CHOLESTEROL INDUCED CHANGES IN THE CHARACTERISTICS OF THE TIME SERIES FROM PLANAR LIPID BILAYER MEMBRANE DURING ELECTROPORATION *

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The electroporation can be used as a non-toxic method for introducing exogenous macromolecules, especially DNA and drugs, into various types of cells. Research into new therapeutic methods based on Long Duration Electroporation (LDE) is of special interest. A new current-clamp method makes possible the electroporation of very long duration with no damage to bio-membranes. In this paper we compare responses of lipid planar bilayer membranes at physiological concentration of KCl, with lipid membranes formed at higher ionic strength, and membranes containing cholesterol. A longer lifespan of the membranes with cholesterol and membranes with increased ionic strength could be observed. Sensitivity of the power spectrum response to the presence of cholesterol, ionic strength, current intensity, and membrane ageing was examined. The membrane memory was analyzed by means of autocorrelation function and rescaled range analysis. We showed that the memory of the system decreases for higher current intensities and this relation is pronounced better at higher ionic strength. At low current intensities all membranes showed slightly persistent type of noise behavior with crossover to Brownian type of noise for higher current value. The transition was much faster for higher ionic strength, where the next transition to anti-persistent response was observed for relatively low currents. Very interesting results were obtained from power spectrum analysis. At low current intensity, all membranes exhibited $1/f$ noise, which disappeared for higher currents, maintaining f^{β} type with rising value of β. Membranes formed at lower ionic strength and with cholesterol showed a pronounced tendency to lose flicker noise while ageing, also with rising β value.

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1. Introduction

Strong electric fields applied to lipid bilayer membranes cause the phenomenon of electroporation [1], which stimulates the molecular transport system and the planar mechanical rupture of the membrane [2]. Currently, the phenomenon is attracting a great interest because of its application to biotechnology and medicine. Electroporation is used as a simple and relatively non-toxic method for introducing exogenous macromolecules (such as DNA, RNA, proteins, antibodies, drugs, and fluorescent probes) into cells of various types [2]. Novel therapeutic methods in cancer treatment can employ electroporation to enhanced topical and transdermal local delivery of chemotherapeutic agents to solid tumors [3]. Electro-chemotherapy (ECT), used for drug delivery, is a new and very promising technique. Recent studies show that ECT can provide an effective treatment of tumors of vital organs like brain, liver, and lungs by means of intra-tumoral injection of low dosage of otherwise marginally effective chemotherapeutic drugs [4]. Also a gene therapy of cancer using electroporation method is being examined and raises a great hope [5]. However, the use of electroporation is limited by incomplete understanding of this phenomenon. Research results on this field may be used to optimize electroporation protocols to enhance DNA uptake and its subsequent expression or improve ECT methods. This can be especially interesting when safe long duration electroporation (LDE) methods can be introduced into therapy [6], which has not been achieved, yet. Electroporation has also been observed during large defibrillation shocks applied to the cardiac tissue [7], and may prove lethal in severe cardiac ischemia cases. Therefore, it is vital to examine how changes in the membrane structure affect the membrane behavior after electroporation.

The knowledge on molecular phenomena responsible for the electroporation is still very limited. Therefore, the use of electroporation for laboratory chemotherapy and transfection is not very widespread. We lack knowledge on the effects of electroporation parameters on molecular uptake and cell viability [8]. We do not know details on microscopic structural rearrangement that take place in membranes during pore formation, contraction and disappearance.

The stochastic nature of pore dynamics could be confirmed only when the current-clamp method was first introduced [9,13]. Unlike voltage-clamp technique, in which the membrane lifespan is very short with a high chance for an irreversible breakdown within milliseconds, the new current-clamp method is the only method in which an electroporated membrane can survive over 1 hour (for high currents an average membrane lifespan decreases), and a stochastic behavior of fluctuating electropores can be observed. The stochastic process underlying electropore fluctuations proved non-stationary

[14] due to the stochastic trend and variable variance, which makes the standard analysis less reliable. However, application of the classical decomposition method improves the stationarity, which can be quantitatively controlled by means of the reverse arrangements statistical test [14]. The spectral analysis of the data showed that electropore fluctuations produce $1/f$ noise at low current intensities with a very interesting dependence on electropore dynamics resulting from the membrane fluidity and the ionic strength of the forming solution. Also the memory of the membranes could be examined.

The reason for the increased lifespan of the membrane under current-clamp conditions lies in a feedback mechanism that decreases the transmembrane potential when pores open, followed by a significant increase in the conductance. This mechanism prevents the membrane breakdown and increases the average lifespan. Therefore, current-clamp technique may provide us with a new safe method for ECT drug delivery. The use of electroporation protocols based on current-clamp approach can also help to design more efficient LDE methods that guarantee longer time for introduction and subsequent expression of DNA in cells. It is very important, however, how the presence of physiological compounds affect the membrane response to the electroporation. In this paper we examine how cholesterol affects the membrane characteristics and susceptibility to the electroporation. To distinguish the effect of ionic strength of the forming solution, two different concentrations of KCl were tested.

Cholesterol is an aromatic compound with a well-developed side chain and a hydroxyl group (Fig. 1). In lipid bilayer membranes cholesterol molecules assume perpendicular orientation to the membrane surface in such

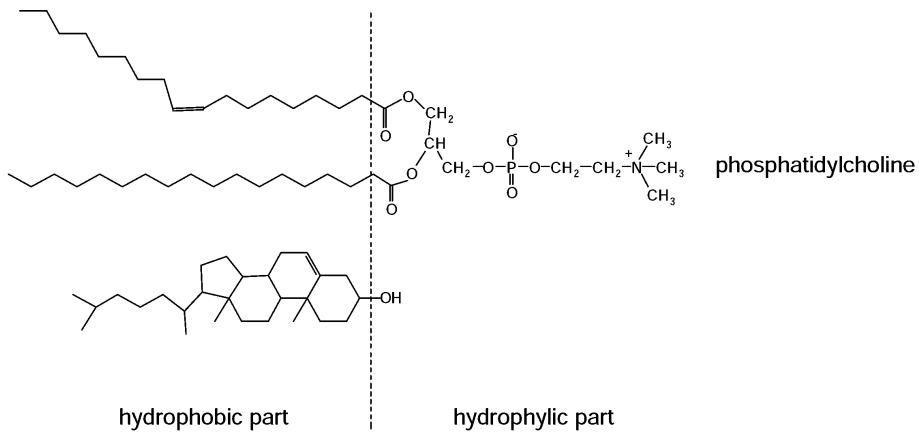

Fig. 1. Structure of cholesterol molecule.

a way that the hydroxyl group is in the immediate vicinity of the phospholipid carbonyl. The cholesterol rings are oriented in parallel to the hydrocarbon chains of the phospholipids. Such orientation of cholesterol molecules restricts free movement of the phospholipid chains. Rigid structure of the cholesterol molecule increases stability of the membrane through improving order of other membrane molecules and suppressing their mobility. High cholesterol content in the membrane, above the gel temperature, decreases its fluidity. Conductivity of the membrane with cholesterol for small cations is also very decreased since cholesterol molecules impede creating of the membrane defects. For the same reason, membranes with cholesterol need higher potential for electroporation [11], which is closely related to randomly emerging defects [12, 16]. The cholesterol molecule does not fit well in the curvature of the pore, and it increases the energy consumed by the pore creation. As an effect the pore diameter is smaller [11]. Cholesterol is a natural component of biological membranes [15], where it improves stability by preventing thermal and mechanical rupture.

Experiments show that cholesterol improves significantly the membrane ability to survive for long time. The same result is caused by the increase in ionic strength. Although the mechanism underlying this effect is identical in both cases, electropores of much smaller diameter are created, properties of these two types of membranes are not identical. The membranes with cholesterol cannot sustain high current intensities ($I > 1$ nA), while membranes formed at higher ionic strength can endure the current up to 50 nA. This difference stems from their fluidity. In this paper we examine how ionic strength and cholesterol affect other properties of the membranes and electropore dynamics.

We compared characteristics of pure lipid planar bilayer membranes at physiological concentration of KCl (0.1 M), the lipid membrane at non-physiologically high, 2 M, concentration of KCl, and the membrane with cholesterol added at its physiological concentration (at 0.1 M KCl). Conditions for the flicker noise to appear and its sensitivity to the presence of cholesterol, ionic strength, current intensity, and membrane ageing were examined. The membrane memory was analyzed by means of autocorrelation function and rescaled range analysis.

2. Materials and methods

Egg yolk phosphatidyl-choline (PC) was purchased from Fluka (Buchs, Switzerland), *n*-decane from Aldrich (Gillinghem-Dorset). Analytical grade KCl was obtained from POCh (Gliwice, Poland). Forming solution for bilayer membranes contained lipids (20 mg/ml) dissolved in *n*-decane. Two types of the electrolyte were used 0.1 M KCl and 2 M KCl, which were

buffered with Hepes (Aldrich, Gillinghem-Dorset) to pH = 7.0. Ultrapure water was prepared with a Milli-Q system (Millipore). Experiments were performed on planar bilayer membranes formed in an aperture of septum separating two electrolyte solutions. The membranes were formed by Mueller-Rudin method in a vessel made of one-piece Teflon, which consisted of two chambers, 10 cm^3 volume each. The septum between the chambers was 0.3 mm thick and the aperture diameter was 1 mm. The process of spontaneous membrane formation was monitored by the membrane capacitance recording, and by visual observation of transmitted light. The experiments were performed at temperature of 23–25° C. Data of 16000 points in each series were collected with the sampling frequency of 100 Hz, which is justified by the signal band [14]. Before the analysis first 2000 points of each series were cut off to allow time for a pore formation or the system to reach equilibrium if the membrane already had a pore. The applied current values ranged from 0.2 nA to 50 nA for membranes at 2 M KCl, and from 0.2 nA to 1 nA for membranes at 0.1 M KCl. The data were differenced with step 2, which improved the stationarity level by subtracting the stochastic trend [14] and removed artifact component of 50 Hz and its multiple.

Chronopotentiometry measurements were performed with four-electrode potentiostat-galvanostat described in earlier paper [17]. The measuring system is fully controlled by PC computer and software working in Windows TM (Microsoft) environment. The system uses two pairs of Ag–AgCl electrodes. One of these pairs applies direct constant intensity current. They are connected to the current supply. Specialized software designed by one of the authors for the purpose of this measurement method controls current supply connected to 12-bit digital-to-analog converter. Two other electrodes connected to the amplifier of high input resistance, measure the transmembrane voltage, which is converted by the 12-bit analog-to-digital converter. Input resistance of the amplifier is higher than $10^{12}\,\Omega$ and input offset current is lower than 0.5 pA.

3. Experimental results

The chronopotentiometric characteristics of lipid bilayer membranes under current-clamp conditions were registered for several direct current values. Some measurements were conducted at the same current value until irreversible breakdown of a membrane, which provided us with relatively long time series. Other measurements were taken in several stages for different current values, in increasing sequence, for the same membrane. Our objective was comparative analysis performed on the data from the same membranes, without averaging on stochastic properties of the membrane [18–20]. Fig. 2 shows a typical chronopotentiometric curve. The curve

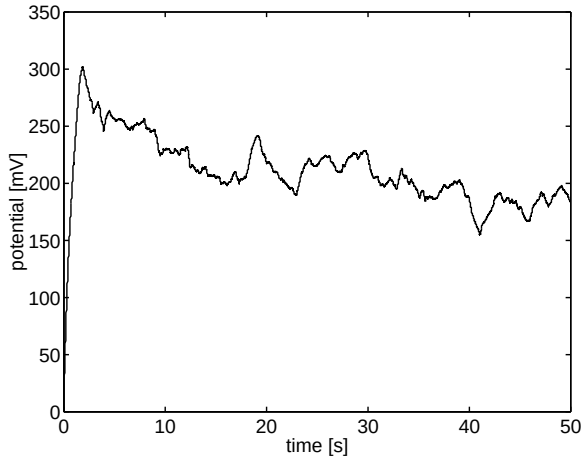

Fig. 2. A chronopotentiometric curve for the membrane with cholesterol (0.1 M KCl) showing dependence of transmembrane potential fluctuations on time, under current-clamp conditions (0.4 nA). An exponential rise of the voltage is followed by rapid decrease of the potential (4 s), which indicates electroporation. The pore fluctuations can be observed afterwards.

begins with an exponential rise of the voltage, for which membrane capacitance is responsible. Then, a sudden decrease of the voltage can be observed. This effect reveals a pore formation. The estimation of pore diameter [10] indicates that usually a single pore is formed, and short breaks in current supply do not let the pore re-seal. The stage of pore formation is followed by stochastic voltage oscillations, which reflect pore size fluctuations. Typically, the curve shape depends strongly on the current value. For low current values no electroporation takes place and the voltage rises exponentially to a constant value. It should be stressed that results similar to those received for membranes at very low current values (no electroporation) were obtained for passive RC circuit, with electric parameters corresponding to the typical planar bilayer membrane formed in our laboratory, for low and high current values. This result reassured us that the registered voltage fluctuations originate in the electroporated membrane processes. We were also very careful so as not to introduce any ionophore into the membrane by an accidental contamination.

4. Memory of the electropore

Estimating autocorrelation function by sample autocorrelation function brings information on the memory of the process. Very slow decrease of the autocorrelation function may also suggest non-stationarity of the process.

After differencing the electropore time series, which remarkably improved the stationarity level [14], the autocorrelation function decrease is significantly faster. The autocorrelation function range can be calculated from the Bartlett's formula. The autocorrelation function, is negligible for $h > q$, *i.e.* with probability 0.95 falls between the bounds

$$\pm 1.96 \sqrt{\frac{(1 + 2\rho^2(1) + ... + 2\rho^2(q))}{n}}.$$

In practice $\pm 1.96\sqrt{n}$ bounds can be used. The same formula can be used for partial autocorrelation function, where the partial correlation function between X_t and X_{t-k} is the correlation function between these two random variables, provided that all variables in the intervening time

$$\{X_{t-1}, X_{t-2}, ..., X_{t-k+1}\}$$

are fixed. If the range of autocorrelation function or partial autocorrelation function is small enough we may try to represent the process by linear ARMA model.

Linear Auto-Regressive Moving Average (ARMA) models [21] include Moving Average (MA) and Auto-Regressive (AR) components. In case of a non-stationary process, which can be made stationary by differencing transform, Auto-regressive Integrated Moving Average (ARIMA) models can be applied. Time series X_t is an ARMA(p, q) process if X_t is stationary and if for every t holds:

$$X_t - \phi_1 X_{t-1} - ... - \phi_p X_{t-p} = Z_t + \theta_1 Z_{t-1} + ... + \theta_p Z_{t-p}\,, \qquad (1)$$

where $\{Z_t\}$ represents white noise with mean 0 and variance σ^2, and the polynomials $(1 - \phi_1 z - ... - \phi_p z^p)$ and $(1 + \theta_1 z + ... + \theta_p z^q)$ have no common factors. Left side of equation (1) represents auto-regressive (AR(p)) part, and right side moving average (MA(q)) component of ARMA (p, q) process. Estimation of the linear model order is possible by evaluation of orders of MA and AR components on the basis of the range of autocorrelation and partial autocorrelation functions derived from Bartlett's formula. Linear modeling is often arbitrarily employed to reduce the problem in expressing the finite series by means of linear white noise parametric functions. Given a detailed model, it may be easier to find a physical interpretation of its components and general properties of the process. Also, a possible prediction of the data series future values can be performed.

It could be observed that neither correlation nor partial autocorrelation function of the differenced fluctuation process falls into the range sufficiently (Fig. 3). It means that the electropore dynamics cannot be modeled

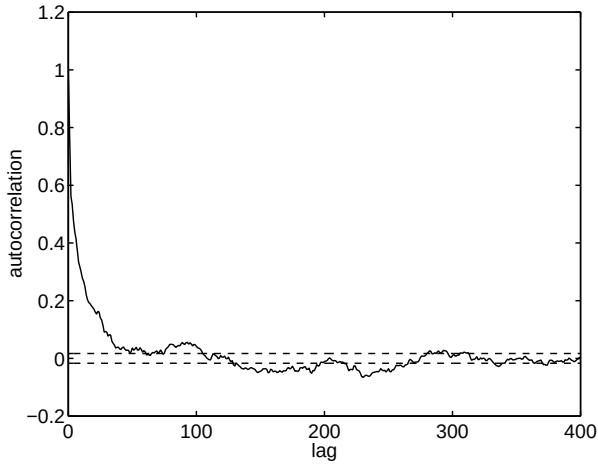

Fig. 3. Autocorrelation function of the membrane with cholesterol (solid line), and the negligibility range derived from the simplified Bartlett's formula (broken line).

by means of a linear ARMA process of a reasonably low order. For both functions, however, there are lag values above which correlation and partial autocorrelation functions oscillate around bounds of the negligibility range. In the following, these values, which proved sensitive to the measurement conditions, will be termed a correlation (partial correlation) range.

Estimating autocorrelation function by sample autocorrelation function, it was observed that the autocorrelation range depends strictly on the current value (Fig. 4), it is significantly lower for high currents. It is more pronounced for the lipid membranes at 2 M KCl. Moreover, the value of autocorrelation range is more repeatable for high currents. Since the autocorrelation function reflects memory of an investigated system, it can be concluded that electropore dynamics has significantly better memory for lower currents. No dependence between correlation range and time was detected.

Analysis of the autocorrelation function showed its oscillations around the bounds of negligibility up to the very large lag. Therefore, we could not model the process with any linear ARMA process. On the other hand the behavior of the autocorrelation function did not bring a definite answer whether the process has really long range correlations. Therefore, another tool was employed — the rescaled range analysis (R/S) invented by Hurst [23]. This heuristic method for analyzing processes with long memory compares the correlations measured at different time scales. For the time series $x = \{x_k\}_{k=1}^{N}$ and any $2 \leq n$, it can be defined:

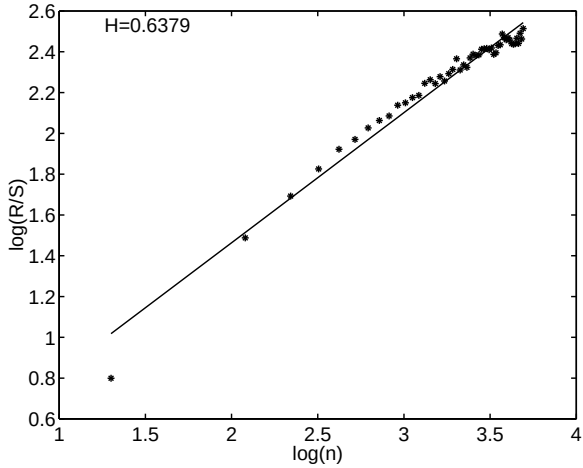

Fig. 4. Evaluation of Hurst exponent, for $I = 0.2$ nA (membrane with the cholesterol), by means of the rescaled range analysis. The slope value $H = 0.64$ indicates a persistent process.

$$\langle x \rangle_n = \frac{1}{n} \sum_{i=1}^{n} x_i \,, \tag{2}$$

$$X\,(i,n) = \sum_{u=1}^{i} \left(x_u - \langle x \rangle_n \right) , \tag{3}$$

$$R(n) = \max_{1 \le i \le n} X(i,n) - \min_{1 \le i \le n} X(i,n) \,, \tag{4}$$

$$S(n) = \left[\frac{1}{n} \sum_{i=1}^{n} \left(x_i - \langle x \rangle_n \right)^2 \right]^{1/2} , \tag{5}$$

$$\frac{R(n)}{S(n)} \sim \left(\frac{n}{2} \right)^H . \tag{6}$$

As a result the Hurst exponent H, $0 < H \le 1$, is obtained, whose value brings the information on the nature of the system correlations. For the series generated by Independent Identically Distributed random variables (IID noise) the Hurst exponent takes the value $H = 0.5$. The exponent $H > 0.5$ informs us of the long-term memory of the process. The process is termed persistent, which means that an increase of the series is more likely to be followed by another increase, and the same tendency occurs for the decrease. By contrast $H < 0.5$ means antipersistent process, where

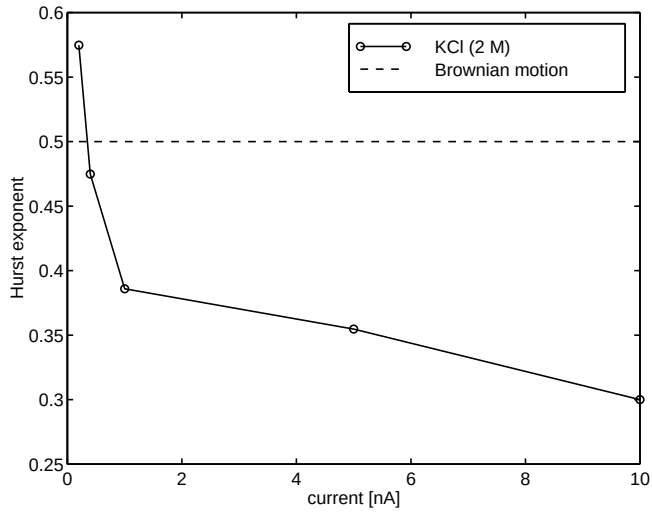

Fig. 5. Dependence of the Hurst exponent on the current value of the lipid membrane at 2 M KCl, showing transition from the persistent process, through Brownian motion, to antipersistent process for higher currents.

the tendency is reversed. However, then the Hurst exponent cannot be interpreted in such an easy way and we can only say that the process changes the direction more often than the Brownian motion during the same time interval.

The Hurst analysis performed on the series reflecting electropore dynamics showed that at low current intensities all membranes showed slightly persistent type of noise behavior (Fig. 5), with crossover to Brownian type of noise for higher current value. This transition was much faster for lipid membranes at 2 M KCl, where the next transition to antipersistent response was observed for relatively low currents (Fig. 6). Hurst exponent was maintained constant throughout the lifetime of the membranes at 2 M KCl, but it showed a slight decrease of value for the membranes at 0.1 M KCl without regard to the cholesterol presence, which indicates change of the memory characteristics related to ageing of the electropores in membranes at lower ionic strength.

Studies on the distribution function for moderate current values by means of histogram of the series [14] indicate Gaussian distribution of the differenced voltage fluctuations with mean 0. The identical distribution for the whole time series, regardless of the current intensity and stationarity level of the interval, means that, however, the series is non-stationary in general sense, it is stationary in the distribution sense, which is sufficient for power spectrum analysis and explains insensitivity of PSD function on the chosen interval.

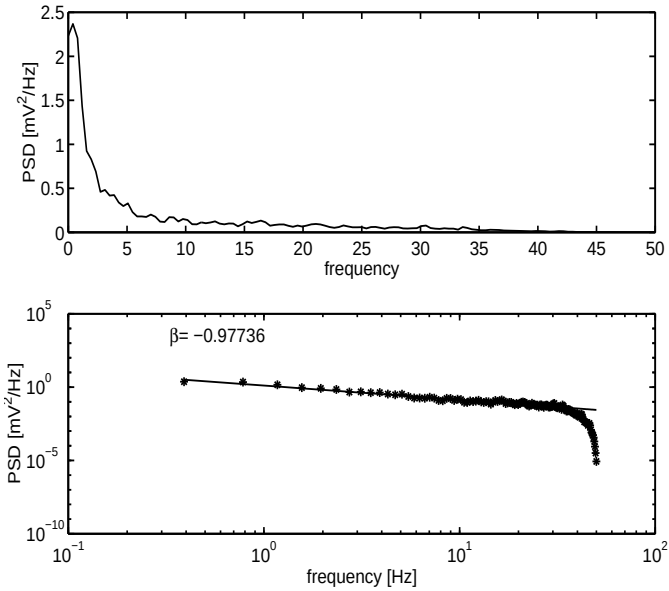

Fig. 6. The power spectrum density (PSD) function, for $I = 0.2$ nA (membrane with cholesterol), in linear and log–log scale showing f^{β} dependence. The slope $\beta \sim -1$ indicates a $1/f$ type of noise.

5. Disappearance of flicker noise

The spectral analysis, which applied for stationary stochastic processes, is the analoge of the Fourier representation of deterministic functions, brings about essential information on the process. It complements the time domain analysis based on the autocorrelation function with the frequency domain analysis. The spectrum analysis, performed on all new membranes at low currents, show a $1/f$ type of the noise (flicker noise) for frequencies in the range of 1–40 Hz (Fig. 7). Conditions for the flicker noise to appear and disappear show very interesting dependence on electropore dynamics resulting from the membrane fluidity and ionic strength of the forming solution.

The phenomenon of $1/f$ noise attracts a great interest since the mechanisms underlying its appearance are still poorly understood. Experimental data from various systems proved that flicker noise does not result from non-optimal data collection or data processing. The flicker noise can be encountered in complex systems whose subsystems produce electrical signals significantly varying in amplitudes and characteristic time scales. Presumably these subsystems are related to each other by some scaling factors [25]. However, the specifics of this scaling, as well as properties of the simplest object still capable of producing the flicker noise, have not been discovered yet. In biology the basic level seems to be represented by various types

of biological membranes, which play essential role in ion fluxes, and control voltage gradients in the cell. Biological membranes can generate $1/f$ noise even under steady-state conditions. It was shown [26] that flicker noise can be generated by artificial planar lipid bilayer membranes with incorporated channel forming compound with fluctuating dynamics. Permanently open ion channels do not produce $1/f$ noise [25]. Similar results were obtained [27] for large aqueous pores and porous synthetic membranes. These experiments show that the flicker noise phenomenon is inherent to the membrane ion transport through the openings of high enough dynamics.

Current-clamp technique allows to observe the flicker noise from much simpler system of the biological membrane without an ionophore. Therefore, the dynamics of the electropore does not depend on the properties of the channel compound. Instead, the straightforward relation between membrane properties, pore dynamics, and flicker noise can be observed. Our experiments showed that, however, power spectrum of the electroporated membrane is always of f^{β} type, β depends on the current value [14], membrane age, and ionic strength of the forming solution. For all membranes the value of β had a rising tendency when the current value was increased (Fig. 8). Time dependence for the value of β was, however, different for each type of the membrane. For the membranes at high ionic strength the value of β did not change with time and the flicker noise was maintained throughout the whole membrane lifetime. Decrease of the ionic strength disturbed the appearance of the flicker noise, which was not observed in all intervals.

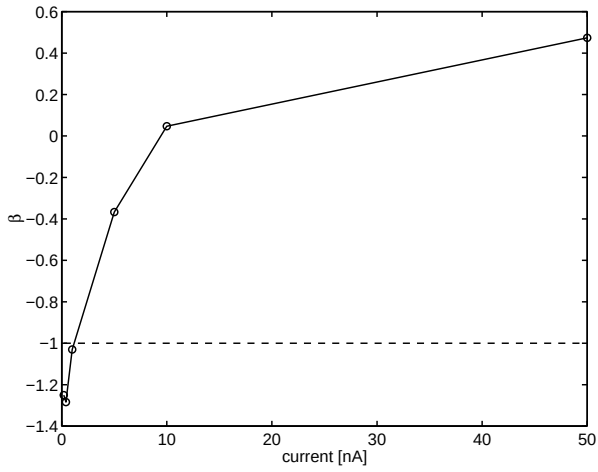

Fig. 7. Dependence of the exponent β, in the PSD function, on the current value for lipid membranes at 2 M KCl, showing disappearance of the flicker noise for higher currents.

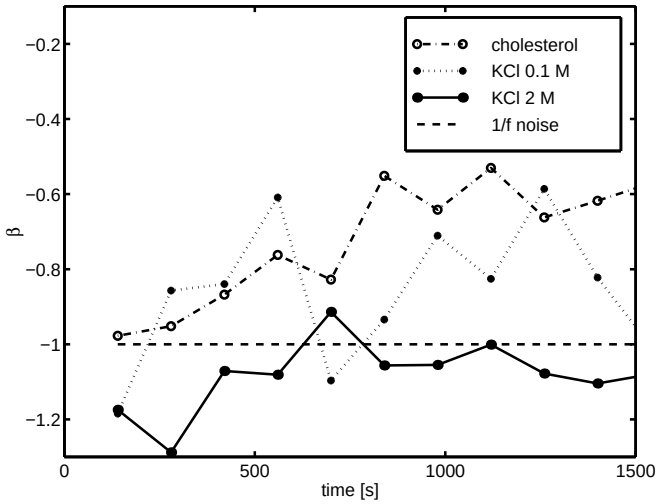

Fig. 8. Time dependence of the exponent β, in PSD function, for three types of biological membrane at 0.2 nA, showing disappearance of the flicker noise for the membranes formed at lower ionic strength.

The membranes with cholesterol showed $1/f$ noise only at the beginning of their lifetime. Afterwards, the exponent β kept increasing its value with the time.

There could be observed that reduction of the electropore diameter extends the membrane lifespan and results in better dynamics of the electropore. This dynamics may underlay appearance of the flicker noise at small currents, for all new membranes. However, only the membranes formed at higher ionic strength can keep this characteristics throughout all their lifetime. It can be concluded that decrease of the electropore dynamics can be prevented only by the elevated ionic strength. More rigid structure of the membrane, introduced by the cholesterol, accounts only for smaller electropore size, which results in extended lifespan. It does not help to maintain the electropore dynamics with time and prevent the ageing process.

6. Discussion and conclusions

In this paper, we presented an analysis of the voltage time series produced by electropores induced in planar lipid bilayer membranes under current-clamp conditions. This experimental method, which seems very promising with regard to development in medical application into ECT and LDE techniques, may also provide us with better understanding of the stochastic processes that take place in the membrane during electroporation.

We showed that linear ARIMA noise processes of reasonably low order could not model the electropore fluctuation process. Sensitivity of the system memory to the current value was proved. The rescaled range analysis revealed decreasing Hurst exponent value and transition from a slightly persistent process for low current intensities, through the Brownian process (with Gaussian PDF function), and to an antipersistent process for high currents. This transition was faster for lipid membranes at higher ionic strength. These membranes maintained also the Hurst exponent value throughout their lifetime. Membranes at lower ionic strength exhibited a slight decrease of Hurst exponent, without regard to the cholesterol presence, which indicated change of the memory characteristics related to faster ageing of the membranes with lower ionic strength.

The spectral analysis of the series demonstrated $1/f$ noise produced by electropore fluctuations at low enough current intensity, for new membranes. The flicker noise disappeared for higher current intensities and during the ageing process of the membranes at low ionic strength, which was more pronounced if the membrane fluidity was changed by the cholesterol. We can conclude therefore that however the cholesterol makes biological membranes more rigid and extends their lifespan, it does not improve the viability of the electropore. This can be achieved by the increase of the ionic strength.

The analysis of data obtained by current-clamp method provided clues to better understanding of the molecular processes of the biological membranes with regard to their fluidity and ionic strength.

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