Bioelectrochemistry 74 (2008) 52–57

Contents lists available at ScienceDirect

Bioelectrochemistry

journal homepage: www.elsevier.com/locate/bioelechem

The temperature effect during pulse application on cell membrane fluidity and permeabilization

M. Kandušer [a], M. Šentjurc [b], D. Miklavčič [a,*]

[a] University of Ljubljana, Faculty of Electrical Engineering, Tržaška 25, SI-1000 Ljubljana, Slovenia
[b] Institute Jozef Stefan, Jamova 39, SI-1000 Ljubljana, Slovenia

ARTICLE INFO

Article history:
Received 30 May 2007
Received in revised form 21 March 2008
Accepted 8 April 2008
Available online 16 April 2008

Keywords:
Electropermeabilization
Membrane order parameter
Temperature
In vitro

ABSTRACT

Cell membrane permeabilization is caused by the application of high intensity electric pulses of short duration. The extent of cell membrane permeabilization depends on electric pulse parameters, characteristics of the electropermeabilization media and properties of cells exposed to electric pulses. In the present study, the temperature effect during pulse application on cell membrane fluidity and permeabilization was determined in two different cell lines: V-79 and B16F-1.
While cell membrane fluidity was determined by electron paramagnetic resonance (EPR) method, the cell membrane electropermeabilization was determined by uptake of bleomycin and clonogenic assay. A train of eight rectangular pulses with the amplitude of 500 V/cm, 700 V/cm and 900 V/cm in the duration of 100 µs and with repetition frequency 1 Hz was applied. Immediately after the pulse application, 50 µl droplet of cell suspension was maintained at room temperature in order to allow cell membrane resealing. The cells were then plated for clonogenic assay. The main finding of this study is that the chilling of cell suspension from physiological temperature (of 37 °C) to 4 °C has significant effect on cell membrane electropermeabilization, leading to lower percent of cell membrane permeabilization. The differences are most pronounced when cells are exposed to electric pulse amplitude of 900 V/cm. At the same time with the decreasing of temperature, the cell membranes become less fluid, with higher order parameters in all three types of domains and higher proportion of domain with highest order parameter. Our results indicate that cell membrane fluidity and domain structure influence the electropermeabilization of cells, however it seems that some other factors may have contributing role.

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1. Introduction

Cell membrane permeabilization of an intact cell (exposed to external electric field) is caused by the application of high intensity electric pulses of short duration. The parameters of electric pulses need be chosen properly in order to obtain desired permeabilization of the cell membrane and at the same time not to significantly affect the cell survival [1,2]. The process of electropermeabilization consists of at least two separate phases: pore formation that takes place during, and resealing which happens after the pulse application [3].

The interest on the temperature effect on electropermeabilization was mainly focused on the Joule heating that takes place during the electric pulse application [4,5,6,7,8]. A significant localized Joule heating was observed, which increased with the voltage and the duration of electropermeabilization pulses in human stratum corneum [5]. For exponential pulses with pulse amplitude 70 V and duration of 1 ms, the temperature rise above physiological temperature for 19 °C has no significant role in stratum corneum permeability increase [7]. In *in vitro* experiments it was found that the spatial temperature gradient that changes with time could be an important factor for post-pulse recovery processes but not for the electropermeabilization. For pulse durations used for electropermeabilization and pulses of rather large amplitude (4.5 kV/cm), no considerable heating was observed when applied to cell suspension at 25 °C [4].

On the other hand, in some studies researchers focused on the chilling effect on electropermeabilization of erythrocytes, alga and porcine skin [9,10,11,12]. Experiments with erythrocyte showed that the temperature had no significant effect on electropermeabilization, as the breakdown voltage was 0.8 V at 25 and 37 °C and 0.87 V at 4 °C [9]. However, in alga Valonia, electropermeabilization at 5 °C required higher voltage, because its breakdown potential was 1 V, while at 35 °C it was 0.65 V [10]. Similar results were obtained with porcine stratum corneum. The chilling of the stratum corneum also had a significant effect on electropermeabilization. Decreasing the temperature from 25 °C to 4 °C required higher voltages for electropermeabilization, suggesting that elevated temperature facilitated the formation of electro pores. Authors further proposed that lipid fluidity, which is

* Corresponding author. Tel.: +386 1 4768456; fax: +386 1 4264658.
E-mail addresses: masa.kanduser@fe.uni-lj.si (M. Kandušer), marjeta.sentjurc@ijs.si (M. Šentjurc), damijan.miklavcic@fe.uni-lj.si (D. Miklavčič).

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doi:10.1016/j.bioelechem.2008.04.012

controlled by the temperature, is the major factor affecting electropermeabilization of the stratum corneum [12].

As the temperature has an effect on cell membrane electropermeabilization and on cell membrane fluidity, the aim of our study was to correlate both effects. We determined the effect of temperature decrease from physiological temperature 37 °C to 4 °C on average cell membrane fluidity and on the membrane domain structure of two different cell lines.

2. Materials and methods

2.1. Cell culture and media

Cell lines V-79 Chinese hamster lung fibroblasts and B16-F1 Murine melanoma were grown in Eagle's minimum essential medium, supplemented with 10% foetal bovine serum (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) in the incubator (Kambič, Slovenia) at 37 °C in a humidified 5% CO_2 atmosphere. From confluent cultures a cell suspension was prepared by 0.25% trypsin/EDTA solution (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany). To obtain cell pellet, cell suspension was centrifuged at 1000 rpm (180 ×g) at 4 °C for 5 min (Sigma, Germany). The cell pellet was resuspended in electropermeabilization medium. As electropermeabilization medium Spinner's modification of Eagle's minimum essential medium (Life Technologies Ltd, Paisley, UK) that does not contain calcium, with pH 7.4, osmolarity 300 mosm/kg and conductivity 1.6 S/m was used.

2.2. Cell membrane fluidity

Electron paramagnetic resonance (EPR) method on X-band EPR spectrometer (Bruker ESP 300) was used to measure cell membrane fluidity. The spin probe methyl ester of 5-doxylpalmitate (MeFASL (10.3)), which is lipophilic and therefore dissolves in the membrane phospholipid bilayer was used. Cells were labelled only for EPR experiments, while electropermeabilization was performed with unlabeled cells.

One ml of cell suspension in electropermeabilization medium (described in detail in cell culture and media section) that contained 20×10^6 cells was incubated for 15 min at the room temperature at constant shaking with the spin probe that was prepared as a thin film on the walls of a glass tube. Cell pellet obtained by the centrifugation at 1000 rpm at room temperature was put in a glass capillary and EPR spectra were measured at 4 °C and 37 °C. Each measurement of cell pellet was repeated three times and three independent experiments were performed.

Data on cell membrane fluidity and proportion of different domain types were obtained by computer simulation of experimental EPR spectra. The model used [13,14] takes into account that the membrane is a heterogeneous structure, 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 domains with different fluidity characteristics. It should be stressed that the lateral motion of the spin probe is slow on the time scale of EPR spectroscopy. Therefore an EPR spectral component describes only the properties of a spin probe's immediate environment, which corresponds to the domains on nm scale. All the domains with the same mode of spin probe motion define a certain domain type and are reflected in one spectral component.

Distinct domain types are characterized with sets of spectral parameters, which describe different modes of spin probe motion. The order parameter (S) describes the ordering of lipid hydrocarbon chains and could vary from 1 to 0. It is inversely proportional to the fluidity of certain membrane domain types. Besides, 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. Since it was found that MeFASL(10.3) is equally distributed between different types of domain throughout the whole temperature region from 4 to 37 °C [17] we can assume that the proportion d correspond to the proportion of domain types with particular fluidity characteristics. The tuning of EPR spectral parameters to obtain the best fit of the calculated to the experimental spectrum was performed by the program EPRsim 2.6 [15] implemented in the software package EPRsim (http://www.ijs.si/ijs/dept/epr/).

2.3. Temperature measurements and cell size

The cell suspension in Eppendorff electropermeabilization cuvett was placed in water bath at 37 °C or on ice. The volume of cell suspension was 400 µl. That was sufficient to maintain the temperature constant for the duration of one minute, which was the time necessary for the electropermeabilization treatment. The time required to obtain the desired temperature of the sample volume was less than five minutes; therefore, the sample was preincubated at desired temperature five minutes before the pulse application. The temperature was measured before and after electric pulse application. Since the electrodes were not cooled or heated during the pulse application, the temperature change was measured before and after the pulse application. The temperature change during the time needed for pulse application and/or due to Joule heating caused by the pulse was between 4 °C and 8 °C. In this range it did not cause any changes in cell membrane fluidity.

For each temperature treatment (4 °C and 37 °C), the cell size was measured under the inverted microscope at the objective magnification 40×. To control the temperature during the experiment, cells were placed in a multiwell plate filled with water of desired temperature. Three wells in the interior of the plate were filled with the cell suspension of desired temperature so that the temperature remained constant during the cell size measurements. The average of 80 cells was measured per each condition and the data is presented as the mean ± standard deviation.

2.4. Electropermeabilization experiments

Cell membrane electropermeabilization was determined by bleomycin method, described in detail in Kotnik et al. [16]. Cell suspension in concentration 20×10^6 cells per ml with 50 nM bleomycin was prepared in Spiner modification of Eagles minimum essential medium. To obtain the desired temperature of the sample, 400 µl of cell suspension in Eppendorff electropermeabilization cuvett was kept on ice (4 °C) or water bath (37 °C) for five minutes before the application of electric pulses. Samples were exposed to electric pulses in Eppendorff electropermeabilization aluminium cuvetts, electrode distance 4 mm. The amplitude of the applied field was 500 V/cm, 700 V/cm or 900 V/cm. A train of eight rectangular pulses with duration of 100 µs and repetition frequency 1 Hz was applied. All the treatments were normalized to a control treatment, which consisted of cells exposed to different temperatures, but were not treated with electric field. We used electric pulse generator Cliniporator (Igea, Italy) for pulse application. The temperature of the sample did not change significantly during the pulse application. Nevertheless, immediately after the pulse application, 50 µl of cell suspension was diluted in 950 µl of electropermeabilization medium and maintained 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 cell membrane was resealed. The cells were plated in concentration of 250 cells per Petri dish for clonogenic assay. Colonies were grown at 37 °C in a humidified 5% CO_2 atmosphere in the incubator and after six days, when visible colonies formed they were fixed with methanol (Merck KGaA, Darmstadt, Germany) and stained with crystal violet (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany). Colonies were counted and results normalized to control. The percentage of survived colonies was

subtracted from 100% to obtain the percentage of bleomycin uptake. At least four experiments were pooled together for each data point. Statistical analysis was performed using Student's *t*-test for comparing of two treatments or one-way ANOVA and all pair wise comparison procedures (Tukey Test) when many treatments were compared.

3. Results

Two cell lines, V79 and B16F1 were used to determine the effect of the temperature on cell membrane fluidity and electropermeabilization at different temperatures. By comparing the experimental EPR spectra measured at 4 °C and 37 °C of both cell lines it is evident that the average cell membrane fluidity significantly decreases (by chilling the cells) (Fig. 1). The spectra at 4 °C are typical for highly restricted motion of spin probe, while the spectra at 37 °C are typical for less restricted motion.

More detailed information about the membrane domain alterations was obtained by computer simulation of EPR spectra. Good fit to the experimental spectra was obtained, if we take into account that the spectra are superposition of three spectral components indicating that the membrane is composed of three types of coexisting membrane domains (Fig. 1, domains 1, 2 and 3).

According to the computer simulation of EPR spectra, the reduced membrane fluidity, due to the chilling of cells to 4 °C, is reflected in higher order parameters in all the domain types that compose cell membrane and higher proportion of domain types with highest order parameter (domain 3) when compared to cell suspension maintained at 37 °C. The same pattern is observed in both cell lines, however it is more pronounced in V79 cell line (Fig. 2). At 37 °C the cell membrane fluidity of studied cell lines differs, being less fluid in B16F1 than in V79 due to higher proportion of less fluid domain type 3 and higher order parameters of domain types 2 and 3 (Fig. 2).

The morphology and size of the cells did not depend on temperature (Fig. 3). Fig. 3 also shows that the average diameter of B16F1 cells is higher than of V79 cells which correlate with the percentage of permeabilized cells at 500 and 700 V/cm (Fig. 4) as the induced transmembrane voltage is size dependent [19]. At 900 V/cm the cell size of V79 is no longer the limiting factor for successful permeabilization. The percentage of permeabilized cells is identical for both cell lines at physiological temperatures and the cell survival is not affected.

The chilling from physiological temperature to 4 °C has significant effect on cell membrane electropermeabilization in both cell lines. The temperature effect on cell membrane permeabilization is presented in Fig. 4. The pulse amplitude, which is one of the crucial factors for electropermeabilization of the cell membrane, affects only the permeabilization of cells exposed to 37 °C during the pulse. At 4 °C, the permeabilization increases only slightly (statistically insignificantly, $P>0.05$) with the pulse amplitude; in cell line V79 from 13% at 500 V/cm to 25% at 900 V/cm. It is slightly higher in B16F1; it increases from 18% at 500 V/cm to 42% at 900 V/cm. At 900 V/cm, where both cell lines are permeabilized to similar extent (around 80%) at physiological temperature, the effect of the temperature is statistically significant for both cell lines ($P<0.001$). If we compare the responses of cell line B16F1 and V79 to the electric pulses, we observe that the difference in permeabilization caused by the temperature is more pronounced in cell line V79 ($P<0.05$). Furthermore, if we compare the extent of electropermeabilization at 4 °C and 900 V/cm

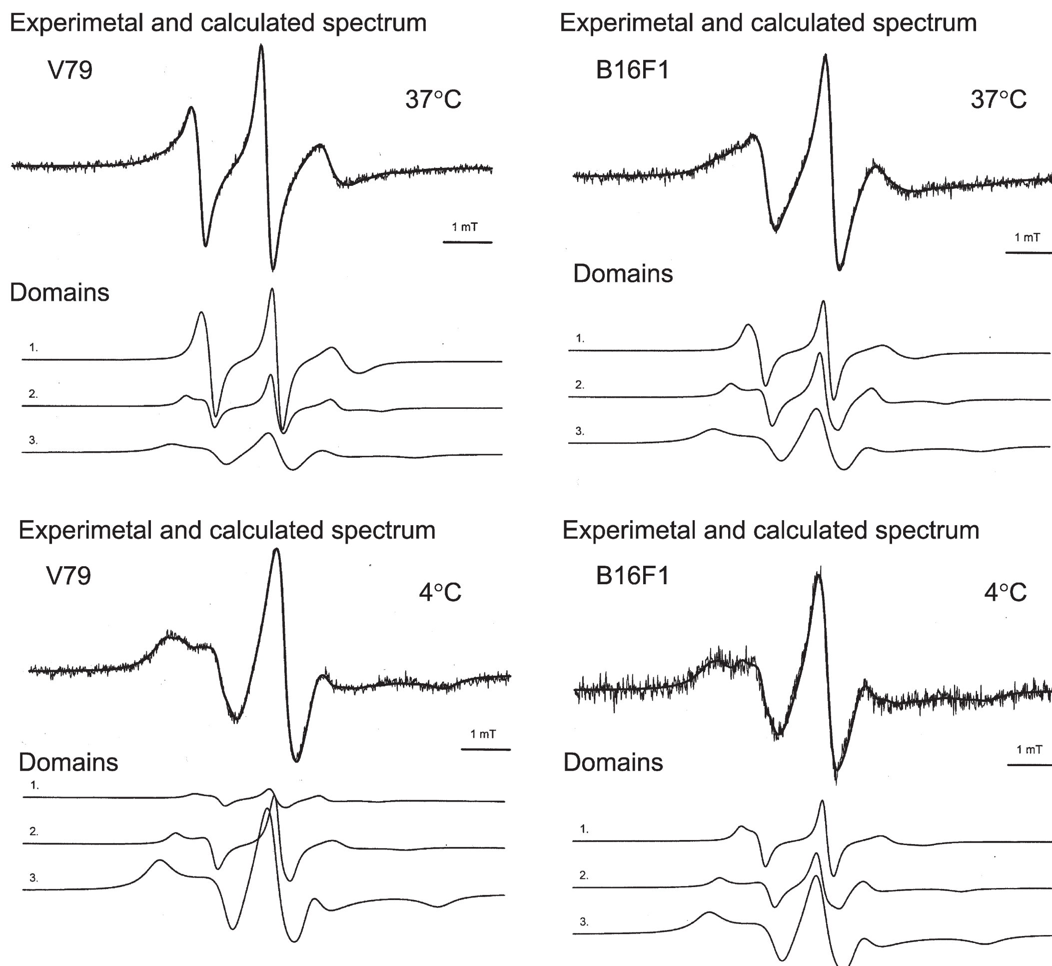

Fig. 1. Experimental spectra of MeFASL (10.3) in the membrane of cell line V79 and B16F1 at 4 °C and 37 °C with their best fits obtained by computer simulation.

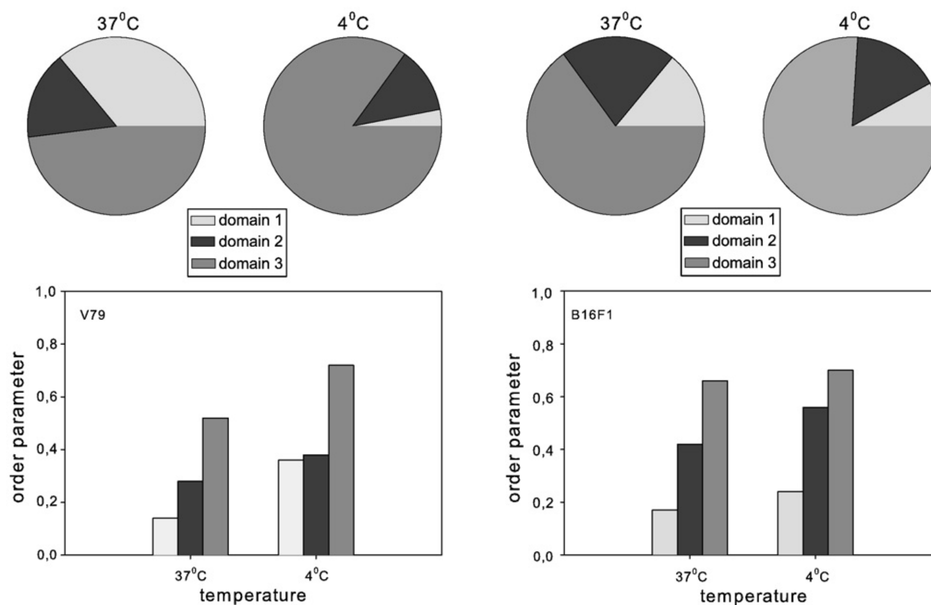

Fig. 2. Weight and order parameters of three coexisting domains (domain1, 2, 3) in the membrane of cell line V79 and B16F1 at 4 °C and 37 °C. Data obtained by computer simulation of the experimental EPR spectra.

we observe that it is higher ($P<0.05$) in cell line B16F1. Observed differences in membrane permeabilization between the two cell lines at highest voltage could be related to differences in cell membrane fluidity and domain composition. In cell line V79, the changes in average membrane fluidity and in membrane domain structure with temperature are more pronounced than for B16F1 (Figs. 1 and 2). In addition, the membrane of V79 is composed predominantly of the less fluid domains; 85% of cell membrane is occupied with the most ordered domain (domain 3, in Fig. 2) at 4 °C which is not easily electropermeabilized, while in B16F1 cells this proportion is lower (76%).

4. Discussion

The main goal of our study was to determine the effect of cell membrane fluidity and membrane domain structure on electropermeabilization by changing the temperature of the cell suspension during the application of electric pulses. Our results show that cell membrane permeabilization depends on the temperature, which has a significant effect on cell membrane fluidity and domain structure.

In discussing the membrane domain structure as measured by EPR, we would like to stress that EPR labelling is not specific for certain type of domains. The reason is that spin probes are distributed uniformly between different domains in the membrane [17] and reflect ordering and dynamics of the membrane phospholipid alkyl chains in their nearest surrounding on the nanometer scale, which depends on the type of phospholipids (saturated, nonsaturated alkyl chains), amount of cholesterol, protein distribution etc. All the regions in the membrane with the same ordering and dynamics (the same fluidity characteristics) are described as one type of membrane domain [18,14].

By lowering the temperature from physiological 37 °C to 4 °C cell membrane fluidity significantly decreases. That is reflected in an increase of the most ordered membrane domain type and increased ordering of all the domains (Figs. 1 and 2). At the same time, cells exposed to 4 °C need higher voltage for electropermeabilization of their membranes, and even at highest pulse amplitudes (900 V/cm), only about 40% of cells in suspension is permeabilized (Fig. 4). On the other hand, the permeabilization of the more fluid membranes of cells exposed to 37 °C is significantly higher, reaching around 80%. Similar results were obtained in other studies that examined the influence of low temperatures on the electropermeabilization. Similarly the results on porcine stratum corneum show that lowering the temperature from 25 °C to 4 °C increases the voltage needed for permeabilization [12]. In addition, in the case of alga Valonia at 5 °C higher voltage is required for electropermeabilization of its membrane than at 35 °C [10].

When cell membrane fluidity differences are caused by other means than temperature, different results are obtained. In the study of Rols et al., where the fluidity was changed by addition of ethanol and lysolecitin the more fluid membrane was permeabilized at lower voltages [20], while in our previous study where cell membrane fluidity of different cell lines was compared, the same tendency was observed, although it was not statistically significant [19]. Even in the present study, if we compare the fluidity of two cell lines at physiological temperature (37 °C) and their relation to electropermeabilization, the results are similar as previously published [19,20].

These findings and the results, where cell membrane fluidity was modified by chilling of the cell suspension from 37 °C to 4 °C (Fig. 3) indicate that there are other factors that average cell membrane fluidity that affect electropermeabilization. Present results (Figs. 1 and 2) as well as previous EPR studies show that in the membranes of live cells, liquid ordered and disordered domains coexist even at low non-physiological temperatures [21,22]. At 4 °C and 900 V/cm cell membrane permeabilization is drastically reduced in both cell lines ($P<0.001$). Nevertheless, the effect is more pronounced in cell line V 79 than in B16F1 (Fig. 4) and

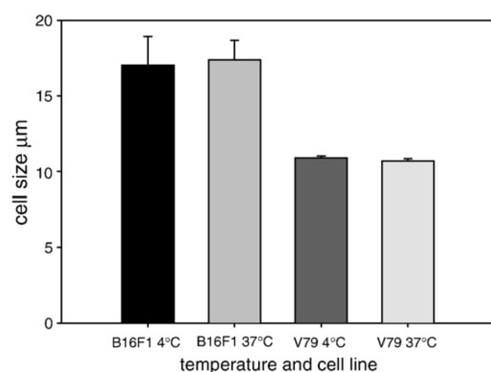

Fig. 3. Cell diameter of cell line V79 and B16F1 at 4 °C and 37 °C. Data are presented as a mean ± standard deviation, the average of 80 cells was measured per each condition.

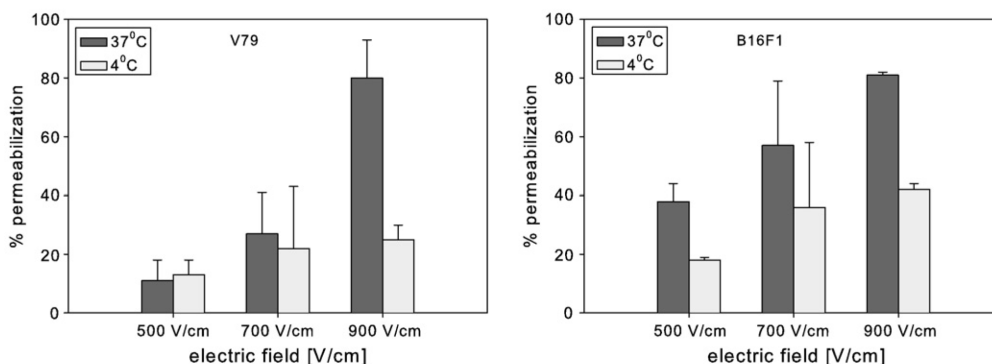

Fig. 4. Cell membrane permeabilization in cell line V79 and B16F1 at 4 °C and 37 °C measured by bleomycin uptake. The train of eight pulses and repetition frequency 1 Hz was applied. Values are means of four independent experiments ± standard deviation. The differences in the percentage of cell membrane permeabilization at 4 °C and 37 °C were statistically significant ($P<0{,}001$) at 900 V/cm for both cell lines determined by Student *t*-test. At the same amplitude, statistically significant ($P<0.05$) differences in the percentage of cell membrane permeabilization are found between two cell lines (V79 and B16F1) at 4 °C (Student's *t*-test).

the difference is statistically significant ($P<0.05$). In this study, we have found that decreasing the temperature from 37 °C to 4 °C affects proportion and order parameter of the most ordered domain 3. The change is more pronounced in cell line V79 than in B16F1 (Figs. 1 and 2). Besides, if we compare the extent of cell membrane permeabilization of two cell lines at 4 °C; it is significantly higher in B16F1 than in V79 ($P<0.05$). This again correlates with the membrane domain alterations. When the membrane is chilled to 4 °C (Fig. 2), the proportion of the most ordered domain type (domain 3, probably liquid ordered domains) increases much more for V79 than for B16F1 cells, indicating important role of this domain type on electropermeabilization. In the case of V79 at 4 °C, almost entire membrane is in liquid ordered state, which is not easily electropermeabilized (only 25% cells at 900 V/cm). Our results as well as previous results on lecithin cholesterol model membranes indicate that not only an average membrane fluidity but rather membrane domain structure is important for membrane permeabilization [23, 24]. It was shown on model lipid membranes from egg yolk phosphatidylcholine, that incorporation of cholesterol at concentration of 50 mol% to 60 mol%, which decreases membrane fluidity of liquid crystal phase bilayers [25], increases the break down voltage for cholesterol containing membranes [24]. Lower concentration of cholesterol (up to 29 mol%) did not affect electropermeabilization of fluid membranes (egg yolk phosphatidylcholine) but reduced the voltage required for electropermeabilization of the membranes which are in the gel phase at the room temperature (dipalmitoyl-sn-glycero-phoshocholine), when cholesterol introduces more fluid domains into the membrane [23]. This is also supported with the results on pure lecithin membranes where it was shown that the critical voltage for electropermeabilization is in the same range for egg yolk phosphatidylcholine membranes, that are in fluid phase, and dipalmitoyl-sn-glycero-phoshocholine membranes, that are in the gel phase at room temperature [23].

Alternatively, the temperature effect on electropermeabilization could be explained by thermal effects during electro pore formation [26,27,28]. A cell membrane domain structure and fluidity could play a role in that process. According to the electroporation theory, hydrophobic pores in the cell membrane are formed spontaneously by lateral thermal fluctuations of the lipid molecules. The structural rearrangements of hydrophobic to hydrophilic pores during electroporation occur when the radius of hydrophobic pore exceeds critical value and when the reorientation of the lipid molecules becomes energetically favourable [26]. The energy needed for reorientation of lipid molecules to form hydrophilic pore is expected to be lower in more fluid cell membrane or membrane domain with lower order parameter.

It needs to be mentioned however that temperature by itself affects physiology and metabolism of the cell. Therefore, the time during which the cells were exposed to 37 °C or 4 °C was reduced to five minutes, as needed to obtain the desired temperature for electropermeabilization. Control treatments were performed so that cells were incubated at 37 °C or 4 °C and then permeabilized at room temperature. The results were compared to the results obtained in our previous study [19], when the cells were permeabilized at room temperatures and no differences were found. Therefore, we concluded that even if there are metabolic and/or physiological changes related to preincubation of cell suspension at 37 °C or 4 °C they do not affect electropermeabilization of these two cell lines. The possible effect of the temperature treatment on the cell size was also determined. As the cell size is an important factor affecting electropermeabilization, cell diameter was measured at different temperatures. We established that the temperature treatment used in our experiment does not affect either morphology or the cell size (Fig. 3).

In conclusion, the temperature of cell suspension during the pulse application that affects cell membrane domain structure consequently changes the electropermeabilization behaviour in both cell lines, V79 and B16F1. From the results obtained in our in vitro study, we can conclude that the optimal temperature for electropermeabilization is physiological temperature and that chilling significantly reduces electropermeabilization effectiveness. We have demonstrated that cell membrane fluidity and the membrane domain structure play an important role in electropermeabilization, however other factors may be involved.

Acknowledgments

This work was financially supported by Slovenian Research Agency (ARRS).

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