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BREAKDOWN OF LIPID BILAYER MEMBRANES IN AN ELECTRIC FIELD

L.V. CHERNOMORDIK, S.I. SUKHAREV, I.G. ABIDOR and Yu.A. CHIZMADZHEV

Institute of Electrochemistry, Academy of Sciences of the U.S.S.R., Leninsky prospect 31, 117071 Moscow (U.S.S.R.)

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The behaviour of lipid bilayer membranes, made of oxidized cholesterol, and UO_2^{2+}-modified azolectin membranes in a high electric field has been investigated using the voltage clamp method. When a voltage pulse is applied to the membrane of these compositions, the mechanical rupture of the membranes is preceded by a gradual conductance increase which remains quite reversible till a certain moment. The voltage drop at this reversible stage of breakdown leads to a very rapid (characteristic time of less than 5 μs) decrease in the membrane conductance. At repeated voltage pulses of the same amplitude with sufficient intervals between them (approx. 10 s), the current oscillograms reflecting the reversible resistance decrease are well reproduced on the same membrane. The time of attainment of the predetermined level of the membrane conductance is strongly dependent on voltage. At different stages of breakdown we have investigated changes in the conductance of UO_2^{2+}-modified membrane after the application of two-step voltage pulses, the kinetics of development of the reversible decrease in the membrane resistance in solutions of univalent and divalent ions, and also the influence of sucrose and hemoglobin on the current evolution. The relationship between the reversible conductance increase, the reversible electrical breakdown [15] and the rupture of membrane in an electric field is discussed. We propose the general interpretation of these phenomena, based on the representation of the potential-dependent appearance in the membrane of pores, the development of which is promoted by an electric field.

Introduction

A sufficiently strong polarization of cell membrane by an electric field leads to a very significant increase in its conductance. From such a high-conductance state, the membrane can return to its initial state if the voltage across it drops rapidly. This phenomenon was termed reversible electrical breakdown [1,2]. If the amplitude or duration of a pulse are large enough, irreversible membrane damage occurs.

Interest in this class of phenomena is determined by their biological significance and by the wide scope of applications they offer. Among biomedical applications we would mention electrostimulated cell fusion [3–5], loading of cells with biologically active compounds [6], and transformation [7]. Electrical breakdown is regarded as one of the mechanisms for the loss of the barrier function of membranes in pathology [8]. The local electrical breakdown seems to play a certain part in the normal functioning of cells [8], in particular, during their fusion [9,10].

In recent years, the nature of the electrical breakdown of membranes has been extensively explored on such convenient models as suspensions of liposomes [11,12] and planar bilayers [13,14]. However, in contrast to biological membranes, in the case of lipid bilayer membranes it has been impossible for a long time to obtain any

considerable increase, induced by an electric field, in conductance which has not been accompanied by mechanical rupture of the membranes. In 1979 Benz et al. [15], using the charge relaxation method, found that during a rapid (approx. 500 ns) charging of oxidized cholesterol membranes up to approx. 1 V their resistance decreases reversibly by nearly nine orders of magnitude. Thus, for the first time in bilayers it has been possible to observe a reversible electrical breakdown. We have discovered a similar phenomenon with bilayers made of azolectin or phosphatidylserine and modified with UO_2^{2+}: with the stepwise application of voltage, the resistance of these membranes decreases reversibly by more than seven orders of magnitude up to a state of 'electrical invisibility' [16,17]. A reversible decrease in the resistance by many orders of magnitude in a strong electric field is also observed on phosphatidylcholine-cholesterol membranes in the presence of the alkaloid, holoturin A [18].

The objective of the present paper is to investigate, using the voltage-clamp method, the effect of a reversible conductance increase, i.e., the development of breakdown in two different systems – membranes of oxidized cholesterol and UO_2^{2+}-modified membranes of azolectin – and to elucidate on this basis the degree of similarity of the given phenomenon. Furthermore, using the familiar data on the phenomenology and the mechanism of rupture of bilayers in an electric field and comparing them with the results given below on the reversible conductance increase, we shall try to establish the relationship between these phenomena.

Materials and Methods

We have investigated bilayers made of azolectin (Associated Concentrates) dissolved in decane (40 mg lipid/ml decane) and membranes made of oxidized cholesterol dissolved in decane (5 mg/ml) and formed by the usual method on a 1 mm opening in the partition of a two-chamber Teflon cell at a temperature of 30°C in 10^{-2}–1 M KCl. Oxidized cholesterol was obtained by the 1.5 h bubbling of air through a cholesterol solution (Calbiochem) in decane (5 mg/ml) at 125°C. The UO_2^{2+} was introduced in the form of a solution of uranyl acetate up to a 10^{-3} M concentration into the two compartments of the cell after the full spreading of the membrane; measurements were initiated following 60–120 min incubation of the membrane in the UO_2^{2+} solution. We have used ordinary Ag|AgCl electrodes directly immersed in the solutions in the cell. A current through the membrane was measured when rectangular voltage pulses had been applied to it. This procedure is equivalent to clamping of the voltage across the membrane as long as its resistance is well in excess of the cell resistance. However, when the resistance of the lipid bilayer membrane becomes comparable with that of the cell (10^2–10^4 Ω), a considerable portion of the voltage applied to the electrodes drop in the solution rather than across the membrane. We shall consider mainly the conductance increase until the membrane resistance reaches a value of 10^6 Ω.

In Refs. 15 and 19–21 the charge relaxation method was used to investigate the reversible breakdown of oxidized cholesterol membranes. This method is characterized by a high time-resolution: within tens of nanoseconds the membrane is charged to a high voltage and then a membrane discharge due to the breakdown is monitored with a resolution of approx. 150 ns. In this case, however, the voltage across the membrane is varied continuously. By this method the voltage dynamics is recorded only at a sufficiently low (up to 5–50 Ω) membrane resistance. The breakdown proper occurs just before the start of recording the discharge: the measured curves of discharge of oxidized cholesterol membranes are strictly exponential, which corresponds to the invariability of their resistance during the measurement time [15].

The voltage clamp method we have used can give additional information on the nature of the reversible breakdown. Its rate of response is limited by the cell time constant $\tau = RC_m$ where R is the total resistance of the measuring system and of the cell without the membrane and C_m is the membrane capacitance. The τ usually constitutes 5–10 μs; consequently, the voltage clamp method is far inferior in the rate of response to the charge relaxation method. However, the voltage clamp method has its advantages because it enables one to monitor changes in the membrane resistance

continuously (up to a cell resistance of about 1 kΩ).

At fairly long pulses, a PAR-175 (Princeton Applied Research) generator served as a voltage source; the current was measured by a Keithley-427 amplifier; for current recording use was made of an NTA-1024 (Orion, EMG) multichannel analyzer in the mode of a digital oscillograph, or a C8-13 storage oscillograph.

For obtaining voltage pulses with a duration of less than 100 μs and for recording of current, use was made of a special potentiostat developed in the Institute of Electrochemistry of the USSR Academy of Sciences by V.V. Kushnev. (The scheme of potentiostatic measurements is essentially equivalent to the voltage-clamp schemes employed in electrophysiological measurements on cell membranes.)

When a train of voltage pulses was applied to membranes, the intervals between pulses were no less than 1 min.

Note that, although the general picture and all the investigated regularities of development of the reversible conductance increase on all membranes of the same composition are qualitatively reproduced, quantitatively the measurement results vary markedly from membrane to membrane. Therefore, subsequently in all cases the oscillograms of the reversible resistance decrease, given in one figure, refer to the same membrane.

The capacitance of bilayers was monitored by a capacitive current with the linear sweeps of the potential at a speed of 100 V/s. For UO_2^{2+}-modified azolectin membranes, the specific capacitance was 0.3 to 0.4 $\mu F/cm^2$. The specific capacitance of oxidized cholesterol bilayer membrane was approximately 0.5 $\mu F/cm^2$.

Results

The reversible conductance increase and the subsequent mechanical rupture of membranes

Fig. 1 presents typical current oscillograms obtained after the application of 700 mV voltage pulses to an oxidized cholesterol membrane. In Fig. 1a–c a conduction current becomes dominating after 5 μs.

At the end of a pulse (20 μs) the conduction current constitutes approx. $5 \cdot 10^{-5}$ A, i.e., the conductance increases by a factor of 10^5–10^6. This drastic increase in the conductance is reversible and is fully reproduced after the repeated application of such pulses (Fig. 1b). If, however, the pulse duration is increased to 40 μs (Fig. 1c), then even at the first pulse the membrane is, as a rule, irreversibly damaged. This comes from the fact that after the application of a repeated pulse (Fig. 1d) the current through the cell is limited by the ohmic resistance of the solution in the cell rather than by the membrane.

The transition from the stage of the reversible conductance increase to the stage of the irreversible rupture is illustrated still more vividly in Fig. 2. In this run, 200 mV voltage pulses of 5 and 10 ms duration were applied to an oxidized cholesterol membrane. On a coarse scale the oscillogram (Fig. 2b, bottom) has the form typical of the breakdown of a membrane of usual composition in an electric field. However, in the case of a higher sensitivity of measurements (Fig. 2a, top) one can see that the process consists of two stages: first the conductance slowly increases * and then undergoes an abrupt jump. As in the case of membranes which exhibit no reversible resistance drop in an electric field, the lifetime of the membranes under study, i.e., the time from the instant of voltage application to the onset of membrane rupture, is a random quantity with a rather wide distribution with respect to the mean value. We stress that the lifetime does not include the duration of the stage of the mechanical membrane rupture proper [22].

Fig. 3a presents the dependence of the mean lifetime of oxidized cholesterol membranes on voltage. Each of the circles corresponds to the mean value with respect to 15 membranes, in which case the lifetime of each membrane was found by recording the current jump at a clamped voltage (see Fig. 2b). Fig. 3b shows the voltage dependence of the mean lifetime of membrane modified with UO_2^{2+} (curve 1).

We now turn to a detailed analysis of the data pertaining to the reversible conductance increase. Fig. 4 shows a current buildup with the applica-

* In sensitive measurements ($I \approx 10^{-8}$ A), marked current fluctuations are visible on both the UO_2^{2+}-modified azolectin membranes [16] and the oxidized cholesterol membranes (Fig. 2).

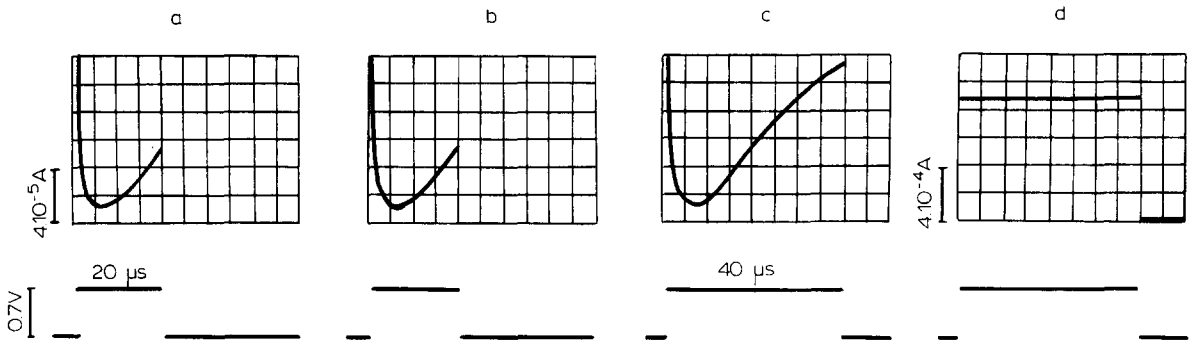

Fig. 1. Current oscillograms of oxidized cholesterol membranes with the successive application to the membrane of 20 μs ((a) and (b)) and 40 μs ((c) and (d)) voltage pulses of $U = 0.7$ V. 1 M KCl.

tion of 15 μs voltage pulses of different amplitude to the membrane. It is worth noting that the amplitude of voltage pulses in Fig. 4a (oxidized cholesterol membrane) is significantly lower than that in Fig. 4b (UO_2^{2+}-modified azolectin bilayers). It is seen from the figure that for both systems at the same pulse duration the rise of voltage magnitude increases not only the maximum value of current but also the rate of its build-up.

The kinetics of the reversible conductance increase are conveniently specified by the time of attainment of the given membrane conductance, G, at different potential values. Such dependences for UO_2^{2+}-modified azolectin membranes are depicted in Fig. 3b (curves 2, 3 and 4). Noteworthy is the fact that the time of conductance build-up decreases with voltage even more abruptly than the mean lifetime. We note that a strong dependence of the time of development of the reversible breakdown on voltage was also observed in the work by Benz and Zimmermann [19].

Of particular interest for elucidating the mechanism of reversible electrical breakdown is the process of restoration of the insulating properties of the bilayer membrane after the breakdown. Fig. 5 presents the I-t curves obtained by applying two-step pulses with the amplitude U_1 and U_2 ($U_1 > U_2$) to the bilayer membrane of both compositions under study. The oscillograms in Fig. 5 differ only

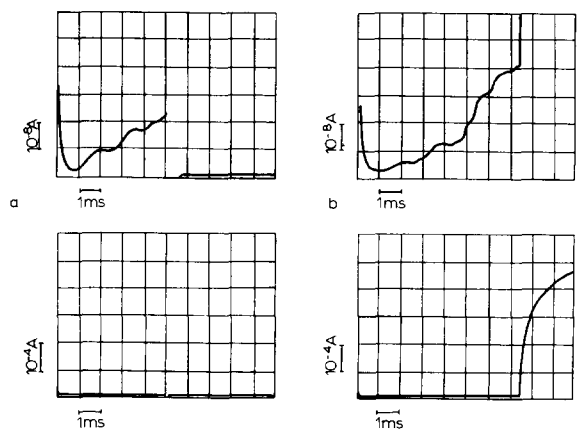

Fig. 2. Reversible increase in the conductance and the subsequent mechanical rupture of the membrane. Current oscillograms of the oxidized cholesterol bilayer membrane with the application to the membrane of voltage pulses of $U = 0.2$ V, $t = 5$ ms (Fig. 2a) and $U = 0.2$ V, $t = 10$ ms (b). Both oscillograms in (a) were obtained simultaneously on different current scales. In the oscillograms (b), obtained in a similar manner, one can see an abrupt current jump corresponding to the beginning of irreversible rupture of the bilayer. 1 M KCl.

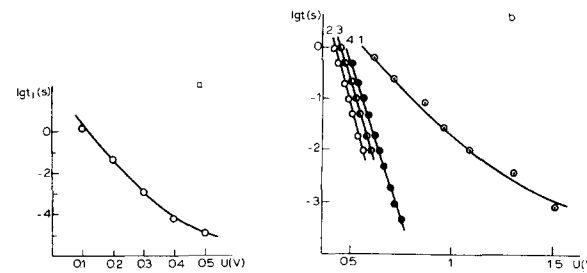

Fig. 3. (a) The logarithm of the mean (at least with respect to ten measurements) lifetime (t_1) of the oxidized cholesterol membranes as a function of voltage. 1 M KCl. (b) The voltage dependences of the logarithm of the lifetime (curve 1; to each point there corresponds the mean with respect to ten measurements) and of the logarithm of the time (t_G) taken for attainment of the given level of conductance G: $1.38 \cdot 10^{-8}\ \Omega^{-1}$ (curve 2); $3.85 \cdot 10^{-7}\ \Omega^{-1}$ (curve 3); and $1.46 \cdot 10^{-6}\ \Omega^{-1}$ (curve 4) for the UO_2^{2+}-modified azolectin membrane. Curves 2, 3 and 4 were obtained on one membrane. 0.1 M KCl.

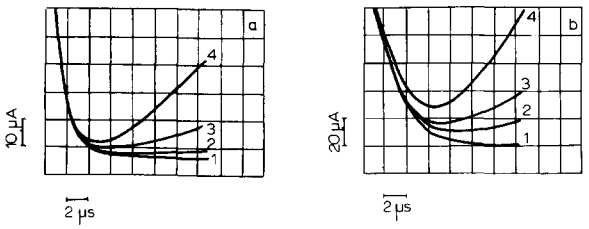

Fig. 4. Current oscillograms during the reversible increase in the bilayer membrane conductance in an electric field. (a) The oxidized cholesterol membrane; 1 M KCl; the voltage across the membrane, U, is 0.60 V (1), 0.64 V (2), 0.68 V (3) and 0.72 V (40). (b) The UO_2^{2+}-modified azolectin membrane; 1 M KCl; the voltage across the membrane is $U = 1.20$ V (1), 1.23 V (2), 1.26 V (3) and 1.30 V (4).

in that in the former case (a) the capacitive current component, which is dominant at the start of the first and second pulse steps, is much better resolved. It is evident from Fig. 5 that with a voltage jump from U_1 to U_2 the current drops, whereupon it continues to decrease gradually or, conversely, to increase as a function of the U_2 value. The stepwise transition from U_1 to U_2 is accompanied by a very rapid ($\tau \approx 5$ μs) increase in the membrane resistance, in which case the difference between the resistance values before and after the voltage jump increases with decreasing U_2 [16]. Benz and Zimmermann [20] have obtained a value of 2 μs for the characteristic time of restoration of the insulating properties of oxidized cholesterol bilayer membrane after reversible breakdown at 20°C.

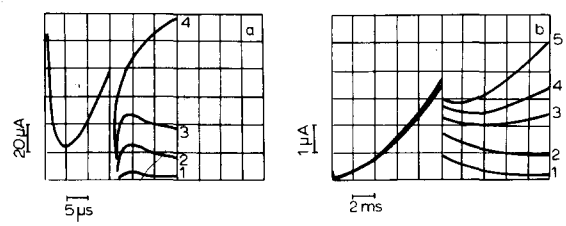

Fig. 5. Changes in the bilayer membrane conductance with the stepwise voltage decrease. (a) Electrical pretreatment of the oxidized cholesterol membrane by a voltage pulse of $U_1 = 0.80$ V before the application of a voltage pulse of $U_2 = 0.30$ V (curve 1), 0.45 V (2), 0.60 V (3) and 0.75 V (4). The pulse durations t_1 and t_2 are 15 μs. 1 M KCl. (b) A similar experiment on the UO_2^{2+}-modified membrane. $U_1 = 0.72$ V, $U_2 = 0.50$ V (curve 1), 0.60 V (2), 0.65 V (3), 0.67 V (4) and 0.68 V (5). The pulse durations t_1 and t_2 are 10 ms. 0.01 M KCl.

The dynamics of the bilayer conductance

Consider the results obtained from the comparison of the membrane conductance at different stages of the reversible breakdown.

Important information on the nature of the reversible breakdown can be extracted by studying the currect dynamics at two-step voltage pulses with a different relation between the durations of the first and second steps. A two-step pulse, $U_1 =$ 600 mV and $U_2 = 500$ mV, was applied to a UO_2^{2+}-modified azolectin membrane. The duration of the first step, t_1, was varied from 5 to 500 ms. We measured the current I_1, at the end of the first step, and the current I_2, at the start of the second step, and then calculated the nonlinearity factor $\alpha = I_1U_2/I_2U_1 = G_1/G_2$ where G_1, G_2 are the respective conductances. Fig. 6 presents the dependence of α on the logarithm of the duration of the first voltage step, t_1. It is evident from Fig. 6 that α is markedly dependent on the duration of the prepulse, in which case, with an increase in the latter, $\alpha \to 1$.

It is interesting to elucidate how the kinetics of development of the reversible resistance decrease depend on the charge of current-carrying ions. For this purpose we have set up an experiment on UO_2^{2+}-modified azolectin membranes in an unsymmetrical system: in one compartment of the cell there was a K_2SO_4 solution and in the other an $Mg(NO_3)_2$ solution (Fig. 7). The experiment was carried out as follows. Membranes were

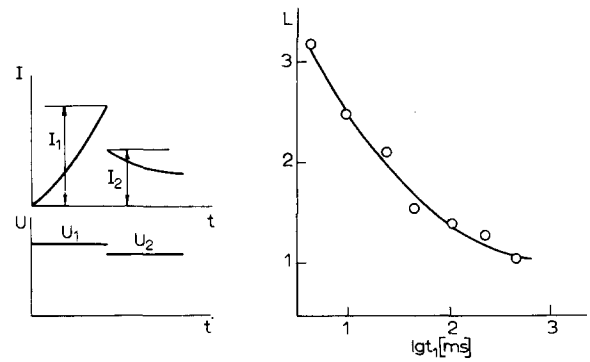

Fig. 6. Dependence of the nonlinearity factor α, reflecting changes in the conductance of the UO_2^{2+}-modified membrane with the stepwise voltage decrease, on the logarithm of the duration of the first step, t_1. $U_1 = 0.6$ V, $U_2 = 0.5$ V and $t_2 = t_1$. 0.1 M KCl.

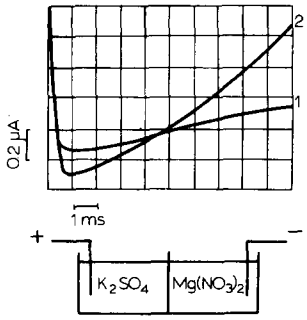

Fig. 7. Current oscillograms with the application of rectangular voltage pulses to the UO_2^{2+}-modified bilayers. On one side of the membrane there is a 0.1 M K_2SO_4 solution and on the other, a 0.1 M $Mg(NO_3)_2$ solution. At $U = +0.4$ V (as shown in the figure) the current through the membrane is determined by singly charged ions (curve 1) and in the opposite polarity ($U = -0.4$ V), by doubly charged ions (curve 2).

formed in 10^{-4} M KCl. After the bilayer formation uranyl acetate was introduced symmetrically into the cell. After 30 min, K_2SO_4 and $Mg(NO_3)_2$ were introduced, respectively, into the different cell compartments up to a concentration of 0.1 M. In this case, the membrane surface potential difference measured by a potentiodynamic method [23], was close to zero. Obviously, under these conditions with the application of a voltage, depending upon its polarity, the membrane current is mainly determined by only univalent or only divalent ions. Fig. 7 presents the oscillograms of the reversible current buildup after the application of $U = 400$ mV (curve 1) and $U = -400$ mV (curve 2) to the membrane. It appears from the figure that in the region of short times the univalent ions pass through the membrane significantly better. However, the rate of current buildup for the divalent ions is appreciably higher. Table I lists the voltage values at which in a unit of time the conductance of the modified bilayer reaches the same value ($9.09 \cdot 10^{-7}$ Ω^{-1}). In the low voltage region, the current of doubly charged ions is built up more rapidly than that of singly charged ions, and vice versa in the high voltage region. These results agree qualitatively with the data obtained by Benz et al. [15], who, using very short charging times (approx. 500 ns), have found that in the case of doubly charged ions the reversible breakdown develops at significantly higher voltages than in the case of singly charged ions (1.7 V against 1 V).

We have also investigated the influence of sucrose (molecule radius approx. 4.3 Å) and hemoglobin (approx. 30 Å) on the change in the membrane conductance in the process of development of the reversible resistance decrease. In 2, 3, 5 and 10 min after the introduction of sucrose into the cell up to a 75 mM concentration *, voltage pulses of $U = 1230$ mV and $t = 20$ μs (current oscillograms 2, 3, 4, and 5 in Fig. 8a) were applied to a membrane modified with UO_2^{2+}. In addition, in 1, 4, 6 and 11 min after the introduction of sucrose, voltage pulses of $U = 620$ mV and $t = 10$ ms (oscillograms 2, 3, 4 and 5 in Fig. 8a) were applied to the same membrane. Current oscillograms 1 (Fig. 8a,b) were obtained before the introduction of sucrose. As is evident from Fig. 8, at short voltage pulses the addition of sucrose leads to a very significant decrease in the membrane conductance value at the end of a pulse (by a factor of approx. 3.3). At the same time, at a longer 10 ms pulse the current through the membrane is decreased by only 1.2-times. Similar results have also been obtained on oxidized cholesterol membranes: here also the blocking effect becomes pronounced at short times only.

* Preliminary experiments have shown that such an amount of sucrose does not affect the background conductance and capacitance of the membrane.

TABLE I

THE AMPLITUDES OF VOLTAGE PULSES (U) OF VARIOUS DURATION, REQUIRED FOR OBTAINING THE CONDUCTANCE $G = 9.09 \cdot 10^{-7}$ Ω^{-1} FOR UNIVALENT AND DIVALENT IONS, IN mV

	Pulse duration (ms):					
	1	2	10	20	50	100
K^+, NO_3^-	440	418	390	382	368	353
Mg^{2+}, SO_4^{2-}	486	460	402	380	350	330

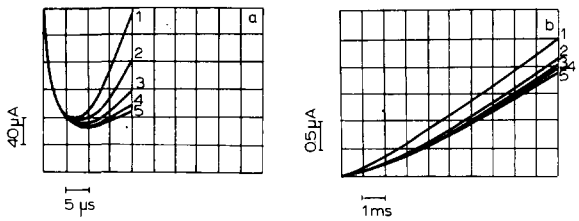

Fig. 8. Influence of sucrose on the current buildup with the reversible increase in the membrane conductance. Current oscillograms in response to pulses of $U = 1.23$ V and $t = 20$ μs in 2 (curve 1), 3 (2), 5 (3) and 10 (4) min after the introduction of sucrose up to a 75 mM concentration (a) and to pulses of $U = 0.62$ V and $t = 10$ ms in 1 (1), 4 (2), 6 (3) and 11 (4) min after the introduction of sucrose.

If, in place of sucrose, 20 μM hemoglobin is introduced into the cell, then the kinetics of the reversible increase in the conductance of UO_2^{2+}-modified azolectin bilayers slow down appreciably only at relatively long times (approx. 10–100 ms). At voltage pulses of 20 μs duration, the current oscillograms in the presence of hemoglobin remain practically unchanged. The strong destabilizing effect of hemoglobin did not allow us to carry out analogous experiments on oxidized cholesterol membranes.

Discussion

The results presented here show that a significant reversible decrease in the resistance of oxidized cholesterol membranes can be observed not only at high voltages (about 1 V) and short times (under 10 μs) [15], but also at relatively long times (approx. 10 ms) and essentially lower voltages. In addition, the reversible breakdown of these membranes is described by common regularities at different ranges of times and voltages. In the realisation of reversible breakdown on the membranes of other compositions, the noticeable quantitative differences are observed. Nevertheless, the comparison of the results obtained from a study, using the voltage-clamp method, of the reversible increase in the conductance of UO_2^{2+}-modified membranes and oxidized cholesterol bilayers as well as the investigation of the reversible decrease in the resistance of cholesterol-containing membranes in the presence of holoturin, performed previously by the same method [18], are indicative, in our opinion, of the similarity of the phenomena considered. The effect of voltage pulses of adequate amplitude and duration on the bilayers of these compositions leads to a very significant continuous and quite reversible increase in their conductance. At repeated voltage pulses with sufficient intervals between them, the current oscillograms of the reversible resistance decrease are reproduced with high accuracy. A nonlinear dependence of current on voltage at the equal time of the field effect is characteristic of all systems investigated. With decreasing voltage across the membrane there occurs a very rapid drop in the membrane conductance. If the voltage across the membrane is maintained for a fairly long time, the reversible conductance increase is followed by an irreversible rupture of the membrane. What is the nature of these phenomena?

The breakdown mechanism – pore development

Exploring the mechanism of reversible membrane conductance increase in electric field, we were interested first of all in a scale of changes in membrane structure during the breakdown, especially during its initial stage. It is evident that a large-scale transformation (i.e., changes in membrane area, thickness or dielectric constant) must be coupled with changes in membrane capacitance. In Ref. 17, measurements were made of the capacitance of UO_2^{2+}-modified azolectin membranes at that stage of the process when the conduction current is still small compared with the capacitive current. In this case it was found that the membrane capacitance in the course of the reversible resistance decrease remains unchanged to within 2%. This unambiguously implies that the whole point is in local defects such as through pores. Estimation from the current value even in the state of 'electrical invisibility', i.e., at a resistance of about 10^4 Ω, gives only a value of approx. 10^{-8} cm^2 for the total area of such pores, which is much less than the area of the whole membrane (approx. 10^{-3} cm^2). The reproducibility of the current oscillograms at the reversible stage with the application of sequential voltage pulses (Fig. 1a,b) and the absence of visible fluctuations at relatively large currents indicates that the law of large numbers is valid: a great number of defects appear in the membrane. The number of defects

and the rate of their accumulation depend on the voltage applied and the time of its effect. At the earlier stage of current evolution ($I \approx 10^{-8}$ A) fluctuations are noticeable, that is, the number of pores at the beginning is relatively small.

The results presented here indicate that the buildup of current with the reversible decrease in the membrane resistance is associated not only with the increase in the number of pores but also with the increase in their mean radius. This conclusion has been reached using three experimental approaches. We have investigated (i) changes in the nonlinear properties of the membrane conductance during the reversible current buildup, (ii) differences in the membrane conductance for univalent and divalent ions, and also (iii) changes in the blocking effect of molecules of different size at the different stages of conductance increase.

(i) A vary rapid (less than 5 μs, Fig. 5a, 2 μs, Ref. 20) drop in the conductance with decreasing voltage can, in principle, be explained by: (1) the decreased number of pores; (2) the decreased mean radius of the pores; and (3) the nonlinear character of the resistance of small pores. The third of these proposed versions of explanation appears to be most probable. In fact, in Ref. 26 it was shown that the specific conductance in the pores of radius $r \leqslant 10$ Å is significantly lower than the bulk one due to the action of image forces. It is physically reasonable to suppose that a narrow pore, in terms of its conducting properties, is like a nonlinear resistor, whereas with increasing radius it becomes similar to an ohmic system. Therefore, in the case of small pores ($0 < r < 10$ Å) the voltage decrease leads to an abrupt drop in the conductance (nonlinearity factor, $\alpha > 1$). On the other hand, if the membrane conductance is determined by large pores, then it might be anticipated that with a stepwise voltage decrease the current drop should be ohmic. And, in fact, at the later stages of resistance decrease, as the contribution of large pores to the total current increases, α approaches unity (Fig. 6).

(ii) The above-mentioned ideas are also applicable to a qualitative interpretation of the data given in Fig. 7 and in Table I. As long as the mean radius remains small (the initial stage of conductance increase), the membrane conductance for univalent ions is, evidently, significantly higher than that for divalent ones [26]. With increasing pore size, the relation between the conductances for univalent and divalent ions is reversed. It may be assumed that divalent ions enhance the growth of pores [27].

(iii) One of the most direct approaches to the estimation of the radii of conducting pores in a membrane may be a study of the influence of molecules of different size on the membrane conductance [28]. If a molecule is of size comparable with a pore it can, to some extent, block the passage of ions. The different blocking effect of sucrose and hemoglobin on the reversible increase in the conductance of the membranes investigated (Fig. 8) bears out the above assumption about an increase in the mean pore radius with the buildup of conductance. In fact, the strong blocking effect of sucrose at short times points to the existence at this stage of a considerable number of pores comparable in radius with sucrose (4.3 Å). At long times, the membrane current during the reversible resistance decrease is primarily determined by the pores whose radius is comparable with that of a hemoglobin molecule (approx. 30 Å). It is not unlikely that sucrose and hemoglobin in our system are not indifferent blocking agents and may affect not only the conductance of the pores but also the kinetics of their growth since, in contrast to the channels having fixed sizes [28], the pores in our case grow with time. Here, too, however, the conductance decrease in the presence of a blocking agent can take place only if in the membrane at a given stage of breakdown development there are pores of size close to the molecular radius of the blocking agent.

Thus, a simultaneous increase in the number and size of the conducting pores is at the basis of the reversible decrease in the bilayer membrane resistance in an electric field. The rates of these processes are determined not only by the nature of the membrane but also by the amplitude and duration of voltage pulses applied to the membrane. The membrane conductance level, equal in magnitude, may correspond to different (in number and in size) populations of pores.

It should be noted that the blocking effect of membrane pores developed in electrical breakdown by molecules of sucrose (or other compounds of suitable size close to the pore diameter)

may enhance a viability of cells during electrofusion according to Zimmermann [3].

The behavior of bilayer in a high electric field

It was shown previously [13] that when a voltage step is applied to membranes of usual composition *, the stage of a rapid current buildup associated with the mechanical membrane rupture is preceded by a latent stage during which the processes occurring in the membrane affect its conductance relatively weakly (or lead to reversible changes). The duration of the latent stage, i.e., the membrane lifetime in the field, is a random quantity. Therefore the current oscillograms of breakdown of each membrane in the field are individual in character [13] and the breakdown phenomenon has to be specified by stochastic quantities, the most important of which – the mean membrane lifetime – is strongly dependent on the voltage across the membrane. The breakdown mechanism * has been explored fairly thoroughly [13,14,24]. Now there is already no doubt that the appearance and development in a membrane of structural defects such as through pores underlies this phenomenon. With respect to the formation of such structural defects, the membrane is a metastable stage. The energy of such a system, being subject to an electric field, has the form [13]:

$$W = 2\pi\gamma r - \pi\sigma r^2 - 0.5\pi C U^2 r^2 \qquad (1)$$

where r is the pore radius, $C = (\varepsilon_s/\varepsilon_m - 1)C_0$ is the change in the capacitance in the region of a defect filled with a polar medium with the dielectric constant ε_s, ε_m is the dielectric constant of the membrane, C_0 is its specific capacitance, γ is the linear tension, and σ is the surface tension. Eqn. 1 suggests that the dependence of the energy of the membrane with a defect on its radius has the form of a curve with a maximum (Fig. 9, right). The

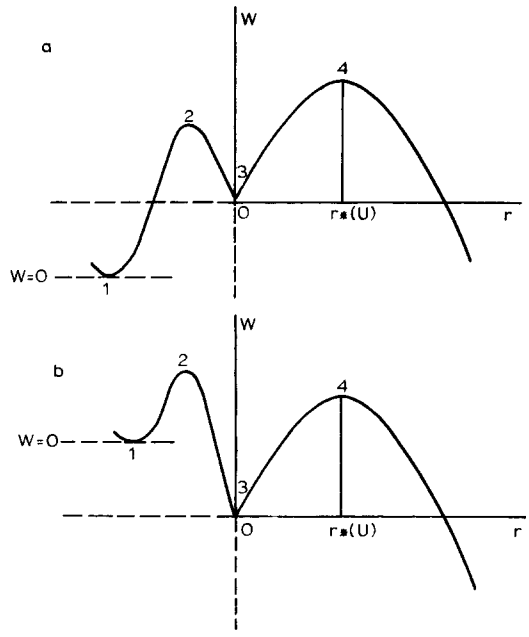

Fig. 9. Changes in the membrane energy, W, with the development of a pore for the membrane voltage U. r is the radius of an inverted pore. (1) The initial defectless membrane state $W = 0$; (2) the barrier of the inverted pore formation; (3) the state of inverted pore $r = 0$; (4) the diffusion barrier. (a) Membranes which do not show a reversible breakdown; (b) 'reversible' membranes. For further explanations see text.

height of the energy barrier and the value of the critical radius r_* abruptly decrease with increasing voltage across the membrane. When $r < r_*$, the membrane is stable but if, as a result of thermal motion of some lipid molecules, the pore size exceeds the critical value r_* the pore will begin spontaneously increasing till the membrane is disrupted. This process can be prevented if the voltage is dropped, for example, to zero as soon as the defect has reached the critical radius because $r_*(0) > r_*(U)$.

The mean lifetime of a metastable membrane having n defects appears as [24]:

$$\bar{t} = \frac{(kT)^{3/2}}{4\pi n D\gamma\left(\sigma + \frac{1}{2}CU^2\right)^{1/2}} \exp\left[\frac{\pi\gamma^2}{kT\left(\sigma + \frac{1}{2}CU^2\right)}\right] \qquad (2)$$

where D is the diffusion coefficient of a defect in the space of the radius and $\gamma \approx \sigma h$ (h is the membrane thickness), that is to say, the internal surface

* We mean membranes made of phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, azolectin, and general lipids of the bovine brain.

* Speaking about the breakdown mechanism, we mean not the later irreversible stage of the mechanical membrane rupture but, on the contrary, the latent period and the dependence of its duration (i.e., the mean lifetime) on the potential jump, and the like.

of the pores, the supercritical development of which results in a mechanical rupture of the membrane, is covered with the polar heads of phospholipids.

The above-mentioned representations are also applicable for describing the process of rupture of the 'reversible' membranes. We begin by observing that the lifetime of oxidized cholesterol membranes and azolectin membranes modified with UO_2^{2+}, as well as usual bilayers having no reversible stage of conductance increase before rupture, is a random quantity. This may suggest that rupture of the 'reversible' membranes as well as the 'irreversible' ones occurs after a single defect such as a through pore has surmounted the energy barrier 2 (Fig. 9). This is indicated by the similar character of the dependence $\bar{t}(U)$ in all the cases studied (see Fig. 3a,b and Eqn. 2).

It is possible to use this approach for the reversible breakdown interpretation. On an energy diagram (Fig. 9) in the region $r > 0$ we have a defect arranged as an inverted pore. On the left side, the barrier corresponding to such a pore formation in a defectless membrane (state 1) is shown. Since the process of pore formation is not quite clear * [13,15], this part of diagram is given only by way of illustration. The reversible conductance increase observed at some membrane compositions is probably concerned with an accumulation of significant number of pores between the barriers 2 and 4. This process requires the realisation of some particular conditions. Firstly, when the field is applied, the energy of state 3 must be lower than the energy of defectless membrane ($W = 0$) (Fig. 9b). Secondly, the height of barrier 4 has to be sufficiently large or (and) the diffusion coefficient of the defect in the space of the radius has to be relatively low. In the opposite case, the development of the first supercritical defect will precede the formation of a number of conducted subcritical pores. The switching-off of the field before the onset of mechanical rupture promotes an increase in barrier 4 and simultaneously a healing of the pores formed. Returning to defectless state 1 requires a certain time. That is why, probably, the current oscillograms during the reversible resistance decrease are reproduced only when certain intervals are maintained between voltage pulses. In fact, in Ref. 17 it was shown that, even though the characteristic times of restoration of the bilayer insulating properties after a voltage decrease are measured in microseconds (Refs. 20, 17 and Fig. 5), the application of a repeated voltage pulse of the same amplitude and duration in less than 2 s gives a larger resistance decrease for UO_2^{2+}-modified membranes than at the first pulse. It is natural to assume that this is due to the existence in the membrane of a considerable number of inverted pores of small radius with a very low conductance for some time after the pulse.

Thus, the rupture of membranes in an electric field as well as reversible breakdown can be explained within the framework of the representations outlined here.

* It is possible that the pore formation process may be relieved in the presence of molecules with a high spontaneous curvature (lysophosphatides) [29].

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