Biochimica et Biophysica Acta, 640 (1981) 169—178

BBA 79035

THE RESEALING PROCESS OF LIPID BILAYERS AFTER REVERSIBLE ELECTRICAL BREAKDOWN

R. BENZ and U. ZIMMERMANN

Fakultät für Biologie der Universität Konstanz, D-7750 Konstanz, and Arbeitsgruppe Membranforschung, Kernforschungsanlage Jülich GmbH, Postfach 1913, D-5170 Jülich (F.R.G.)

(Received May 22nd, 1980)
(Revised manuscript received August 25th, 1980)

Key words: Lipid bilayer; Electrical breakdown; Pore formation; Resealing process; Lipid diffusion

Summary

The resealing process of lipid bilayer membranes after reversible electrical breakdown was investigated using two voltage pulses switched on together. Electrical breakdown of the membranes was induced with a voltage pulse of high intensity and short duration. The time course of the change in membrane conductance after the application of the high (short) voltage pulse was measured with a longer voltage pulse of low amplitude. The decrease in membrane conductance during the resealing process could be fitted to a single exponential curve with a time constant of 10—2 μs in the temperature range between 2 and 20°C. The activation energy for this exponential decay process was found to be about 50 kJ/mol, which might indicate a diffusion process. Above 25°C the resealing process is controlled by two exponential processes.

The data obtained for the time course of the resealing process can be explained in terms of pore formation in the membranes in response to the high electrical field strength. A radius of about 4 nm is calculated for the initial pore size. From the assumed exponential change of the pore area with progressive resealing time a diffusion constant of 10^{-8} cm^2/s for lateral lipid diffusion can be estimated.

Introduction

The effect of high electric fields on cell membranes and lipid bilayers has been studied in a number of recent publications [1—5]. It has been found that both types of membranes show a dramatic increase in conductance, if the

membranes are exposed very rapidly to a voltage of the order of 1 V [1—5]. By analogy to similar effects in solid state physics, the high conductance state in membranes has been termed electrical breakdown [1]. The electrical breakdown of membranes is both reproducible and reversible [1—5], therefore, for lipid bilayer membranes it has to be distinguished very carefully from the irreversible mechanical breakdown which occurs if the membranes are kept at several hundred mV for a longer time [6].

Up to now it has not been clear what molecular event is responsible for the electrical breakdown in membranes. However, the high conductance increase (up to nine orders of magnitude in the case of the lipid bilayer membranes, i.e., from 10^{-8} to 0.1 $\Omega \cdot cm^2$ for the specific resistance of the membranes) makes it very likely that the electrical breakdown leads to a formation of pores [3]. If this is the case, the pores form in a time shorter than 10 ns [4,5]. Recently, some evidence has been presented to indicate that the mechanical breakdown (rupture) of lipid bilayers is caused by the formation of pores in response to the action of the electrical field [6]. However, it seems that for the irreversible breakdown the pores must remain open for a much longer time (milliseconds) than in the case of the reversible breakdown.

Electrical breakdown is a very useful tool for membrane research and the encapsulation of hydrophilic molecules into cells [7,8]. The latter process especially is of great importance for the loading of cells with drugs and the controlled release of these drugs in organisms without the stimulation of immunological response.

The life-time of the voltage-induced pore during the breakdown phenomenon is of great interest for the encapsulation of drugs into cells. From the charge-pulse experiments with lipid bilayer and cell membranes, only a rough estimate for the pore life-time could be derived [3]. In this publication, we describe experiments in which the decay of the membrane conductance with time after breakdown was measured. For this purpose a double-pulse generator was used. The electrical breakdown of the membranes was induced by a high-intensity pulse of short duration. A lower intensity voltage pulse of longer duration was used to measure the actual conductance of the membrane after breakdown had occurred. In addition, the resealing process was investigated as a function of temperature.

Materials and Methods

Black lipid bilayer membranes were made from a 1—2% (w/v) solution of oxidized cholesterol or oxidized cholesterol/lipid mixtures dissolved in *n*-decane. The oxidized cholesterol was prepared as described previously [3]. The other lipids (egg lecithin and brain phosphatidylserine) were isolated according to standard methods [9,10]. Only membranes from oxidized cholesterol or from oxidized cholesterol/lipid mixtures with a molar ratio greater than 2 : 1 showed reversible electrical breakdown. Membranes with a higher content of phospholipid did not survive the breakdown experiments and broke mechanically. The cell used for bilayer formation was made of Teflon; the circular hole in the wall between the two aqueous compartments had an area of about 2 mm^2 [11]. The membrane cell was attached to a thermostatically

controlled metal block, allowing the temperature in the cell to be varied between 0 and 60°C. The membranes were formed in a 1 M KCl solution (Merck, Darmstadt, F.R.G., analytical grade) in double-distilled water.

The membrane experiments were performed either with the four-electrode method (two voltage and two current electrodes) or with only two electrodes (silver/silver chloride/platinum black). However, because of a potential drop in the aqueous solution between the membrane and the electrode at high membrane conductances, the actual membrane voltage was overestimated by about 10—20% even in the four-electrode arrangement. A double-pulse generator (built in our electronic workshop) was used in the experiments. The output voltage of the short (high) pulse (with a rise time of 100 ns) could be varied between 0 and 8 V (resistance 50 Ω) and the duration between 200 ns and 8 μs. The low-voltage pulse (switched on together with the high-voltage pulse) had an output voltage between 0 and 200 mV (output resistance 50 Ω) and a duration between 10 μs and 100 ms.

The time courses of voltage and current were recorded simultaneously with a Tektronix 7633-storage oscilloscope. The actual membrane voltage was measured with a 7 A 13 plug-in amplifier (80 MHz bandwidth), whereas the current through the membrane was detected as a voltage drop across a 50 Ω resistance in series with the pulse generator and membrane with a 7 A 13 (80 MHz bandwidth) or a 7 A 22 plug-in amplifier (1 MHz bandwidth).

Results

The time course of the resealing process

The resealing process of lipid bilayer membranes after electrical breakdown was investigated in the following way. A high pulse generated by the double-pulse generator caused the electrical breakdown of the membrane. This high pulse was switched on together with a pulse of lower amplitude (of the order of 10—200 mV). This smaller pulse was used to measure the resistance of the membrane in the high-conductance state and the resealing process of the membrane with time.

The oscillographic record of such an experiment is presented in Fig. 1, in which four double pulses were applied to the same membrane. The short (high) pulse had in all runs an output voltage of 8 V, whereas its duration was varied between 300 ns (trace 1) and 500 ns (trace 4). The long (lower) pulse had in all cases an amplitude of 65 mV and a duration of 50 μs. As indicated in Fig. 1, there is almost no electrical breakdown detectable in the first experiment (trace 1). In this experiment, the curves for current and voltage reflect more or less the discharge process of the membrane from the high voltage (several hundred millivolts) down to the low value (65 mV), although there is some indication in trace 1 that a small conductance increase occurred. In the second experiment (380 ns duration for the high pulse), the membrane conductance is so high that the discharge process of the membrane is very rapid (trace 2). In addition, a high membrane current is observed, which drops down to zero within about 15 μs. The membrane conductance is so high that the voltage across the membrane cannot be kept at 65 mV. These findings for current and voltage are more strongly pronounced in traces 3 and 4, indicat-

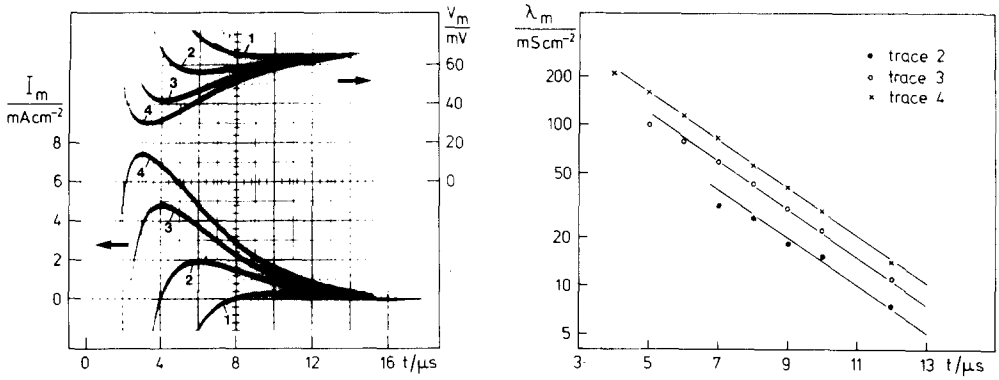


Fig. 1. Double-pulse experiments performed on a membrane from oxidized cholesterol/*n*-decane. The duration of the high pulse having an output voltage of 8 V ranged between 300 ns (trace 1) and 500 ns (trace 4), whereas the duration of the long pulse was in all cases 50 μs (output voltage 65 mV). T = 10°C; 1 M KCl.

Fig. 2. Semilogarithmic plot of membrane conductance vs. time of the data given in Fig. 1. The relaxation time constant of the exponential decay of the membrane conductance during the resealing process was in all cases about 3 μs. The initial specific conductance (at the end of the high pulse) ranged between 0.37 S · cm^{-2} (trace 2) and 0.76 S · cm^{-2} (trace 4).

ing an even greater electrical breakdown of the membrane.

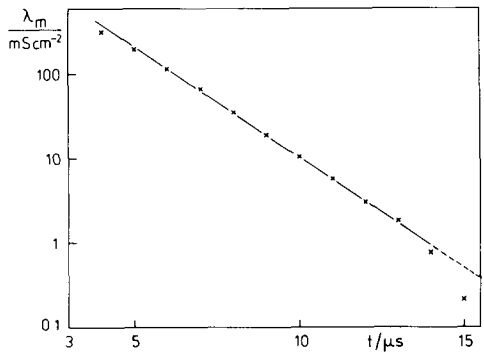
Fig. 2 shows a semilogarithmic plot of the specific membrane conductance vs. time derived from traces 2, 3 and 4. As can be seen from the data presented in Fig. 2, there is almost no difference between the slopes of the three curves, which can be approximated by a single exponential curve. This result indicates that the resealing process after breakdown shows a similar time course if the durations of the high pulses (which induce breakdown) do not differ greatly.

A semilogarithmic plot of an extended analysis of the resealing process of lipid bilayer membranes after electrical breakdown is given in Fig. 3. As can be seen from Fig. 3, there is a rapid drop of the membrane conductance below 0.1 mS · cm^{-2}.

It is interesting to note that the conductance vs. time curve during the resealing process can be approximated with a high accuracy by a single exponential curve. The time course of the conductance follows this exponential curve for more than two orders of magnitude.

Only in the time range between 13 and 15 μs is there a somewhat greater than exponential decay of the membrane conductance. If the electrical breakdown is caused by the formation of pores, then the resealing process may well reflect the closing of the aqueous channels. If the size of the pores is of the order of penetrating ions, there would be some interaction between the pores and the ions. This interaction would lead to a much faster than exponential decay of the conductance with time.

In order to check whether the amplitude of the low pulse has any influence on the time course of the resealing process, experiments were performed with the same magnitude and duration of the short (high) pulse but at different voltages for the long (lower) pulse. Fig. 4 shows such an experiment in which



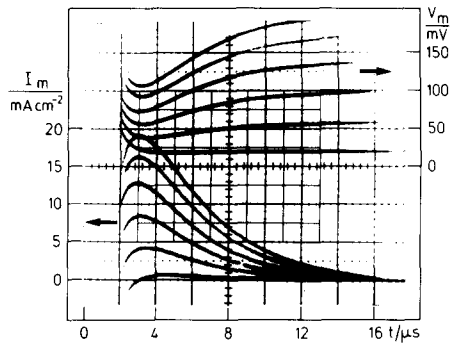


Fig. 3. Semilogarithmic plot of the membrane conductance vs. time as derived from an extended analysis of the resealing process. The time constant of the exponential decay of the membrane conductance was about 3 μs. The initial membrane conductance, estimated from extrapolation to the end of the high pulse, was 3.3 $S \cdot cm^{-2}$. Note that the change in membrane conductance follows an exponential decay only up to 13 μs. $T = 20°C$.

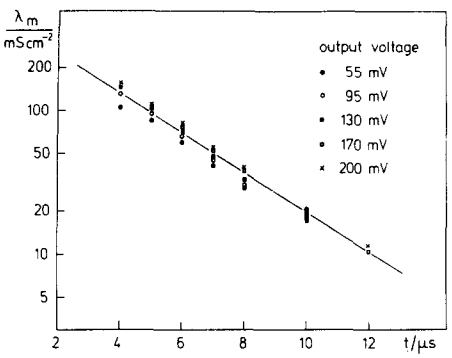
Fig. 4. Double-pulse experiments performed on a membrane from oxidized cholesterol/*n*-decane. The durations of the high pulses (output voltage 8 V, not shown) and the low pulses were 500 ns and 50 μs, respectively. The output voltages of the low pulses were varied between 20 and 200 mV. $T = 12°C$, 1 M KCl.

the output voltage of the long pulse was varied between 20 and 200 mV whereas the high pulse always had an amplitude of 8 V and a duration of 500 ns.

Fig. 5 represents the semilogarithmic plot of the data given in Fig. 4. As can be seen from Fig. 5, there is almost no voltage-induced effect on the time courses of the different resealing processes and, in this case also, they can be approximated by a single exponential curve. Further analysis of the data given in Fig. 4 shows that there is a linear relationship between membrane current and voltage at a given time after the end of the high pulse. In all cases investigated here, a linear current-voltage relationship was found for voltages up to 180 mV. Such behaviour is expected in principle if the electrical breakdown leads to the formation of aqueous channels in the membrane which close during the resealing process.

It is interesting to note that the time course of the resealing process is not independent of the duration of the high pulse (here, 500 ns throughout). For durations of the high pulse of the order of several microseconds, the time constant for the resealing process is considerably larger than that for the short pulse. The difference may be explained in principle by the formation of larger pores in response to long high pulses. On the other hand, rearrangement of the lipid matrix around the hole can occur due to the high current density within the pores once breakdown has occurred (in less than 100 ns).

The use of phospholipid/oxidized cholesterol mixtures instead of oxidized cholesterol for membrane formation did not change the resealing kinetics of membranes once breakdown has occurred. Single exponential decays of the membrane conductance were also observed throughout these experiments up to 25°C.



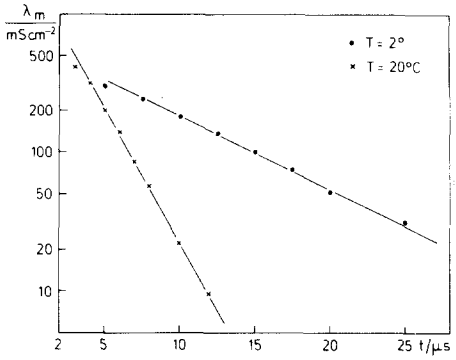
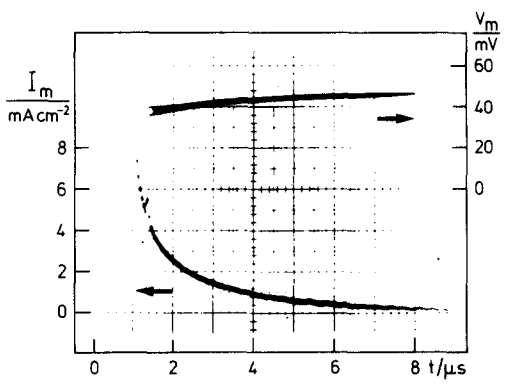


Fig. 5. Semilogarithmic plot of the specific membrane conductance as a function of time (derived from the data given in Fig. 4). The time constant of the resealing process was about 3 μs and the initial membrane conductance at the end of the high pulse had a value of 0.50 S · cm^{-2}. Note that the exponential decay of the membrane conductance is almost independent of the output voltage of the low pulse.

Fig. 6. Semilogarithmic plot of membrane conductance versus time of resealing processes of the same membrane measured at two different temperatures. The time constant of the exponential decays were about 8 μs (2°C) and 2 μs (20°C). The initial membrane conductance had values of 0.58 S · cm^{-2} (2°C) and 1.5 S · cm^{-1} (20°C).

Temperature dependence of the resealing process

The resealing process of the lipid bilayer membranes after electrical breakdown was investigated as a function of temperature. Semilogarithmic plots of the data of such experiments taken from a single membrane at 2 and 20°C, respectively (Fig. 6), demonstrate the resealing process can also be approximated by a single exponential function in this temperature range. However, for experiments at higher temperatures in the range between 30 and 60°C, the time course of the resealing process cannot be fitted to a single exponential curve. At least two exponential decays have to be assumed in order to



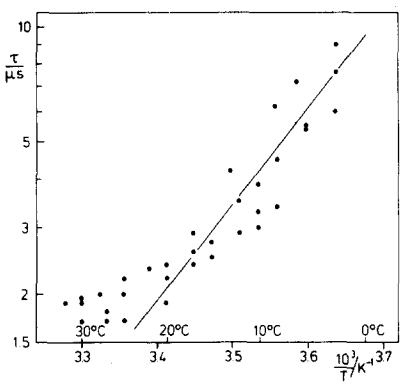


Fig. 7. Double-pulse experiments performed on a membrane from oxidized cholesterol/*n*-decane. The durations of the high pulse (output voltage 48 mV) were 500 ns and 50 μs, respectively. T = 50°C; 1 M KCl. Note that the decay of the membrane conductance with time cannot be fitted to a single exponential function.

Fig. 8. Arrhenius plot of the relaxation time constant of the resealing process. The straight line corresponds to an activation energy of about —50 kJ/mol. For further explanation see text.

obtain a reasonable fit of the experimental data. A typical experiment performed at 50°C is illustrated in Fig. 7. At this temperature, the resealing process can be fitted to two exponential decays, a slow one with a time constant of approx. 2 μs and a rapid one with a time constant of about 350 ns. Due to the very rapid decay, the time constant of the latter process cannot be resolved with sufficient accuracy.

Fig. 8 shows an Arrhenius plot of the time constants, τ, of the resealing process. The data between 0 and 20°C can be fitted to a straight line. Above 20°C, a saturation occurs, presumably because of the above-mentioned splitting of the resealing process into two exponentially decaying process. According to the relationship:

$$\tau = \tau_o e^{-E_a/RT} \tag{1}$$

(where R is the gas constant), an activation energy, E_a, for the resealing process can be calculated from the fit. The activation energy has a value of about - 50 kJ/mol which corresponds to 50 kJ/mol for the activation energy of the process described by the rate constant $k = 1/\tau$ of a first-order reaction.

Discussion

The results presented here indicate that the relaxation time of the resealing process and thus the life-time of the electric field-induced pores are strongly temperature-dependent. The time constants range from about 10 μs at 2°C to about 2 μs at 20°C. The activation energy of the resealing process (50 kJ/mol) is comparable with the value determined for the resealing process in ghost cell membranes after breakdown and electrical haemolysis has occurred. As has been shown by Zimmermann et al [12], the activation energy for the resealing process in red blood cell membranes is about 60 kJ/mol, when it is assumed that the electrically induced haemolysis kinetics are rate-controlled by the diffusion of univalent ions into the cells after the breakdown of the membrane. The slightly higher value for the activation energy for the resealing process in red blood cell membranes in comparison to that measured for lipid bilayer membranes made up of oxidized cholesterol may thus be explained either by the inaccuracy of the more indirect method used in the breakdown experiments of red blood cells or by the different pulse lengths of the applied electric field. In the bilayer experiments reported here, the pulse length was always of the order of 500 ns, whereas the red blood cells were exposed to the critical field strength for 40 μs. Further experiments on lipid bilayer membranes are required in which the postulated dependence of the activation energy on the pulse duration has to be proved in more detail. However, in the present state of information, it cannot also be excluded that the slight differences in the values of the activation energy between lipid bilayer membranes and cell membranes arise from the different composition of both membranes.

A value of 50 kJ/mol for the activation energy suggests that the rate of the resealing process is controlled by the diffusion of the lipid molecules within the plane of the bilayer membranes [13]. Careful analysis of the data presented in Fig. 3 support this assumption.

As shown in the following consideration, in addition to the calculation of the diffusion coefficient of the lateral lipid movement, an estimate of the radius of the pores induced by the breakdown of the membrane can be obtained from the curve shown in Fig. 3. The estimation is based on the assumption (mentioned above) that the more rapid (than exponential) decrease in membrane conductance in the time range between 13 and 15 μs can be interpreted in terms of a pore size which is comparable to the size of the penetrating ions (see above). As the decrease in membrane conductance with time follows an exponential curve in a certain time range, the following equation holds for the specific membrane conductance λ_m:

$$\lambda_m = \lambda_m^o e^{-t/\tau} \tag{2}$$

where λ_m^o is the initial membrane conductance after the end of the high pulse (resulting in breakdown). Assuming that N_o pores per unit area are formed, then λ_m^o can be rewritten as:

$$\lambda_m^0 = N_0 \cdot \sigma \cdot \frac{A_0}{d} \tag{3}$$

whereby A_o is the initial surface area of a single pore, σ the specific conductance of the aqueous phase in the pore and d its length (membrane thickness).

If we assume that the electrolyte in the pore has the same specific conductance as the bulk aqueous phase (i.e., $\sigma = 116$ mS $\cdot$ cm^{-1} for 1 M KCl [16] and that the pore spans the membrane (i.e., $d = 3.3$ nm [3]), the total surface area of the pores, $N_o \cdot A_o$, is calculated from $\lambda_m^o = 3.3$ S $\cdot$ cm^{-2} (Fig. 3) to be:

$$N_o \cdot A_o = 9.4 \cdot 10^{-6} \tag{4}$$

If we assume that the exponential decay in membrane conductivity up to 13 μs is caused by an exponential decay in the area, A, of the individual pores due to the resealing process and not by a decrease in the number of pores, N_o, we can arrive at an expression for the decay of the area of a single pore:

$$A = A_o e^{-t/\tau} \tag{5}$$

As can be seen from Fig. 3, the decrease in conductance becomes more rapid towards the end of the decay curve than would be predicted from a purely exponential decay. It is assumed that it is at this critical point that the area, A, of the single pores becomes about the same size as the hydrated penetrating ions. If a value for the size of these ions is assumed, extrapolation back along the exponential decay curve to time zero provides a value for A_o. If the ions have radii of 0.2 nm, this critical area for the pore is $1.3 \cdot 10^{-15}$ cm^2. A_o is then given by $(1.3 \cdot 10^{-15}$ cm$^2) \cdot e^6 = 5.1 \cdot 10^{-13}$ cm^2, a value which corresponds to an initial radius of 4 nm for a circular pore cross-section.

Introduction of this value into Eqn. 4 shows that the number of field-induced pores is of the order of 10^7/cm^2 which is in the range recently postulated by the authors [3]. A pore radius of 4 nm is in agreement with results obtained with red blood cell membranes. As shown by Zimmermann et al. [14], bovine serum albumin which has a radius of 3.7 nm [15] and haemoglobin (radius 3.1 nm [15]) can pass through the membrane very rapidly

when the cells are exposed to slightly supercritical field strengths in the discharge chamber [14]. Furthermore, Zimmermann et al. [12] have recently estimated the field-induced pore radius, from the analysis of haemolysis kinetics in the presence of sucrose and mannitol, to be 3 nm. The radius of the field-induced pore in the lipid bilayer membrane should increase with increasing length of the applied voltage pulse as was shown for the breakdown in red blood cell membranes [12]. This hypothesis has to be proved in further experiments. However, the stability of the lipid bilayer membrane may become a limiting factor in such experiments [3].

With the knowledge of the initial area of the field-induced pore and its exponential decrease with time, the diffusion coefficient of the lipid molecules can be estimated by assuming (as mentioned above) that the lateral diffusion of the lipid molecules is responsible for the closing of the field-induced pores.

The diffusion coefficient, D, of the lipid molecules is given by the Einstein relationship:

$$D = \frac{\Delta x^2}{2\tau} \tag{6}$$

where Δx is given by the decrease in radius of the pore when one relaxation time, τ, has been passed. The decrease in the pore radius calculated according to Eqn. 5 is 1.6 nm for one relaxation time.

From Eqn. 6 the diffusion coefficient is estimated to be about $1 \cdot 10^{-8}$ $cm^2 \cdot s^{-1}$. This value is close to those reported for lipid molecules in the literature, suggesting that the resealing process is indeed determined by lateral lipid movement [13]. If this interpretation is correct, compounds which alter the membrane fluidity should change the relaxation time of the resealing process.

The estimations are based on the mentioned assumption that the number of field-induced pores remains constant. We feel that this assumption may be valid for temperatures up to 20° C, whereas at higher temperatures pores of different life-times may be generated. This conclusion is supported by the finding that the dependence of the relaxation time of the resealing process on temperature cannot be described by an Arrhenius plot above temperatures of 25°C (Fig. 8). However, it cannot be excluded that other molecular processes are responsible for the splitting of the straight line in the Arrhenius plot into the two branches observed at about 20—30° C. It seems also likely that the resealing process which exhibits a lower activation energy as observed experimentally at very high temperatures is also present in the lower temperature range, but masked by the process which requires an activation energy of 50 kJ/mol. The nature of these two resealing processes which may arise from two different types of pores is not known.

Finally, we would like to point out that the primary process of electrical breakdown is still under discussion. From the results presented here, we are not able to distinguish between the different mechanism of electrical breakdown, the electromechanical compression and/or the Born energy [1,3]. In any case, we have presented here evidence that the primary process leads

to a formation of pores which may also be responsible for the mechanical breakdown occurring at a longer time scale (milliseconds to seconds).

Acknowledgements

The authors would like to thank Dr. P. Läuger for many helpful discussions, Dr. D. Tomos, University of North Wales, Bangor, for a critical reading of the manuscript and Mr. W. Plaga for expert technical assistance. This work has been supported by the Deutsche Forschungsgemeinschaft (SFB 138 to R.B.) and by a grant from the BMFT (BCT 112 to U.Z.).

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