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Rapid Report

Use of irreversible electrical breakdown of lipid bilayers for the study of interaction of membranes with surface active molecules

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Lipid bilayers were formed in the presence of different macromolecules and high electrical fields were used to induce mechanical rupture of the membranes. The kinetics of pore formation during irreversible breakdown was studied as a function of the macromolecules. We observed that macromolecules having a strong binding affinity to the membrane alter the time course of pore formation significantly. We propose this method as a simple test for adsorption of macromolecules to membranes.

External electrical fields are widely used for manipulation of artificial and biological membranes. Electroinjection of macromolecules into living cells and electrofusion of cells are nowadays a well established method for genetic engineering and somatic hybridization [1,2]. Despite the widespread use of these field pulse techniques in biology, medicine and biotechnology the underlying mechanism of the electrical breakdown or electroporation of cell membranes is not yet satisfactory understood. This means that more detailed information is required to improve the present field pulse techniques for applications such as electrochemotherapy [3] and in fields of industrial interest.

Recently, we have investigated the kinetics of the irreversible breakdown in lipid bilayer membranes [4]. In a large number of experimental conditions using various lipids and electrolytes, we have shown that the characteristic opening process of the pore is independent of the actual membrane potential and electrolyte concentration. The experimental results furthermore suggest that the mechanism responsible for the widening of the pore is basically determined by the intrinsic material properties of the bilayer and the pore radius is a linear function of time. Following our theoretical treatment this indicates that the velocity of the pore wall is damped by the inertia and not limited only by the viscosity of the membrane.

The main interest in this report is to show that macromolecules adsorbed to or incorporated into the membrane will change the mass of the film and its surface tension. As a consequence, the velocity of the pore rim during irreversible breakdown will change. In the present work we studied the influence of two different types of macromolecules. In a first set of experimental conditions we used Pluronic F-68 and CPCl as typical macromolecules with a strong binding affinity towards membranes. In a second series of experiments we added PAA to the aqueous phase bathing lipid bilayer membranes. This compound is hydrophilic and does not adsorb to membranes.

Qualitative and quantitative knowledge of the interaction of macromolecules with membranes is of basic interest for the understanding for the applications of these molecules in cell cultivation or liposome drug delivery systems [5,6]. As the proposed method is easy to handle we suggest the study of irreversible electrical breakdown as a new tool for a qualitative study of membrane adsorption of macromolecules.

Due to the finite contact angle at the edge of the Teflon rim the lipid film is held under surface tension σ. This tension will favour the opening of pores in the membrane. On the other hand, creating a pore of radius a requires the energy per length, Γ, to form an

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Abbreviations: CPCl, cetyl pyridinium chloride (mol. mass 358 Da); DPhPC, diphytanoylphosphatidylcholine (mol. mass 846 Da); PAA, polyacrylamide (mol. mass 200000 Da); Pluronic F-68, α-hydro-ω-hydroxypoly(oxyethylene)-poly(oxypropylene)-poly(oxyethylene) (mol. mass 8400 Da).

edge. The total energy of a pore is given by these two opposite contributions:

$$E_{\text{pore}} = 2\pi a\Gamma - \pi a^2\sigma \quad (1)$$

Obviously pores with radii smaller than $a < a^* = \Gamma/\sigma$ reseal, while for radii larger than a^* the area of the pores will increase to infinity. Recent investigation on the irreversible electrical breakdown of lipid bilayer membranes [4] has shown that short charge pulses that create a sufficient electrical field across membranes decrease the energy barrier for pore formation. A few μs after the pore area starts to increase, the electric potential difference across the membrane drops down fast and the contribution of the electrical field to the increase of the pore size becomes negligible. After initiation of a pore the kinetics will be determined only by the material properties of the membrane. As shown in our earlier study [4] one can neglect the viscosity of the lipid film and the kinetics of the widening of the pore is driven by the finite surface tension and controlled by the inertia of the film. Balancing the decrease in elastic energy during the widening of the pore by inertia and dissipation yields the following time dependence for the pore radius:

$$a(t) = a_0 + \sqrt{\frac{\Phi\sigma}{d\rho}} \cdot t = a_0 + \alpha t \quad (2)$$

with Φ as a parameter depending on the unknown material flow [4], σ represents the lipid density and d the membrane thickness. Knowledge of the pore radius allows the calculation of the pore conductance, $G(t)$, as a function of time. Assuming a single pore in a thin membrane we obtain:

$$G(t) = 1/R_{\text{pore}} = 2\kappa(a_0 + \alpha t) \quad (3)$$

with κ as the specific conductivity of the aqueous solution.

Black lipid bilayer membranes were formed of a 10 mg/ml solution of DPhPC (Avanti Polar Lipids, Birmingham, AL) in n-decane (Fluka AG, Buchs, Switzerland) or a 10 mg/ml solution of DPhPC and a 10 mg/ml solution of CPCl in n-decane. The membranes were spread across a circular hole with a diameter of about 1 mm^2 in a wall separating two aqueous phases in a Teflon cell (see Fig. 1). The electrolyte, an unbuffered 100 mM solution of potassium chloride (Merck, Darmstadt, Germany), was prepared using twice distilled water.

The detergent Pluronic F-68 (mol. mass 8400 Da) and PAA (mol. mass 200 000 Da) were added to the aqueous phase in concentrations of 2 mg/ml and 3 mg/ml, respectively. The specific conductivity of the aqueous solutions was $\kappa = 1.23$ S/m and their pH was around 6. The temperature was always kept at 20°C.

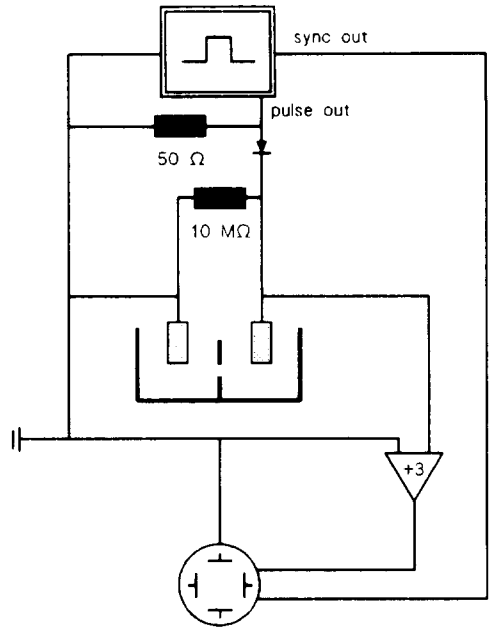

Fig. 1. Scheme of the charge-pulse instrumentation used for the measurement of electric field-induced irreversible breakdown of lipid bilayer membranes.

The detection system consisted of extremely low impedance Ag/AgCl platinum black electrodes (Annex Instruments, Santa Anna, CA), one connected to a fast pulse generator (Hewlett Packard 214B) through a transistor used as a fast diode (reverse resistance $\gg 10^{11}$ Ω) and the other electrode grounded (Fig. 1). The voltage between these two electrodes was measured with a high-input-resistance voltage amplifier manufactured on the basis of a operational amplifier (Analog Devices 3554; bandwidth 50 MHz; gain 3-fold) and a digital storage oscilloscope (Nicolet 4094A). The voltage relaxations were analyzed with a personal computer. For each membrane we determined the capacitance. This was done by charging the membrane with a short charge pulse of 20 μs duration to an initial voltage of about 10 mV and measuring the exponential discharge process through a known resistor. The value of the membrane capacitance was calculated from the *RC*-time constant. The actual membrane area was measured with an eyepiece micrometer.

The irreversible breakdown of the membranes was induced by charging them to high initial voltages in the range of 300 to 600 mV with charge pulses of 20 μs duration. In contrast to our previous study we raised the amplitudes of the current pulses in small steps until we reached the critical voltage initiating mechanical rupture. This was done to ensure that only one single pore was induced in the membranes since we have shown previously that high initial amplitudes increase the probability for causing multiple pores [4].

Fig. 2 shows a typical time course of the potential across the membrane following a brief charge pulse (20

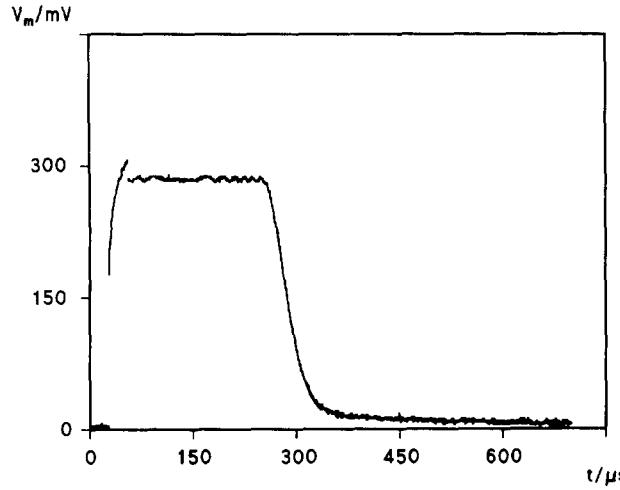

Fig. 2. Time course of membrane vóltage $V_m(t)$ during electric field-induced irreversible breakdown of a membrane formed of DPhPC. The length of the charge pulse was 20 μs. The aqueous phase contained 100 mM KCl; T = 20°C.

μs). The membrane voltage decayed somewhat after the end of the pulse probably due to the shunt resistor of 10 MΩ in parallel to the membrane capacitance and to a minor capacitance relaxation caused by the high membrane potential [8]. Then a much stronger decay occurred which was caused by irreversible breakdown of the lipid bilayer membrane. During this process, the actual membrane conductance, $G(t)$, increased because of the formation of a pore. $G(t)$ was calculated under the assumption that the membrane capacitance C (derived from the experiment described above) was independent of time:with $U(t)$ as the actual membrane voltage.

$$G(t) = I(t)/U(t) = \frac{1}{U(t)} \cdot \frac{\delta Q(t)}{\delta t} = \frac{1}{U(t)} \cdot \frac{\delta (C \cdot U(t))}{\delta t}$$

$$= \frac{C}{U(t)} \cdot \frac{\delta U(t)}{\delta t} \qquad (4)$$

In a first set of experimental conditions we measured the irreversible breakdown of lipid bilayer membranes formed of pure DPhPC in the absence of macromolecules. Fig. 3 shows a plot of the conductance as a function of time. In agreement with a previous study [4], we observed a linear conductance versus time relationship for all systems which indeed indicated that the inertia of the film has a dominating influence on the kinetic of pore increase during electrical breakdown. The time delay between the end of the pulse and the begin of the widening of the pore is due to the statistical origin of the pore and had no influence on the slope of the conductance–time curve. According to Eqn. 3 we can attribute its slope to the presence of a single pore within the membrane. The rim of the pore moved with a velocity, α = 0.2 m/s (curve 1 of Fig. 3).

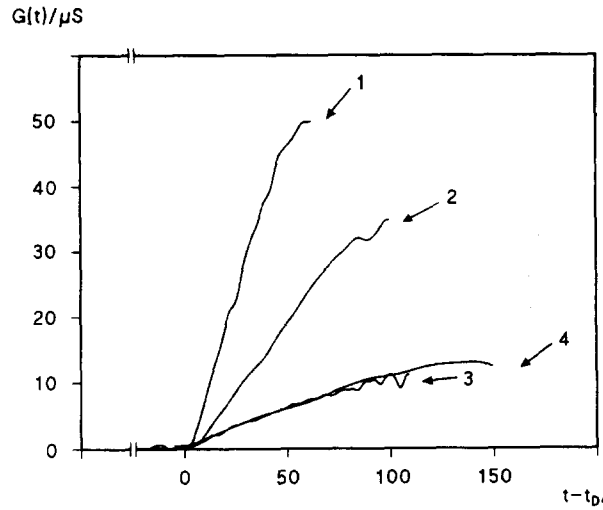

Fig. 3. Conductance $G(t)$ versus time curves during irreversible breakdown of membranes made of: 1: DPhPC, curve slope 0.52 S/s (0.21 m/s); 2: DPhPC and 0.3 mg/ml PAA added to the aqueous phase, curve slope 0.52 S/s (0.21 m/s); 3: DPhPC and 0.2 mg/ml Pluronic F-68 added to the aqueous phase, curve slope 0.42 S/s (0.17 m/s); 4: mixed DPhPC/CPCl bilayer, curve slope 0.20 S/s (0.08 m/s); Note: the significant part we fitted covers the first 50 s. $G(t)$ was calculated according to Eqn. 4 by using the capacitance of the individual membranes and the velocity of the pore rim by using Eqn. 3. The length of the charge pulses was 20 μs. Because of the considerable scatter of the signal the data have been averaged by standard methods [4]. The aqueous phase contained 100 mM KCl; T = 20°C.

The slopes derived from the irreversible breakdown of a large number of membranes are given in Table I. In most cases we observed a velocity, α, around 0.2 m/s (mean value ± S.D.: 0.21 ± 0.05 m/s for 51 experiments). However, occasionally we obtained a larger velocity for the increase of pore size [4] which may indicate the formation of more than one pore at a given time (data not shown).

In a second set of experimental conditions we added 2 mg/ml Pluronic F-68 to the aqueous phase. This compound slowed down the thinning of the lipid bilayer membranes. Bilayer formation may be accelerated by the application of a voltage across the lipid lamella. After formation of black films we applied the same protocol as described above which means that we

TABLE I

Velocity of pore increase during irreversible breakdown of lipid bilayer membranes made of pure and detergent-endowed DPhPC

System	α(m/s)	C_m(nF/cm^2)	n
DPhPC	0.21 ± 0.05	366	51
DPhPC/CPCl	0.06 ± 0.02	310	20
DPhPC, 3 mg/ml PAA [a]	0.21 ± 0.06	366	16
DPhPC, 2 mg/ml Pluronic F-68 [a]	0.17 ± 0.04	450	19

[a] In the aqueous phase.

measured the membrane capacitance and induced then irreversible breakdown with a charge pulse of high amplitude. A typical time course of the pore conductance under these conditions is represented by curve 3 in Fig. 3 superposed to that of pure lipid. The slope of curve 3 ($\alpha = 0.17$ m/s) is visibly different from that of DPhPC. This change was significant as the results of Table I clearly indicate (mean value $\pm$ S.D.: 0.17 ± 0.04 m/s for 19 experiments).

In another set of experiments we added 3 mg/ml PAA to the aqueous phase. In contrast to the experiments with Pluronic F-68, we could not observe any difference of the slope ($\alpha = 0.21 \pm 0.06$ m/s for 16 experiments) of the conductance versus time curves (see curve 2 of Fig. 3). This results clearly indicated that PAA does not bind to the membrane. Then we added CPCl in a concentration of 10 mg/ml to the membrane-forming solution (5 mg/ml DPhPC). Interestingly, the slope of the conductance versus time curve during irreversible electrical breakdown decreased significantly ($\alpha = 0.06 \pm 0.02$ m/s for 20 experiments; see also curve 4 of Fig. 3). This may be caused by a decrease of the surface tension of the mixed DPhPC/CPCl membranes (see Eqn. 2) since it is well known that positively charged CPCl acts as a surfactant and lowers the surface tension [9].

Our experimental results clearly indicate that our method allows to measure in a fast and reliable way whether macromolecules added either to the aqueous phase or to the membrane-forming solution change the properties of the membrane, such as surface tension or the inertia of the membrane itself. Pluronic F-68 adsorbs obviously to the membrane surface and forms there a structure similar to the cytoskeleton of biological membranes [5]. This structure increases the inertia of the lipid bilayer membranes and the irreversible breakdown of the membranes is slowed down. CPCl is a compound known to change the surface tension of membranes. The surface tension has a strong influence on the mechanical properties of membranes and controls in particular the velocity of the pore rim during mechanical rupture. We expect to develop the irreversible breakdown of lipid bilayer membranes as a method for a quantitative analysis of mechanical membrane properties in further studies.

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