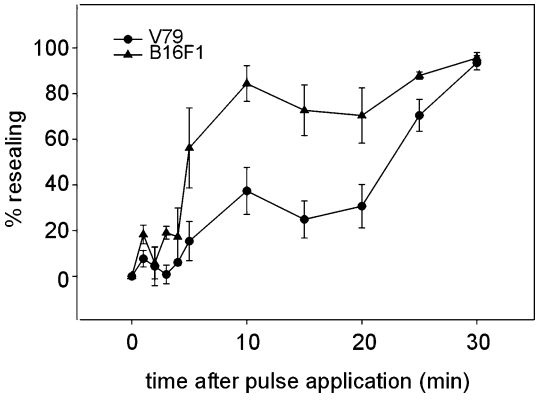

Fig. 3 Membrane resealing after pulse application for cell lines V-79 and B16-F1. The train of eight pulses, 100 μs and repetition frequency 1 Hz was applied. The voltage applied to 2 mm electrodes was 147 V for V-79 and 107 V for B16-F1. Values are means of five independent experiments ± standard error

Cell membrane fluidity

The empirical rotational correlation time (τ_{emp}) at 25°C, which is related to the overall cell membrane fluidity, was 2.5 ± 0.4 ns for V-79, 2.8 ± 0.2 ns for DC-3F and 3.0 ± 0.4 ns for B16-F1 (Table 1). The differences show a tendency towards decreased membrane fluidity from the cell line V-79 to the cell line B16-F1.

More detailed information about the cell membrane characteristics in different cell lines was obtained by computer simulation of the experimental EPR spectra. Good agreement between the calculated and experimental spectrum was obtained only if we took into account the fact that the spectrum is composed of three spectral components with different modes of spin probe motion (Fig. 4). Each spectral component corresponds to the spin probes in the domains, which exhibit equal fluidity characteristics, defined with order parameter and correlation time. According to our results the cell membrane is composed of at least three different types of domains with different fluidity characteristics (Table 1). For the cell lines V-79 and DC-3F no significant differences are observed between the order parameters and correlation times; only the portion of the less ordered domain (domain 1) is slightly larger for V-79 cell lines. A larger proportion of less ordered domains (i.e. the most fluid domains) is reflected also in larger average membrane fluidity, which is expressed with slightly larger empirical correlation time (τ_{emp}) for V-79 cell lines in comparison to the other two cell lines. The differences are more pronounced with respect to the cell line B16-F1. Significant difference is observed in the proportion of domain 2, which is 0.24 for DC-3F cells and 0.3 for B16-F1 cells, while the proportion of the less ordered domain (domain 1) is lower (Table 1). At the same time, the order parameter of the less ordered domain is slightly higher. As the less ordered domains correspond to the most fluid regions in the membrane, the lowest portion as well as the highest order parameter of this domain in the B16-F1 cell line means that the overall fluidity of B16-F1 membrane is larger as compared to the membranes of V-79 or DC-3F cell lines. This is as expressed also in the largest τ_{emp} for this cell line.

Discussion

The aim of our study was to investigate the effect of intrinsic cell membrane fluidity of three different cell lines on electroporation behaviour. Electroporation behaviour was defined as the reversible and irreversible electroporation of a given cell line and its cell membrane resealing after the application of electric pulses.

To establish the relation of intrinsic cell membrane fluidity with electroporation behaviour of cell lines, induced transmembrane voltage was taken into account as cell size determines the maximal induced transmembrane voltage (Sale and Hamilton 1967; Zimmermann 1982; Teissié et al. 1999). Cell membrane fluidity was measured

Table 1 Fluidity characteristics of membrane domains 1–3 of three different cell lines (V-79, DC-3F and B16-F1) (determined with order parameter S, rotational correlation time τ_c, and proportion of the coexisting domain types d, obtained as the best fit of the calculated to the experimental spectra of the lipophilic spin probe MeFASL(10.3) dissolved in the membrane of the cell lines at 25°C

	Domain 1	Domain 2	Domain 3
V-79 $\tau_{emp} = 2.5 \pm 0.4$ ns			
Order parameter S	0.11 ± 0.01	0.33 ± 0.01	0.60 ± 0.01
Rot. corr. time τ_c (ns)	1.8 ± 0.1	1.1 ± 0.1	0.31 ± 0.07
Proportion d	0.27 ± 0.01	0.22 ± 0.03	0.51 ± 0.03
DC-3F $\tau_{emp} = 2.8 \pm 0.2$ ns			
Order parameter S	0.11 ± 0.01	0.32 ± 0.01	0.60 ± 0.01
Rot. corr. time τ_c (ns)	1.9 ± 0.1	1.3 ± 0.1	0.28 ± 0.04
Proportion d	0.24 ± 0.01	0.24 ± 0.02	0.52 ± 0.02
B16-F1 $\tau_{emp} = 3.0 \pm 0.4$ ns			
Order parameter S	0.13 ± 0.01	0.33 ± 0.01	0.61 ± 0.08
Rot. corr. time τ_c (ns)	1.8 ± 0.1	1.3 ± 0.1	0.20 ± 0.06
Proportion d	0.20 ± 0.01	0.30 ± 0.03	0.50 ± 0.03

The error of each calculated parameter is estimated through covariance matrix analysis (Filipič et al. 2001). The corresponding empirical correlation times are derived directly from the experimental spectra and are related to the overall membrane fluidity (mean values of three experiments ± standard deviation)

by the EPR method using the spin probe methyl ester of 5-doxylpalmitate which dissolves in the membrane phospholipid bilayer. In our study three different cell lines were chosen that could be cultured under identical conditions. Results revealed that for the largest cell line B16-F1, reversible electroporation took place at a lower voltage than for the smallest two, V-79 and DC-3F (Fig. 2a), which is in agreement with the theory (Eq. 1), but the differences were not statistically significant and diminished when induced transmembrane voltage was taken into account (Fig. 2c). Another factor that was reported to affect reversible electroporation is cell membrane fluidity (Rols et al. 1990). Our results show (Fig. 2, Table 1, Fig. 4) the same tendency as described by Rols et al. on Chinese hamster ovary cells, where the less fluid cell membranes were permeabilised at lower voltages than the more fluid ones (Rols et al. 1990). However, in the study of Rols et al. the fluidity of cell membranes was changed by the addition of ethanol and lysolecitin both of which incorporate into the cell membrane and change its characteristics, as discussed later. Cell membrane fluidity of a given cell line can be altered by the addition of chemical compounds, as in the study of Rols et al. or it can be changed by exposure of cells to different temperatures. However, in the existing literature on the effect of temperature on electroporation, one finds conflicting reports (Kinoshita and Tsong 1977; Zimmerman 1982). In our study we observed that the temperature has significant effect on cell membrane fluidity (data not shown), and that when the temperature of a drop of cell suspension placed between electrodes was measured, it was observed that it reached room temperature (electrode temperature) in less than a minute. The temperature of cell suspension exposed to electric pulses was therefore room temperature, the same temperature at which EPR spectra were recorded. Cell membrane fluidity was measured by the EPR method with X-band EPR spectrometer using the spin probe methyl ester of 5-doxylpalmitate, which is lipophilic and therefore dissolves in the membrane phospholipid bilayer. Since the nitroxide groups of the spin probes inside the cell are reduced by the oxy–redoxy systems to the EPR invisible hydroxyl amines (Chen and Morse 1988) the main contribution to the EPR spectrum is from the spin probes in the plasma membrane. Overall membrane fluidity, expressed with the empirical correlation time (τ_{emp}), was determined by rough estimation directly from the EPR spectra by Eq. 2, an expression which is valid only for fast isotropic motion in solution. In the membrane it can be used only to compare relative differences in spin probe dynamics between different cell lines and different treatments (Marsh 1981). More precise information was obtained by computer simulation of the experimental spectra (Fig. 4); changes in the order parameter, correlation times and proportion of the coexisting domain types define the overall membrane fluidity more precisely. Both methods, rough estimation and computer simulation of the experimental spectra confirmed that cell line B16-F1 has the least fluid membrane. From computer simulations it is also evident that the domain types with low and medium order parameter influence the most fluidity characteristics, since the proportion of these two domain types differs the most between the investigated cell lines, while the domain type with the highest order parameter is almost the same (Table 1). The effect of cell membrane fluidity and domain type, where reversible electroporation takes place, could not be established, as the differences for reversible electroporation were not significant among the cell lines. The differences however were more pronounced for irreversible electroporation (Fig. 2b). As irreversible electroporation was determined by cell survival, we could not distinguish between the effects of the electric field application from the effect of different time courses of membrane resealing. Cell membrane resealing in cell line B16-F1 was completed in 50% of the cells in a population 5 min after pulse application, while in cell line V-79 it took more than 20 min to reach the same value. Even though

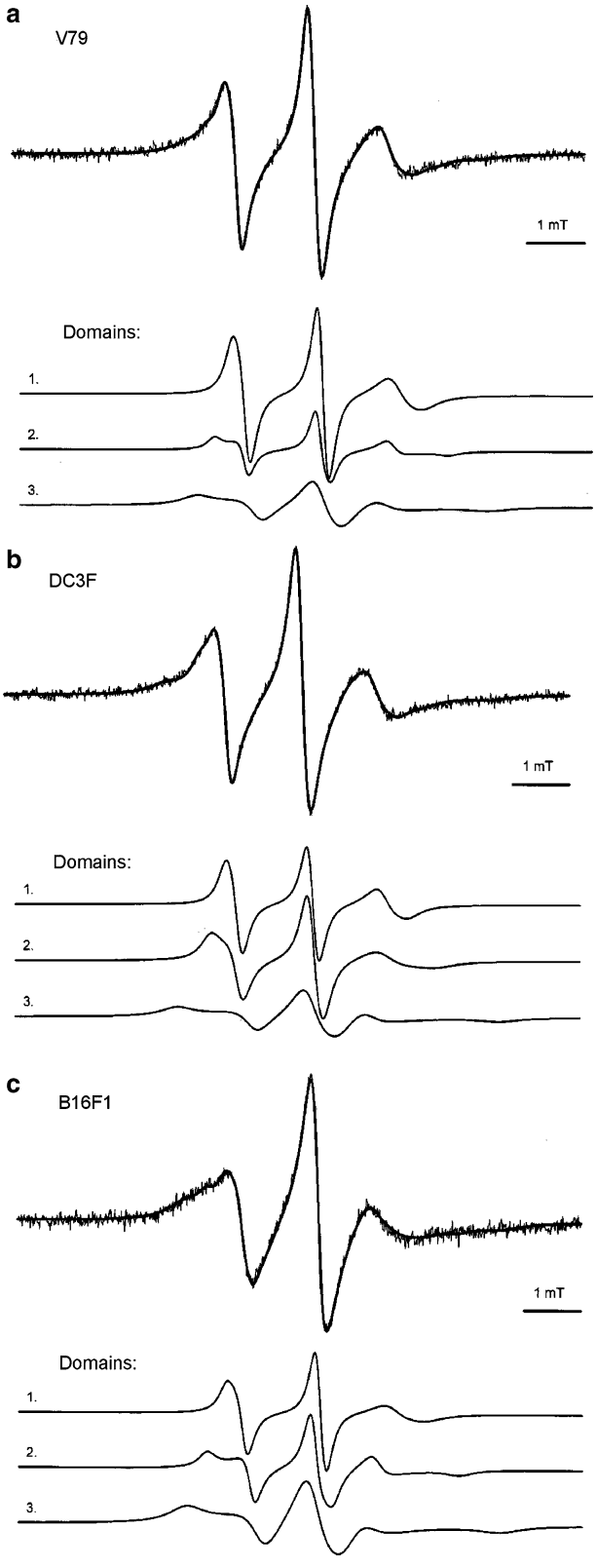

Fig. 4 Sums of six experimental spectra of MeFASL(10.3) in the membrane of V-79, DC-3F and B16-F1 cell lines measured at 25°C with their best fits, taking into account that the spectrum is superposition of three spectral components, which correspond to the spin probes in three coexisting domain types (domains 1–3)

all the cells in population reseal within 30 min in both cell lines, the differences between the resealing of the two cell lines were more than 15 min (Fig. 3), which could have an important effect on cell survival. Nevertheless, the question of cell membrane fluidity having an effect on irreversible electroporation, or affecting cell membrane resealing could not be answered, and neither could the effect of cell membranes domains be established. The effect of cell size on induced transmembrane voltage did not significantly diminish the differences among cell lines for irreversible electroporation (Fig. 2c). Cell line B16-F1 was able to survive at higher electric field strengths and significantly higher induced transmembrane voltage than the other two cell lines. Nevertheless, in this cell line, with the methods used, it was not possible to separate irreversible electroporation from the ability of the cell line to reseal membranes. A permeabilised membrane, apart from transport of extra cellular compounds to the cell, also allows depletion of organic compounds from the cell (Rols and Teissié 1992). Namely, we suppose that the cells that reseal faster have better chances of survival, and therefore, by the method used, it was not possible to draw a direct conclusion that the cells with less fluid membranes can survive a higher applied voltage. The effect of cell membrane fluidity on irreversible electroporation is therefore masked with the ability of this cell line to reseal its membrane faster than the other two cell lines. The membrane resealing that takes place after the pulse application is an active process (Kinoshita and Tsong 1979). Even though the process of resealing is not yet completely understood, it is suggested that the cell cytoskeleton is involved (Rols and Teissié 1992). The resealing of cell lines B16-F1 and V-79 was studied after the application of different amplitudes of an applied electric field (Fig. 3), since the resealing rate constant does not depend on the electric field intensity in our experiment. The time course of resealing in B16-F1 was not modified when 147 V, the amplitude that was used for V-79, was applied (data not shown), which is in accordance with the published data on CHO cell lines exposed to electric fields with comparable pulse number, duration and amplitudes (Rols and Teissié 1992).

The differences in the time course of resealing could be explained by biological differences among cell lines. Even though that from this point of view the work performed with different cell lines can be seen as a drawback, we chose this design of experiment in order to avoid the use of chemical compounds that incorporate into the membrane and change its characteristics. In our previous study we demonstrated that some chemical compounds, like $C_{12}E_8$, known to have a fluidising effect on the cell membrane, also stabilise pores that are formed during electroporation even at concentrations that are too low to alter the cell membrane fluidity (Kandušer et al. 2003). In the case of ethanol-induced change of membrane fluidity, one should consider the toxic effect of ethanol on cells like free radical formation and related lipid peroxidation, induction of apoptosis

and the disordering effect of ethanol on the cell membrane that affects the function of the molecules embedded in the cell membrane (Meshar et al. 1996; Li et al. 2000; Jordão et al. 2004; Neuman 2002; Neuman et al. 2002; Chen et al. 1996, 2000). With our experimental design, we were able to study the effect of cell membrane fluidity on electroporation behaviour of cell lines where differences in cell membrane fluidity were the inherent property of cell membranes.

In conclusion, our results show that differences in cell membrane fluidity observed for the cell lines investigated are not sufficient to provide evidence for the effect on reversible electroporation, although there is a tendency that, with increased membrane fluidity, the voltage required for reversible electroporation decreases. On the other hand, cell membrane fluidity is associated with a lower irreversible electroporation voltage; though its effect is masked with faster resealing of the cell membrane of the B16-F1 cell line.

Acknowledgements This research was supported by the Ministry of Education, Science and Sport of the Republic of Slovenia. The authors wish to express their thanks to Zorka Stolič from the J. Stefan Institute, Ljubljana, Slovenia, for technical assistance.

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