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Simulation of electroporated cell by chronopotentiometry

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Abstract

Chronopotentiometry on planar lipid bilayer (BLM) is proposed as a method for modeling the electrical phenomena in electroporated cell. Two techniques are discussed: constant-current and linear-current chronopotentiometry. It is proposed that the constant-current chronopotentiometry may provide basis for modeling the electroporated cell shortly after the removal of the electric field, when activity of cellular pumps counteracts ionic fluxes through the electropore and ionic channels. The linear-current method can be considered for modeling the cell in the later stage after electroporation, when energetical resources of the cell are gradually getting exhausted and the activity of pumps decreases. Based on this idea, it may be postulated that the electropore in the cell has fluctuating dynamics whose stochastic characteristics, similarly as biological channels, shows $1/f$ noise. The model implies that the fluctuations would disappear leaving the electropore with a constant resistance when efficiency of the pumps becomes very small. The results of chronopotentiometry also may suggest that opening time, conductivity and selectivity of the electropore can be controlled by the cell environment or membrane composition.

Keywords: Bilayer lipid membrane; Electroporation; Chronopotentiometry; Current–clamp experiment; Electropore resealing

1. Introduction

Application of intense electric field increases membrane permeability which results in appearance of hydrophobic and subsequently hydrophilic electropores [1]. If the field intensity is too high or applied for long time without control over the electropore size, an irreversible breakdown of the membrane occurs [2]. The phenomenon of electroporation, although still not fully understood, is important in practical applications, especially for administration of biologically active compounds directly into the cell, e.g., anti-tumoral drugs [3,4]. It is difficult, however, to control the process of cell electroporation. Abrupt changes of the electropore diameter and a high risk of a complete membrane destruction limit the use of electroporation procedures. Currently, in medical applications a method based on very short-time impulse field is used. This technique ensures that electropores do not grow exceedingly. The duration of electric pulses is usually in the range of micro- or milliseconds. Another factor influencing the electroporation is the pulse shape. The exponential (charge-pulse) and rectangular pulses are most frequently used [2,5]. Unfortunately, in this method, a control over the size and opening time of the electropores is rather limited [5]. Therefore, size of the molecules flowing into and out of the cell and duration of this process is practically beyond the control.

Another important aspect, still poorly explored, is functioning of a cell injured by electroporation. Direct observation of the electropore and its effect on the cell is very difficult [1,6], theoretical models are still inaccurate. A cell is a complex structure, surrounded by inhomogeneous lipid membrane with incorporated proteins. Natural transmembrane potential of the cell in the physiological state may reach $U_m = 100$ mV [7] even in non-excitable cells. After electroporation the membrane potential may change but it still affects the electropores. Better understanding of the processes in electroporated cell would help to design a safer and optimized drug administration. It may also help to reveal the actual role of electroporation in cardiac

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defibrillation — so that we could determine whether to treat the electropore appearance as a dangerous side effect or rather as a necessary factor for a successive emergency treatment [8].

In this study we focus on the processes in electroporated cell and chances for their better control or modification. A method to model a cell with an electropore, based on experiments on BLM with controlled current-source, is proposed and tested. By this method, the influence of non-native ions and compounds from extracellular fluid on the characteristics of electroporated cell may be investigated. The method also may be used to model the response from cellular ion pumps and channels and their functioning altered by electroporation.

1.1. Ionic fluxes in an electroporated cell and modeling the electropore

Under physiological conditions, ionic channels and pumps, mainly electrogenic Na^+/K^+-ATPase, regulate the natural transmembrane potential of a non-excitable cell (state 1, Table 1). This pump exchanges 3 Na^+ for 2 K^+ ions between intracellular and extracellular compartments, which generates a transmembrane potential characteristic for a particular cell.

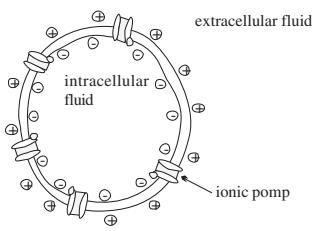
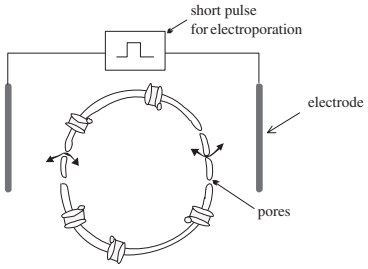
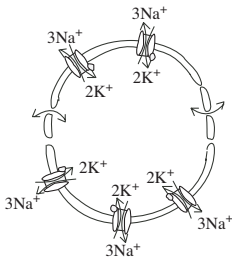
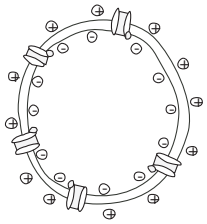
Application of an electric pulse of sufficiently high amplitude and duration creates a conductive hydrophilic electropore (state 2, Table 1) [9]. If the generated transmembrane potential exceeds a breakdown potential U_B, the probability of the membrane to break up abruptly rises. The value of U_B depends on the cell type, although it is typically lower than 0.5 V [1,2]. The electropore probably appears in the area with a defect or increased local fluidity resulting from the field application. Monte Carlo simulations based on modified Pink model show changes in local conformation of the membrane and its fluidity, which may lead to defects [10]. Another possible reason for increased probability of breaking up the membrane is the electropermeabilization following local thermal fluctuations [1,11].

When an electropore appears, the plasma membrane loses its most important function as a barrier, resulting in non-selective flow of ions and the depolarization of the membrane. There are two opposite processes in electroporated cell with no external field applied (state 3, Table 1). On the one hand the decreased potential allows for gradual closing of the electropore. On the other hand ionic pumps in the membrane increase their efficiency, trying to restore the characteristic value of the membrane potential. The increased activity of ionic pumps generates a higher electric field that counteracts the resealing. The final stage of electroporation in the cell is eventual electropore resealing, a very slow process which may take up to several hours [11–13] (state 4, Table 1). The resealing time is variable for each cell and difficult to predict.

Electrically, an ion pump in the plasma membrane is analogous to a controlled current source activated when transmembrane potential drops below the value characteristic for the cell (Fig. 1). In the electroporated cell, ions move freely across the plasma membrane, thereby reducing the concentration gradients and, consequently, the potential. Simultaneously, electrogenic pumps of the cell (e.g. Na^+/K^+ pump) transport specific ions between intra- and extracellular fluids to restore the gradients. A systematic observation of these processes in situ is very difficult because of the small size of the electropores. An alternative solution is to develop a less complex model system whose parameters are easier to control. We propose chronopotentiometry as a method to model the electroporated cell under physiological or other specific conditions (Fig. 2).

Chronopotentiometry provides time series of transmembrane potential U from experiments with a controlled current source (Fig. 3). Electropores generated by this method have a very long lifetime (up to 4 h) [14–16], which has been considered as a

Table 1
States of the cell before, during and after electroporation

1		Sodium–potassium pumps maintain a negative potential inside the cell and appropriate Na^+ and K^+ concentrations. The characteristic potential and the concentration gradient play an important part in the inward transport of organic substrates. ATP synthesis requires high transmembrane potential.
2		Application of a short-time intense electric impulse to the cell generates electropores in the plasma membrane resulting in nonselective flow of ions through the membrane.
3		When the electric field is removed the electropore is still open and nonselective flow of ions continues depolarising the cell and destroying ionic gradients. Ionic pumps tend to restore the gradients and potential, excessively consuming ATP supplies. The process is not yet fully understood.
4		Restoration of the membrane structure which may take up to a few hours. The process is not yet fully understood.

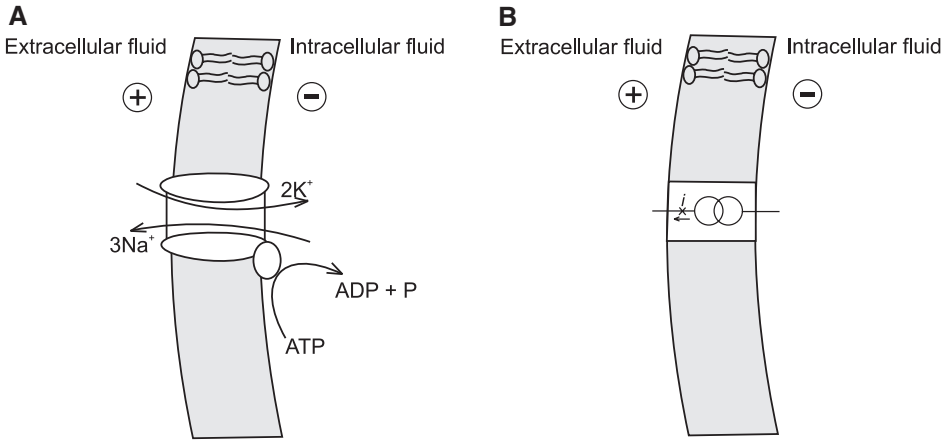


Fig. 1. Ion pump (A) as a regulated current source in current–clamp mode (B).

main asset of this method. Two possible chronopotentiometric experiments are discussed here: constant-current and linear-current methods. In the constant-current measurements, a current with a constant value is applied. In these experiments, the membrane initially accumulates the charge due to its capacitance, which causes the voltage rise exponentially (Fig. 4). Then, a sudden decrease in potential occurs, which indicates an appearance of an electropore. The stage of electropore formation is followed by stochastic fluctuations of the voltage. The fluctuations and the long lifespan of the membrane are due to a negative feedback mechanism. When the electropore opens excessively, the transmembrane potential drops. The electropore size, which is potential dependent, decreases so the conductivity of the electropore is reduced. As an effect the membrane potential increases and the electropore is likely to increase its size again. Then the cycle repeats again. The feedback hampers uncontrolled growth of the electropore and elicits fluctuations. The chronopotentiometric method allows the maintenance of the electroporated membrane for a long time under well-controlled conditions. We propose that it also may provide basis for modeling ionic transport in electroporated cell.

In the cell in state 3 (Table 1), two opposite processes can be observed: non-physiological ionic flux through the electropore, adding to the physiological flux through the channels, and the action of the ion pumps trying to restore the physiological transmembrane potential. In the chronopotentiometric experiment on the membrane with the electropore, the controlled current source can be considered as a model for the action of the ion pumps.

It could be expected that when the electropore stays open for too long, elevated energy consumption by the pumps reduces cellular energy resources, which leads to decreased activity of

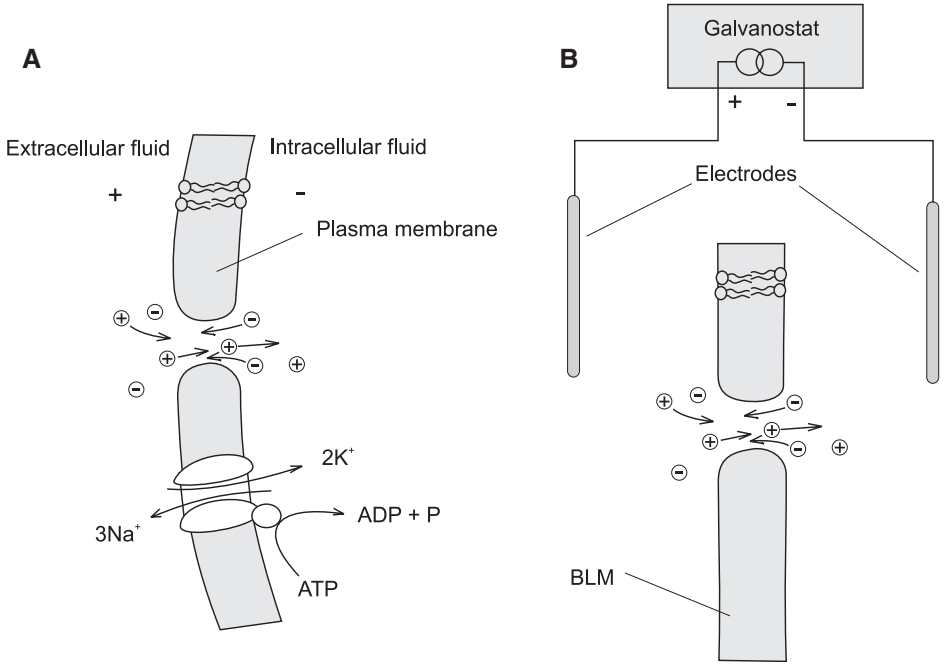


Fig. 2. Ion transport through electroporated plasma membrane (A) an equivalent model system with a planar lipid membrane and a galvanostat (B).

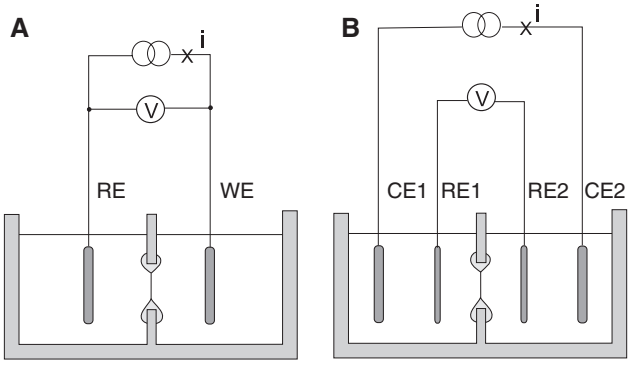


Fig. 3. Chronopotentiometric experiments in 2-electrode (A) and 4-electrode (B) modes. Flow of the constant current through RE–WE electrodes (2-electrode mode) or CE1–CE2 electrode (4-electrode mode) is controlled by galvanostat. Transmembrane potential is measured between reference and working electrodes (RE–WE, 2-electrode mode) (A) or two control electrodes (RE1–RE2, 4-electrode mode) (B).

the pumps. This hypothesis may explain why the tendency for resealing eventually prevails in the cell. As an effect the electropore closes instead of remaining in the fluctuating state, as observed in constant-current chronopotentiometry on planar lipid membranes. It may be expected that supplying the cell with additional energy precursors (e.g., glucose or fatty acids in cardiac cells) should extend the electropore lifetime. On the other hand, it suggests that electropores in anoxic cells should close faster.

Based on this idea, it is proposed that the linear-current chronopotentiometry can be considered for modeling and studying the resealing of the electropore. A cell in state 4 (Table 1) is trying to restore the membrane structure, gradually closing the electropore. Resealing of the electropore was previously investigated by means of two voltage pulses switched on at the same time instant [11]. Electrical breakdown of the membrane was induced with a voltage pulse of high intensity and short duration. The time course of the changes in membrane conductance after application of high and short-time voltage pulse was measured with a longer voltage pulse of low amplitude. However, this method may disturb the natural mechanism of electropore sealing.

The observations from chronopotentiometric experiments may provide more insight into the processes of electroporated cell in vivo due to limitations in available experimental techniques. This approach does not take into account other factors affecting electropore dynamics, for example the mechanical influence of the cellular cytoskeleton has not been considered. However, the model may help to understand the electrical phenomena in electroporated cell in various, also non-physiological, conditions.

2. Materials and methods

2.1. Chemicals

Egg yolk phosphatidylcholine (PC) and phosphatidylserine (PS) were obtained from Fluka (Buchs, Switzerland), cholesterol (Chol) obtained from Sigma (St. Louis, USA), analytical-grade KCl and $AlCl_3$ obtained from POCH (Gliwice, Poland) and *n*-decane from Aldrich (Gillinghem-Dorset). The chemicals were used without additional purification. The electrolytes (0.1 M KCl) were buffered with HEPES (Aldrich, Gillinghem-Dorset) to pH = 7.0. Ultrapure water was prepared in the Milli-Q system (Millipore).

2.2. Measurements

The experiments were performed on planar lipid bilayer membranes formed by the Mueller–Rudin method [17]. The forming solution contained phosphatidylcholine (PC) dissolved in *n*-decane (20 mg/ml). The membranes were formed in round aperture, 1.0 mm in diameter, in two Teflon hydrophobic septum separated cells filled with 10 ml of electrolyte. The experiments were performed at temperature $T = 22 \pm 1$ °C.

The measuring system was fully controlled by a PC computer and software developed in our laboratory. A programmable chronopotentiometry with scheduled current–time dependence was available [18]. Electrical measurements were performed with a four-electrode (Ag/AgCl) capacitance meter and potentiostat–galvanostat described in our previous papers [19,20]. A schematic of experimental setup for chronopotentiometric experiments is shown in Fig. 3.

The process of membrane formation was monitored by visual observation of transmitted light and recorded membrane capacitance. Since the membrane capacitance C rises when the bilayer is forming, it was assumed that the membrane was fully formed when the capacitance drift was lower than 10 pF/min. The membrane resistance was calculated based on voltammetric curves, recorded in the range of $\Delta U = \pm 50$ mV, with the least squares method. The data was processed and analyzed with Excel (Microsoft) and the software from our laboratory.

2.3. Calculation of electropore conductance and diameter

During current–clamp experiment a pore with fluctuating size is created. Electropore conductance G_p at any selected point

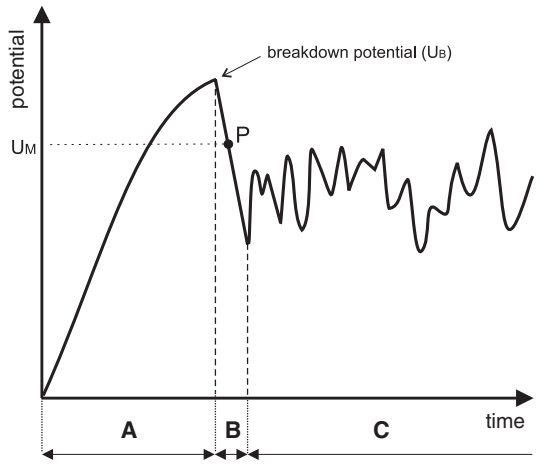


Fig. 4. Reversible electroporation in current–clamp chronopotentiometry. A — exponential charging of the membrane; B — pore creation; C — potential fluctuations. P — point selected for the calculation of pore conductance.

P (Fig. 4) was calculated by the method described previously [21] as:

$$G_p = \left(\frac{i}{U_M} - \frac{1}{R_M}\right) \cdot \left(1 - \frac{V_p}{V}\right) \quad (1)$$

where i is total current flowing through the electrodes and membrane, U_M is the membrane potential, R_M is the membrane resistance, V and V_p are the slopes of the chronopotentiometric curve (dU/dt) at the potential U_M for the membrane without and with the electropore, respectively. This method takes into account the resistance of the membranes without the electropore and changes in the membrane capacitance caused by the membrane potential [21].

The electropore diameter was calculated based on the following assumptions: (a) the shape of the electropore is cylindrical, (b) the electropore is filled with the same electrolyte as a bulk electrolyte, (c) the change in the temperature inside the electropore, resulting from the current flow, did not significantly change the electropore conductance. These assumptions, which are made for analytical purposes, may not reflect the reality in the cell plasma membrane. Based on the assumed cylindrical shape, the electropore diameter d was calculated as:

$$d = 2 \cdot \sqrt{\frac{G_p \cdot L}{\kappa \cdot \pi}} \quad (2)$$

where G_p is electropore conductance; L is membrane thickness; κ is specific conductance of the electrolyte. While calculating the electropore diameter (Table 2), it was assumed that the thickness of each membrane was $L = 7$ nm [22].

3. Results and discussion

3.1. Constant-current chronopotentiometry as a model of electroporated cell in state 3 (Table 1)

A typical chronopotentiometric curve obtained from planar lipid bilayer in a constant-current experiment is shown in Fig. 4. The process begins with an exponential rise of the potential, for which the membrane capacitance and conductance are responsible. Then, a sudden decrease of the potential value can be observed, although it rarely reaches zero. This effect is a sign of an electropore formation. Estimation of the electropore diameter indicates that usually a single nanopore is formed [21]. Short breaks in current supply do not let the electropore reseal [18]. In our experiments the average electropore diameter ranged from 3.8 nm (0.2 nA) to 7.5 nm (1 nA) for PC membranes in 0.1 M KCl [23]. The stage of electropore formation is followed by stochastic voltage oscillations. Typically, the potential oscillates in the range between 70–140 mV, which is too low for subsequent electropores to form.

Table 2
Pore conductance and diameter calculated for electroporated BLM in current–clamp chronopotentiometry — exemplary data for a membrane and electrolyte of different composition

Composition of the membrane	Composition of the electrolyte	Breakdown potential, U_B (mV)	Level of conductance oscillations, G (nS)	Pore diameter, d (nm)
PC	Typical	255±20	2.10±0.30	3.8
PC/Chol (molar ratio 1 : 1)	Typical	280±25	1.90±0.34	3.3
PC/PS (molar ratio 1 : 1)	Typical	300±30	2.04±0.60	3.8
PC/PS (molar ratio 1 : 1)	Typical + 1 mM Al^{3+}	297±30	0.86±0.15	2.4

Forming solution (typical) — phosphatidylcholine in *n*-decane (PC), electrolyte — 0.1 M KCl, HEPES pH = 7.0. The conductance was calculated as arithmetic mean ± standard deviation of at least 8 membranes. Current intensity $I = 0.2$ nA.

Chronopotentiometric characteristics strongly depend on experimental conditions, such as the current value, ionic strength, concentration and type of ions or molecules in the solution, and lipid composition in the membrane (Fig. 5, Table 2). Breakdown potential, electropore diameter and stochastic characteristics of the fluctuations depend on experimental conditions.

Higher intensity of the field speeds up the electroporation and increases the diameter of the electropore. Electropore diameter is also strongly sensitive to ionic strength, decreasing in higher concentrations [24]. In our experiments the average diameter ranged from 0.93 (0.2 nA) to 4.2 nm (5 nA) for PC membranes in 2 M KCl. A Monte Carlo simulation study on the influence of ionic strength on the membrane, showed a higher integrity of the membrane in higher ionic strength [25]. Molecular Dynamics (MD) simulations also show that diffusion coefficient is lower in high ionic strength also due to creation of cation-lipid complexes [26]. Lipid composition may be relevant, too (Table 2). Adding cholesterol slightly decreased the electropore size. Almost no effect was observed when phosphatidylserine (PS) was present although it affects the ability of the membrane to bind multivalent ions. Breakdown potential is also sensitive to electrolyte and lipid composition. An influence of cholesterol and phosphatidylserine on the value of potential U_B was shown (Table 2).

Dynamics of the electropore, represented by oscillatory part of the chronopotentiometric characteristics, can be studied based on the amplitude, level, and the stochastic model of fluctuations [27,28]. It was shown that the conductance oscillations are usually a self-similar stochastic process with long memory [24]. However, the process cannot be classified as a fractional Brownian motion and a stable motion was suggested. Electropores in PC membranes with diameter below 1 nm produce a flicker noise $1/f^{\beta}$, where β is close to 1. The parameter β can be considered as a quantitative measure of ionic concentrations [29] and, possibly, lipid composition. Interestingly, it turns out that stochastic characteristics of electropore and ion channels [30] are more similar than expected since $1/f$ noise was observed in both cases.

A strong effect of multivalent ions on the plasma membrane, such as Al^{3+}, can be studied by chronopotentiometry. The influence of Al^{3+} is interesting due to the neurotoxicity of

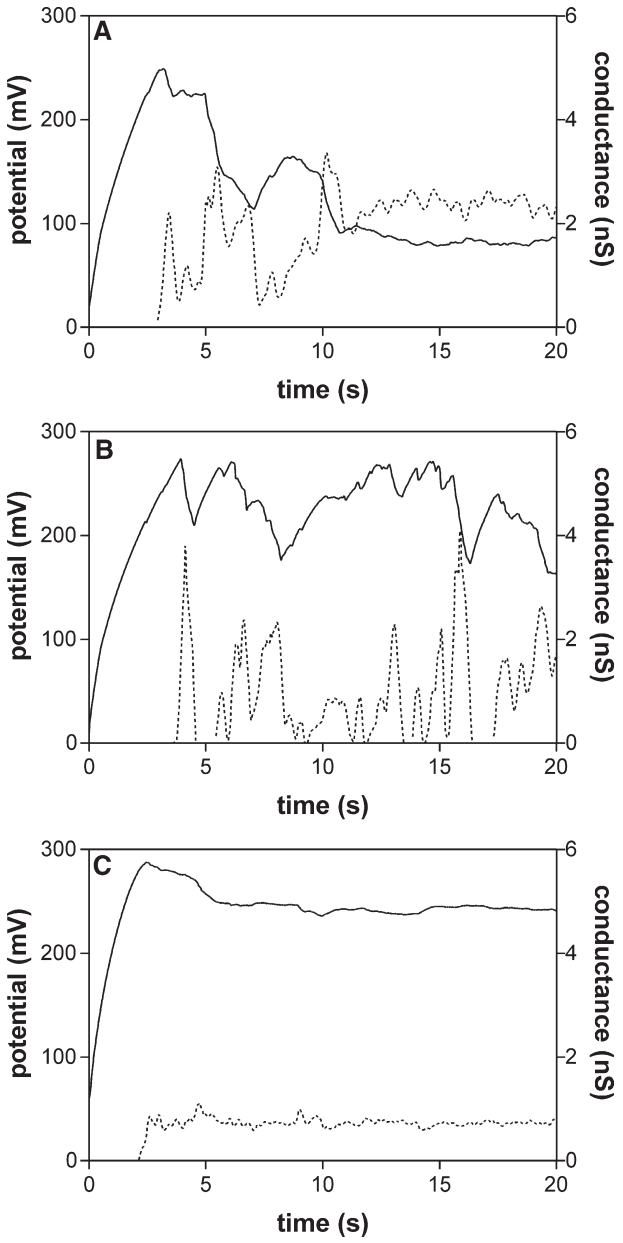


Fig. 5. Chronopotentiometric curves for electroporated BLM registered at $I=0.2$ nA (solid line, left axis) and calculated pore conductance (dashed line, right axis). The forming solutions contained: (A) PC, (B) PC/Chol (1 : 1 mol/mol), (C) PC/PS (1 : 1 mol/mol). Electrolyte: 0.1 M KCl, 10 mM HEPES, pH = 7.0, and in (C) additionally 1 mM $AlCl_3$. The conductance was calculated from chronopotentiometric curve according to Eq. (1).

aluminium ion and its possible relevance to Alzheimer's disease and Parkinson-dementia [31]. Chronopotentiometric study showed that Al^{3+} decreases electropore size and blocks fluctuations almost completely (Table 2, Fig. 5). This effect is attributed to diminished elasticity of the membrane and ordering effect of Al^{3+}.

In the cell, appearance of an electropore could be compared with an appearance of a large non-selective channel. Chronopotentiometric experiments may mimic the situation of a cell in state 3 (Table 1), in which the opening of the electropore allows all ions of a suitable size to move freely across the plasma membrane, tending to reduce the concentration gradients and consequently the potential. Simultaneously, electrogenic pumps of the cell transport specific ions between intra- and extracellular fluids to restore the gradients. Efficiency of ionic pumps is comparable to currents applied in constant-current experiments. For example, efficiency of the pumps in human lens epithelium cell, with transmembrane voltage $U=80$ mV, is about 1–2 nA [32]. Therefore, it is very possible that chronopotentiometric experiments may show the effects observed in a real electroporated cell.

There are two consequences of this approach. First, if we know the net ionic flux J_{cell} through plasma membrane of the cell, we can more accurately obtain size of the electropore in specific conditions, based on the chronopotentiometric experiments. Although the accuracy of our method is limited by the assumptions taken for calculation of the electropore diameter, other currently available techniques give even less accurate estimates.

Secondly, the constant-current experiments on planar lipid membranes show oscillations of the electropore, which may suggest the fluctuating dynamics of the electropore in cell, too. Interestingly, the stochastic characteristics of the fluctuating electropore, showing $1/f$ noise, implicate certain resemblance between the electropore and ion channels. This aspect has not been considered in modeling electroporated cell in state 3, yet. Models of the phenomena in which cells are injured by electroporation, like in cardiac defibrillation [33], also do not take into account the oscillatory character of the electropores.

3.2. Linear-current chronopotentiometry as a model of electroporated cell in state 4 (Table 1)

The linear-current experiment can imitate the resealing electropore in the cell in state 4 (Table 1). It has been postulated that the pumps are gradually loosing their efficiency due to extended time and level of their activity, exhausting energetical resources of the cell. The action of ionic pumps is modeled by decreasing current intensity.

Chronopotentiometric experiment with linearly time dependent current is shown in Fig. 6. In the first 25 s of the

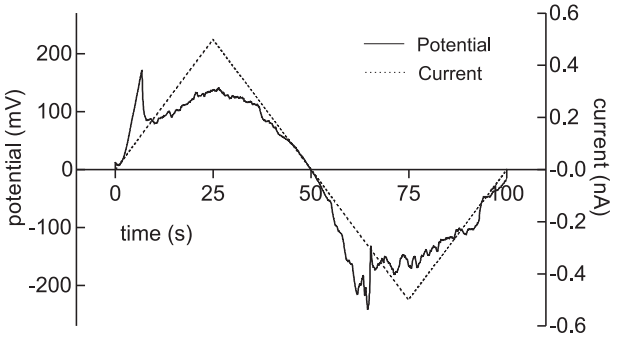


Fig. 6. Chronopotentiometric curve (solid line) registered at linear-current (dashed line). The forming solutions contained PC. Electrolyte: 0.1 M KCl, 10 mM HEPES, pH = 7.0.

cycle the current intensity increases from $I=0$ to $I=0.5$ nA. Then a decrease to $I=0$ within the same period is executed. Afterwards, the current changes its direction. The initial part of the potential curve in linear-current experiment is very similar to the constant-current characteristics (Fig. 4). An increase of the membrane potential to the breakdown potential U_B leads to an electropore formation. Then, due to the increase in membrane conductivity, the membrane potential decreases. A decrease in current value to about 0.1 nA results in the potential decrease to about $50 \div 100$ mV and disappearance of its oscillations. For low current intensity, we observed almost linear dependence between potential and applied current. This indicates the constant resistance of the electropore. In other words, this part of the curve shows the electropore with a relatively invariable average size.

Further increasing of the current intensity causes a slow re-increase of the potential. In voltage–current characteristics it can be observed a distinct region where a significant potential increase is followed by an abrupt potential drop (Fig. 6). This region may suggest a gradual electropore resealing followed by its re-opening at the potential close to U_B. Our results show that the electropore closes when the potential is below U_B. The process is not immediate since even at $U=0$ resealing may last from seconds to tens of minutes, which is in agreement with the previous work [13,34].

4. Summary

Chronopotentiometry with controlled current source applied to planar lipid membrane was proposed as a method to model electroporated cell in its natural conditions. Two possible chronopotentiometric experiments were discussed: constant-current and linear-current methods. In the constant-current technique the current value is fixed. In the linear-current method the electropore is created under a linearly changing current. Both varieties of the chronopotentiometric method maintain the electroporated membrane for long time under well-controlled conditions. Electrical phenomena in the electroporated cell were modeled in the situation when activity of cell pumps counteracts ionic fluxes through the electropore (state 3, Table 1). This case was modeled by constant-current chronopotentiometry. The other modeling attempt involved a cell whose energy resources diminish and the activity of the pumps decreases, leading to the gradual electropore resealing (state 4, Table 1). This activity was modeled by linear-current chronopotentiometry with decreasing current.

The results of chronopotentiometric experiments indicate that the electropore behavior depends on the composition of the membrane and surrounding fluids. More accurate estimation of the electropore size could be based on the chronopotentiometric experiments on planar lipid membranes in specific conditions, corresponding to the cell under study. The results of the chronopotentiometric model may suggest fluctuating dynamics of the electropore in the cell, with characteristics $1/f$. Based on the model, it can be also postulated that, when the efficiency of the pumps becomes very small, fluctuations disappear and the electropore assumes a constant resistance.

The chronopotentiometry can be considered as a method to advance our understanding of electroporation in the cell, and contribute to elaborating more accurate models including the influence of environment. The results of chronopotentiometric experiments may suggest that the electropore can be controlled by composition of lipids or extracellular fluids. Manipulating the environment of the cell may allow for modulation of the electropore conductivity and improve its selectivity.

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