Electro-mechanical permeabilization of lipid vesicles
Role of membrane tension and compressibility

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ABSTRACT A simple micropipet technique was used to determine the critical electric field strength for membrane breakdown as a function of the applied membrane tension for three different reconstituted membranes: stearoyl-oleoylphosphatidylcholine (SOPC), red blood cell (RBC) lipid extract, and SOPC cholesterol (CHOL), 1:1. For these membranes the elastic area expansivity modulus increases from ~200 to 600 dyn/cm, and the tension at lysis increases from 5.7 to 13.2 dyn/cm, i.e., the membranes become more cohesive with increasing cholesterol content. The critical membrane voltage, V_c, required for breakdown was also found to increase with increasing cholesterol from 1.1 to 1.8 V at zero membrane tension. We have modeled the behavior in terms of the bilayer expansivity. Membrane area can be increased by either tensile or electrocompressive stresses. Both can store elastic energy in the membrane and eventually cause breakdown at a critical area dilation or critical energy. The model predicts a relation between tension and voltage at breakdown and this relation is verified experimentally for the three reconstituted membrane systems studied here.

INTRODUCTION

The application of a short (microseconds to milliseconds) critical D.C. electric field pulse to natural cell membranes or to reconstituted lipid bilayers causes a breakdown in membrane integrity and a permeabilized state. The terms breakdown, poration, and permeabilization have all been used to indicate this instability phenomenon. The permeabilized state is characterized by an increased passage of water, ions, small molecules, and macromolecules through the membrane. It is not clear whether this transient membrane state comprises a single large hole, many small holes, or regions of perturbed lipid (Zimmermann, 1988; Dimitrov and Jain, 1984). It is possible that all three may occur depending on the permeabilizing conditions during the short D.C. pulse. A permeabilized state lasting for much longer times than the breakdown pulse has even been reported (Sowers, 1986).

The techniques of electric field induced membrane permeabilization and cell fusion in a variety of cell types are currently being developed as tools for cell loading and genetic hybridization (Neumann et al., 1982; Knutsen and Yee, 1987; Zimmermann, 1986 and 1988 for review). One major criterion is that after permeabilization or fusion the cells should maintain their natural integrity in subsequent suspension or culture. Conditions are therefore sought which elicit minimum membrane damage (reversible breakdown) but maximal loading or fusion. The actual molecular mechanism of electrically induced permeability and its relation to the membrane disruption process and fusion is at present not known.

Early characterizations of lipid bilayer structure and properties used (among others) electrical methods on black lipid membranes (BLMS) (Hanai et al., 1964). Critical field strengths were found that caused increased conductance and breakage and prompted further experimental and theoretical analyses of electrocompression and breakdown (Crowley, 1973; Requena et al., 1975; Abidor, 1979). The model proposed by Crowley (1973) balances the stresses due to electrocompression by an elastic thinning of the membrane. Once the membrane thins to a certain point the model predicts a point of instability where the membrane suddenly collapses. Crowley's model forms the basis for later treatments which include the involvement of turgor pressures as well as electrocompressive stresses (Zimmermann et al., 1977, 1980). Other models include the involvment of electrocompressive forces acting on thermal, mechanical, and physical defects (Zimmermann et al., 1977; Pastushenko and Petrov, 1984); phospholipid and solvent dipole re-orientation (Sugar and Neumann, 1984); the action of disjoining pressure in a viscoelastic film (Dimitrov, 1984) and the redistribution of proteins (Schwister and Deutike, 1985).

Parallel to and independent of the studies of electrocompression and permeabilization of membranes have been studies of membrane expansion and lysis caused by aspirating part of a nearly spherical red cell or vesicle into a micropipet (Evans et al., 1976; Evans and Waugh, 1977; Kwok and Evans, 1981). The aspiration pressure causes the membrane external to the pipet to sphere and creates a uniform isotropic membrane tension and surface

expansion before failure. When the membrane fails, water is rapidly expelled from the cell or vesicle at the point of failure and the entire cell is aspirated rapidly into the pipet. These studies permit the amount of area expansion to be measured and indicate that the membrane can only expand by ~2-3% before it fails. This is in contrast to the models of Crowley (1973) and Zimmermann et al. (1977, 1980) which indicate (theoretically) a much larger thickness change and concommittant area expansion before membrane failure.

We postulate here that membrane deformation and subsequent failure is an intrinsic property of the membrane material. Thus, the criterion for failure is the same regardless of whether the membrane is expanded to the point of failure by an isotropic tension or an electrocompressive stress. Both will store energy in the membrane according to the elastic model of Evans et al. (1976) for the isotropic surface expansion of a membrane.

Because it is expected that breakdown occurs in the lipid phase of membranes, it is desirable to study simple, single-component, single-phase phospholipid bilayers in order to gain any basic understanding of electric field-induced membrane permeabilization. The only studies to date, that have used reconstituted lipid bilayer membranes other than solvent containing membranes (where pore formation, persistence and closing may be influenced to a large extent by the hydrocarbon solvent) were by Tessie and Tsong (1981) and Buschl et al. (1982). Tessie and Tsong used 100 μm diam dipalmitoylphosphatidylcholine liposomes and measured the reversible electric field induced leakage of internal sucrose. The measurements were, however, carried out at temperatures below the gel-liquid crystalline phase transition of the lipid and so the lipid was in a frozen state. Buschl et al., (1982) investigated dielectrophoretic behavior and fusion with changes in membrane composition of a giant (20 μm) liposome preparation. Field strengths to cause fusion of the liposomes were found to be some 10-20 times greater than those which elicited breakdown in BLM systems, and were therefore inconsistent with these planar membrane studies. The involvement of multibilayers and/or insulating lipid films on the electrodes were proposed to account for the discrepancies.

Our approach in this study is to reconstitute lipid membranes of well defined composition and material properties and use a micropipet technique to position a single lipid vesicle between two pulse electrodes in order to determine the critical field strength for membrane breakdown as a function of applied membrane tension. We analyze the results in terms of a model which includes a single material property (the elastic area expansivity modulus K), a geometric parameter (the critical area of a dilation α_c), and the applied stresses (the isotropic tension T and the electrocompressive stress σ_e). We show the

response to mechanical tension and electrocompression for membranes of different compositions whose moduli range from 200 to 600 dyn/cm and we demonstrate the dependence of membrane failure on the product of the expansivity modulus K and the critical area α_c.

EXPERIMENTAL AND THEORETICAL METHODS

Vesicle preparation

Giant lipid vesicles were prepared by gentle rehydration of evacuated lipid lamellae, formed on a teflon substrate by evaporation of the solubilizing chloroform-methanol solvent. The method was based on that first reported by Reeves and Dowben (1969) and was recently modified to give a higher yield of the desired, large (20-40 μm diam) single-walled vesicles (Needham and Evans, 1988). For neutral lipids, such giant unilamellar structures are only formed in nonelectrolyte or dilute electrolyte (<1 mM) solution. The inclusion of small amounts of negatively charged lipid dioleoylphosphatidylglycerol (DOPG) allows the mutual attraction due to van der Waals forces between bilayers to be overcome by electrostatic repulsion in electrolyte solutions. In order to make vesicles in electrolyte solutions that were suitable for electropermeabilization studies, 5 mol % DOPG was incorporated into the otherwise neutral lipid preparations. Three lipid compositions were studied, stearoyloleoylphosphatidylcholine (SOPC)(DOPG), SOPC(:DOPG):cholesterol (CHOL) (Avanti Polar Lipds, Inc., Birmingham, AL) and extracted red blood cell (RBC) lipids (D. Needham and R. S. Nunn, 1989, manuscript submitted for publication) containing ~33 mol % cholesterol by chemical analysis, and estimated to have ~15 mol % charged lipid (van Deenen and de Gier, 1967).

For the main permeabilization studies vesicles were formed by gentle rehydration of the dried lipid lamellae with a solution of 200 mM sucrose containing 10 mM NaCl. The specific resistance of this solution was measured to be 1.1 kΩ cm. The loose lipid cloud that formed in suspension was gently diluted into iso-osmotic media to provide a suitable working concentration, i.e., dilute enough so as not to degrade the optical image. This suspending solution contained 200 mM glucose plus 10 mM NaCl and 0.2% albumin and was measured to have a specific resistance of 1.23 kΩ cm. For the other studies that used the neutral lipids SOPC:CHOL and SOPC, the rehydrating media was 200 mOsm sucrose and the suspending media was 200 mOsm glucose plus 1 mM NaCl and 0.2g% albumin. The measured specific resistances for these solutions were 450 k Ω cm (sucrose) and 7.1 k Ω cm (glucose plus 1 mM NaCl). Vesicle suspensions were then injected into a simple microchamber constructed from plexiglass and thin strips of cover glass, as shown in Fig. 1. The advantage of using refractile solutes such as sucrose and resuspending the vesicles in iso-osmotic glucose was that the refractive index difference between internal and external solutions greatly enhanced visualization in the interference contrast microscope (Hoffman Modulation Optics, Greenvale, NY), as shown in Fig. 2. Also, the density difference collected the vesicles on the bottom of the microchamber for ease of identification and capture. A further advantage was that the presence of trapped solutes maintained a constant vesicle volume (prevented filtration of water) since the osmotic pressure of these solutions far exceeded the micropipet suction pressures used in the micromechanical tests.

Micropipet manipulation

Micropipets were made from 1mm capillary glass tubing, pulled to a fine point and broken by quick fracture to give flat tips of desired internal diameter (typically 8-10 μm). Micropipets were filled with

isotonic, iso-osmotic solution and were mounted in DeFonbrune, model C micromanipulators on the microscope stage. Suction pressure at the pipet tip was applied hydrostatically by means of a water filled manometer with fine micrometer driven displacement. The height of the reservoir was adjusted with respect to the pipet tip to give a condition of "no-flow" in the pipet as judged by the movement of small particulate material. This zero pressure condition was checked frequently because small amounts of evaporation of solution can cause the curvature of the menisci to change thereby changing the pressure in the chamber relative to atmospheric pressure. Micrometer driven movement of the reservoir and the use of syringes allowed control of pipet suction pressure in the range 10^{-6} to 10^{-1} atm (1 dyn/cm^2 − 100,000 dyn/cm^2). Experiments were viewed directly and recorded for subsequent geometric analysis via video camera, recorder and monitor, along with time and suction pressure which were multiplexed into the video image. Subsequent geometric analysis of vesicle and pipet was carried out using calibrated video calipers.

Electropermeabilization experiment

The double microchamber used for the electropermeabilization measurements is shown in Fig. 1. A single lipid vesicle was selected from the suspension chamber (1), using the manipulation pipet (2), and was transferred into the adjacent electrode chamber (3). The manipulation pipet and vesicle were shielded across the air gap with a larger transfer pipet (4). The vesicle was then positioned between the platinum strip electrodes (5) (0.83 mm gap separation). For these experiments an approximately square pulse was used of 60 μs duration. The pulse circuit was essentially the same as that used previously by Miles and Hochmuth (1987) except that a potentiometer was included to give finer control ($\pm$ 10 V/cm) of pulse height. The pulse length and voltage were accurately measured by an oscilloscope. The electric field strength was then simply determined from this voltage and the distance between the electrodes, which was measured with a calibrated, low powered microscope to be 0.83 mm.

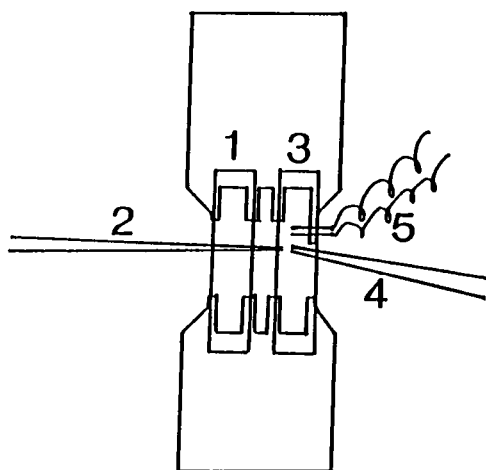Fig. 2 a shows a typical single bilayer vesicle aspirated by a micropi-

FIGURE 1 Electropermeabilization/fusion microchamber. 1, vesicle suspension chamber; 2, manipulation and suction micropipet ~8 μm diameter; 3, electrode chamber; 4, larger transfer pipet; 5, platinum strip electrodes 0.83 mm apart connected to pulse generating circuit.

pet under subcritical suction pressure. Pressurization in this way induced a tension in the membrane $\overline{T}$, which was uniform over the entire vesicle surface. The tension $\overline{T}$ is given by the pipet suction pressure P and the pipet and vesicle geometry.

$$\overline{T} = \frac{PR_p/2}{1 - R_p/R_o},$$ (1)

where R_p is the pipet radius and R_o is the radius of the outer spherical portion of the vesicle (Evans et al., 1976). Thus, vesicle membrane tensions were accurately controlled and determined.

Two experiments were carried out: (a) Vesicles of almost all the same size (20–30 μm diam) were positioned by micropipet between the platinum electrodes and held at a fixed suction pressure. The pulsed electric field strength was sequentially increased in steps of 20 V/cm until the vesicle permeabilized. Vesicle permeabilization was easily assayed. At high suction pressures the vesicle was immediately sucked up the pipet ($<1/60$ s); at low suction pressures permeabilization caused a release of internal pressure and the flaccid membrane was also aspirated as shown in Fig. 2 b; at very low suction pressures the permeabilizing pulse produced small measurable displacements of the projection in the pipet corresponding to small volume displacements through the transiently permeable membrane. Several suction pressures were used which covered the whole range from 100 dyn/cm^2 to the value that caused vesicle lysis at zero electric field strength. (b) At constant low suction pressure the critical electric field strength for permeabilization E_c was determined for a wide range of vesicle sizes (19–60 μm diam).

The first experiment was carried out on vesicles composed of the three lipid mixtures: SOPC(:DOPG):CHOL, RBC lipids, and SOPC(:DOPG) in sugar solutions made conducting by the addition of 10 mM NaCl. Measurements were also made with vesicles prepared from the neutral lipids SOPC:CHOL (1:1), and SOPC, containing only sucrose (and trace amounts of salt) suspended in 1 mM NaCl/glucose solution.

The second experiment was carried out on the SOPC:CHOL(1:1) vesicle preparation in low conducting media.

Measurements of the elastic area compressibility modulus K and the membrane tension required to lyse (rupture) the lipid bilayer have recently been made on these, and other lipid bilayers of different compositions (Kwok and Evans, 1981; Evans and Needham, 1988, a and b; Needham et al., 1988; D. Needham and R. S. Nunn, manuscript submitted for publication). Details of this measurement are given elsewhere (Kwok and Evans, 1981). The elastic modulus and lysis tension were however measured for each of the vesicle preparations used in these studies as a check on the values themselves and as a way of making sure that only single walled vesicles were being used for permeabilization measurements.

Methods of analysis and results

In the first experiment, which is the main theme of this paper, the critical field strength E_c (and hence critical membrane voltage V_c) for membrane breakdown was determined as a function of membrane tension. The membrane tension was obtained from Eq. 1 and the membrane voltage V was evaluated by using the well known expression (Kinosita et al., 1988):

$$V = 1.5 \, ER_o \cos \theta \, (1 - e^{-t/\tau}),$$ (2)

where E is the electric field strength, R_o is the vesicle radius, θ is the angle between the surface normal and the field direction and τ is the time constant for membrane charging.
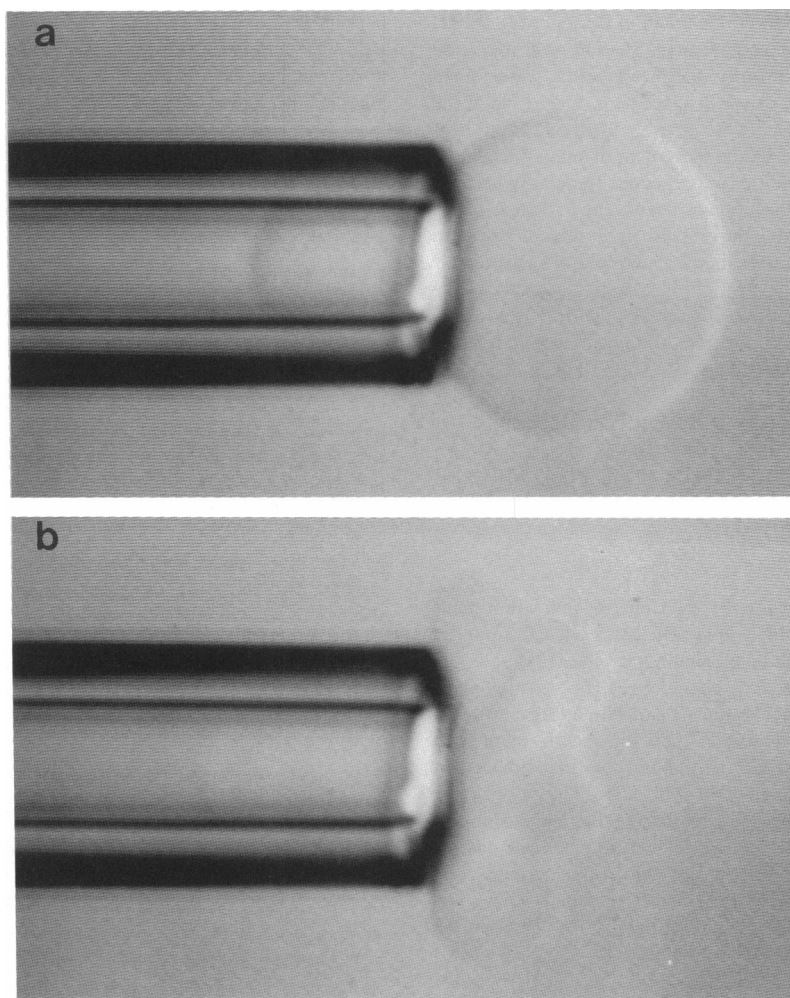
FIGURE 2. Videomicrographs of vesicle permeabilization experiment. (a) single walled lipid vesicle (~20 μm diam) aspirated by micropipet (~8 μm diam) under a low suction pressure which is less than that required to break the membrane under conditions of zero voltage. (b) critical field pulse has been applied and permeabilization of membrane causes volume displacement, depressurization and slow aspiration up the pipet.

The expression for τ is

$$\tau = R_o C(r_i + r_e/2),\qquad(3)$$

where C is the membrane capacitance, taken to be 1 $\mu F/cm^2$, and r_i and r_e are the resistivities of the internal and external solutions.

For membranes of a given composition the critical membrane voltage V_c for breakdown at membrane sites oriented in the field direction should be a single value. In the conducting solutions where the time constant for membrane charging is small compared with the duration of the electric field, Eq. 2 becomes

$$V_c = 1.5E_c R_o.\qquad(4)$$

Thus, we determined the critical membrane voltage over the whole range of membrane tensions for the three lipid mixtures SOPC(:DOPG):CHOL, RBC lipids, and SOPC:(DOPG). The results are shown in Fig. 3. The points represent averages of several vesicles and

the error bars show the range of values. Filled data points are measurements carried out in conducting solutions (sucrose/glucose plus 10 mM NaCl). These preparations necessitated the incorporation of small amounts (5%) of charged lipid, DOPG, in the membranes. Our checks on the elastic modulus K and mechanical lysis tension at zero field showed that such small amounts of DOPG did not change the values from those of the neutral preparations made previously (Evans and Needham, 1988a; D. Needham and R. S. Nunn, manuscript submitted for publication). The open symbols represent data collected on neutral lipid vesicles in low conducting media. Under these conditions Eq. 2 was used with τ (Eq. 3) being calculated from the measured resistivities for bathing and internal solutions. When solution resistivities were taken into account the neutral and charged preparations showed the same behavior, i.e., the same critical membrane voltage at breakdown. The red blood cell lipid vesicles were likely to contain ~15 mol % charged lipid (van Deenen and de Gier, 1964).

For a given lipid composition the critical membrane voltage showed a simple relation to the isotropic membrane tension. The intercepts on
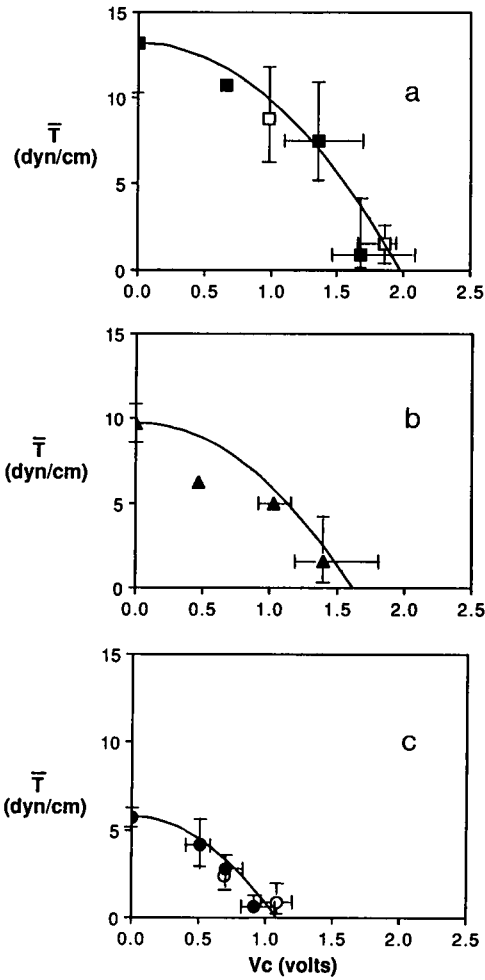
FIGURE 4 Critical electric field strength for permeabilization, E_c versus reciprocal of vesicle radius $1/R_o$, for SOPC:CHOL.

FIGURE 3 Critical voltages for membrane permeabilization V_c as a function of applied membrane tension $\overline{T}$. (a) SOPC(:DOPG):CHOL and SOPC:CHOL, (b) RBC lipids, (c) SOPC(:DOPG) and SOPC. (*Filled symbols*) vesicles that contained sucrose plus 10 mM NaCl and had small amounts of charged lipid DOPG in their membranes. (*Open symbols*) neutral lipid vesicles that contained only sucrose. The solid curves are plots of Eq. 14.

both the tension and voltage axes decreased in the series SOPC:CHOL > RBC lipids > SOPC.

The second experiment was designed to simply test, in our system, that the critical field strength was linearly dependent on vesicle size as dictated by Eq. 2, for a given membrane and solutions. The results are shown in Fig. 4. For SOPC:CHOL the critical field strength E_c does in fact show a linear dependence on $1/R_0$ as expected with a slope of 4 V. According to Eq. 2 this slope is given by

$$\text{slope} = \frac{V_{\text{crit}}}{1.5(1 - e^{-t/\tau})}. \tag{5}$$

Hence, V_c is ~2.4 V, because $(1 - e^{-t/\tau})$ ~0.4 due to the low solution resistivities. This value is in approximate agreement with the intercept on the V_c axis for SOPC:CHOL in Fig. 3.
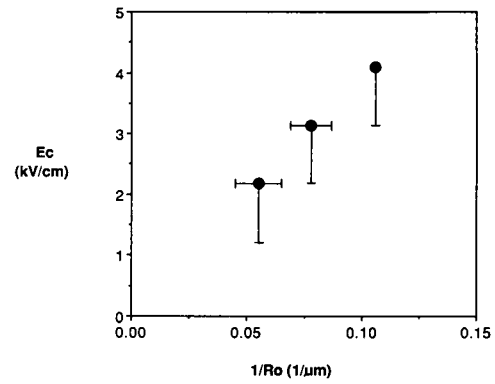
Theoretical model

The simple dependence of critical voltage V_c on membrane tension $\overline{T}$ as shown in Fig. 3 and the shifts in the intercepts with membrane composition and thus with area expansivity modulus K suggest a relationship between these parameters. In order to derive such a relation we employ a simple thermodynamic approach similar to that used by Evans and Hochmuth (1978) in their study of the elastic properties of biomembranes.

Consider a portion of a lipid bilayer membrane with thickness h, dielectric thickness h_e, and area A, as shown in Fig. 5. Aspiration of the vesicle into a pipet creates an isotropic tension $\overline{T}$ along the surface of the membrane and the application of an electrical field creates a compressive stress σ_e perpendicular to the surface. The differential work done on the membrane by the tension and normal stress is

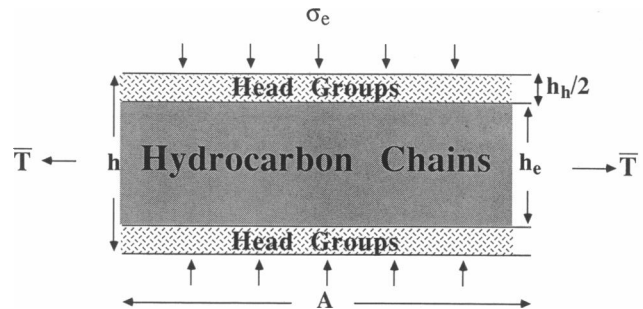$$dW = \overline{T}dA - \sigma_e Adh. \tag{6}$$

FIGURE 5 Schematic diagram of bilayer showing geometric parameters and action of mechanical and electrocompressive stresses. $\overline{T}$ is the isotropic membrane tension; σ_e is the electrocompressive stress; h is total bilayer thickness from x-ray measurements (McIntosh and Simon, 1986); h_e is the dielectric thickness (hydrocarbon) from combined electron density profiles and capacitance and optical measurements (Simon and McIntosh, 1986); h_h is the headgroup thickness (Simon and McIntosh, 1986); A is the area of the membrane (Lecuyer and Derivichian, 1969).

In Eq. 6, the work done by the ambient pressure ($-Pd$ volume) is zero because it is assumed that the liquid membrane is incompressible.

If the membrane behaves as a capacitor, then

$$\sigma_e = \frac{1}{2} \epsilon\epsilon_0 \cdot \left(\frac{V}{h_e}\right)^2, \tag{7}$$

where V is the potential drop across the membrane, ϵ_0 is the permitivity and ϵ is the relative dielectric constant for the membrane. Also, because the membrane is incompressible,

$$Adh = -hdA. \tag{8}$$

The substitution of Eqs. 7 and 8 into Eq. 6 gives

$$dW = \left[\overline{T} + \frac{1}{2}\epsilon\epsilon_0\left(\frac{V}{h_e}\right)^2 \cdot h\right] dA. \tag{9}$$

In general, for any equilibrium process, i.e., when the dissipative (viscous) effects are negligible, the isothermal work done at the boundary of a system is equal to the change in the Helmholtz free energy:

$$dW = dF)_T. \tag{10}$$

If the membrane remains flat during deformation, such that there is no increase in free energy from bending, then

$$dF)_T = K\alpha dA, \tag{11}$$

where the relative increase in area is given by

$$\alpha = \frac{A - A_0}{A_0} = \frac{A}{A_0} - 1$$

and A_0 refers to the membrane in the stress-free state. The constant K in Eq. 6 is called the elastic area compressibility (or expansivity) modulus (Evans et al., 1976; Evans and Waugh, 1977; Evans and Hochmuth, 1978).

The substitution of Eqs. 9 and 11 into Eq. 10 and the subsequent cancellation of the differential area term dA gives

$$\overline{T} + \frac{1}{2}\epsilon\epsilon_0\left(\frac{V}{h_e}\right)^2 \cdot h = K\alpha. \tag{12}$$

Let $h = h_e + h_h$ where h_h is the thickness of the head group region, and h_e

is the dielectric thickness. Then, because the membrane is incompressible

$$h = \frac{h_o}{1 + \alpha}, \qquad h_e = \frac{h_{e_o}}{1 + \alpha},$$

and

$$\overline{T} + \frac{1}{2}\epsilon\epsilon_0 \cdot \frac{V^2}{h_{e_o}} \cdot \left(\frac{h_o}{h_{e_o}}\right)(1 + \alpha) = K\alpha, \tag{13}$$

where h_o and h_{e_o} are values for the membrane thicknesses in the stress-free state. Because $\alpha \ll 1$ (Evans et al., 1976; Evans and Waugh, 1977; Kwok and Evans, 1981; Evans and Needham, 1988a and the results of this study), Eq. 13 becomes

$$\overline{T} + \frac{1}{2}\epsilon\epsilon_0 \cdot \left(\frac{h_o}{h_{e_o}}\right) \cdot \frac{V^2}{h_{e_o}} = K\alpha. \tag{14}$$

Eqs. 13 and 14 simply state that the area of the membrane can be increased either by using pipet aspiration to create an isotropic tension along the surface of the membrane or by using an electrical field to create a compressive stress perpendicular to the surface. We postulate that regardless of the way in which the area is increased, a membrane of given composition will break down at a critical value for α, i.e., at α_c. Both α_c and K have been determined for the three lipid compositions studied here (D. Needham and R. S. Nunn, manuscript submitted for publication). The values along with the other parameters in Eq. 14 and the critical energy per mole are shown in Table 1. At zero voltage $K\alpha_c$ is simply the isotropic tension at lysis $\overline{T}_c$.

It was important in the model to recognize that the electrical compressive stress acts across the dielectric thickness of the bilayer rather than total bilayer thickness. The initial dielectric thickness h_{e_o} therefore corresponds to that obtained from capacitance measurements on planar bilayers. In the absence of such measurements for the actual lipid systems used in the present study we used the values determined for the closely related systems of bacterial phosphatidylethanolamine (BPE) and BPE:cholesterol 1:1 (Simon and McIntosh, 1986). The effective dielectric bilayer thicknesses h_{e_o} for SOPC (taken as that for BPE) and SOPC:CHOL (taken as that for BPE:CHOL, 1:1) was estimated from these capacitance measurements to be 28 Å and 34 Å respectively (Simon and McIntosh, 1986). The same relative dielectric constant ϵ of 2.2 was assumed for all bilayers (Fettiplace et al., 1971).

The initial bilayer thickness h_o for SOPC was taken to be that measured by x-ray methods (39 Å) (Rand et al., 1988) whilst the value used for SOPC:CHOL, 1:1 was taken to be the same as that measured for egg phosphatidylcholine:cholesterol, 1:1 (40 Å) (McIntosh et al.,

TABLE 1 Mechanical and geometric parameters for the lipid bilayer systems studied, including critical energy per mole for failure

Lipid system	K	$\overline{T}_c(= K\alpha_c)$	α_c	$\dfrac{K\alpha_c^2 \cdot \overline{A}_0 N_0}{2}$	h_o	h_{e_o}
	dyn/cm	dyn/cm		$cal/mol.$	$\times 10^8\ cm$	$\times 10^8\ cm$
SOPC	193 ± 20	5.7 ± 0.2	0.030	8.1	39	28
RBC lipids	420 ± 36	9.7 ± 0.8	0.023		40	32
SOPC:CHOL	594 ± 99	13.2 ± 2.2	0.022	9.1	40	34

$\overline{A}_0$ is the average area per molecule and N_0 is Avogadros' number. We estimate the following values from x-ray data on lecithin and lecithin:CHOL (Lecuyer and Derivichian, 1969). For SOPC, $\overline{A}_0$ has been taken to be 65Å^2 and for SOPC:CHOL it is estimated to be 44Å^2. $\epsilon = 2.2$ for all cases. K values are taken from D. Needham and R. S. Nunn, manuscript submitted for publication).

1989). The values h_o and h_{∞} for RBC lipids at ~35% cholesterol were interpolated on a linear mol % CHOL basis from the SOPC and SOPC:CHOL, 1:1 values.

Thus, for a lipid bilayer of given composition, an imposed tension that is $K\alpha_c$ $(=\overline{T}_c)$ allows the calculation of the critical membrane potential for breakdown using Eq. 14 because the constants ϵ_o, ϵ_r, h_o, and h_{∞} are known or can be fairly well estimated for these lipid systems. The predicted values for the critical membrane voltage for a given value of membrane tension are shown in Fig. 3 for comparison with the experimentally determined values of V_c. The theoretical relationship, represented as a solid line, shows a close fit with the measured data for all three lipid compositions.

A further illustration of the good fit between experiment and the simple theoretical model is shown in Fig. 6 in which the data are collapsed onto a "1–1" plot by dividing the mechanical and electrical stresses by $K\alpha_c$. The theoretical prediction for this plot shown as a solid line has intercepts of 1.0 and a slope of 1.0. All of the experimental data agree with this prediction.

DISCUSSION

A simple model based on storage of elastic energy as a result of work done at the boundaries of the system by either tensile or electrocompressive stresses fits the experimental data very well. The model is not only good for a given bilayer but also predicts the shift in breakdown voltage as a result of changing the bilayer compressibility. In these experiments a threefold change in bilayer compressibility was achieved by incorporation of cholesterol into the reconstituted lipid bilayers. Of special interest is the value for V_c predicted by Eq. 14 when $\overline{T}$ = 0. For natural membranes under zero mechanical tension the permeabilization voltage would be expected to increase with increasing cholesterol content. It has recently
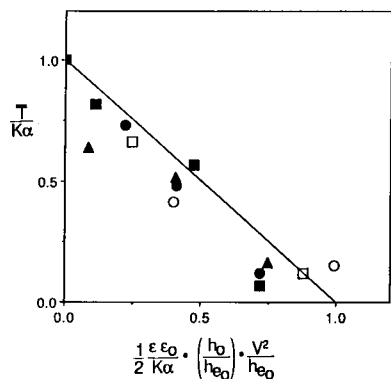

FIGURE 6. "One-one" plot for experiment and theory. All the data from each of the three bilayers are collapsed onto a single plot according to Eq. 14. ■ SOPC(:DOPG):CHOL; □ SOPC:CHOL; ▲ RBC lipids; ● SOPC(:DOPG); ○ SOPC. Filled symbols are for vesicles containing 200 mM sucrose plus 10 mM NaCl and open symbols are for vesicles containing only 200 mM sucrose.

become clear from this and other work (Evans and Needham, 1988a; Needham et al., 1988; D. Needham and R. S. Nunn, manuscript submitted for publication) that the presence of cholesterol is the single most influential factor in determining liquid bilayer cohesion and so directly affects the critical voltage for membrane breakdown.

In Eq. 14 all the parameters have been determined experimentally. Unlike earlier studies, which attempted to control membrane tension or "turgor" pressure by osmotic forces (Zhelev et al., 1987; Zimmermann et al., 1977, 1980), the micropipet manipulation technique allows the membrane tension to be accurately set. Also, the critical membrane voltage for breakdown was shown to be independent of vesicle size and electrolyte concentration when solution resistivities were taken into account. This is an important conclusion for interpretation of membrane fusion experiments that are carried out using the neutral lipids and therefore must be formed in low conducting media (D. Needham, unpublished results).

Our results indicate that failure of a given membrane does not depend on the particular mechanisms by which a membrane is stressed and deformed. Also, the values shown in Table 1 indicate that a general failure criterion for all membranes based on a critical surface energy density $(K\alpha_c^2 \cdot N_o\overline{A_o}/\ 2)$ may be better representation of breakdown than one based on a critical surface area (α_c). Failure is clearly not given by a single critical stress for all membranes. For the three lipid systems studied here, α_c decreases from 0.03 (SOPC) to 0.022 (SOPC:CHOL), a decrease of 27%. The value for the energy per unit area at failure increases from 0.083 to 0.14 ergs/cm², an increase of 70%. On a per mole basis, the energy densities are much closer. For SOPC the energy density at failure is 8.1 cal/mol, and for SOPC:CHOL 1:1 it is 9.1 cal/mol. The membranes containing cholesterol (RBC lipids, SOPC:CHOL) are the toughest, yet they undergo the lesser amount of relative area dilation before failing. Although we measure the value for α at failure, it should be possible to predict this value either with a continuum visco-elastic model based on the stability of thin films (Dimitrov, 1984) or a stochastic and thermodynamic model based on the formation of membrane pores of a critical size (Sugar and Neumann, 1984).

It is interesting to compare our theoretical development (Eqs. 10–14) to Crowley's model, in which an electrocompressive stress elastically thins a membrane, and the extension of this model by Zimmermann and co-workers to account for a membrane stress caused by a turgor pressure. This model is stated as (Zimmermann et al., 1977; 1980):

$$P + P_e + P_m = 0, \tag{15}$$

where P_e is the electrical compressive force (with units of stress or force/area), P_m is the elastic restoring "force" and P is the mechanical compressive force resulting from a turgor pressure. With the expression for P_e (see Eq. 11) and Crowley's expression for P_m, Eq. 15 becomes

$$P + \frac{\epsilon \epsilon_0}{2} \left(\frac{V}{h}\right)^2 + Y \ln\left(\frac{h}{h_0}\right) = 0, \qquad (16)$$

where Y (or "E" in Crowley's work) is a bulk elastic modulus for the membrane. (Note that our expression for the electrocompressive stress is based on the dielectric thickness h_e instead of the total thickness h). Crowley (1973) and Zimmermann et al. (1977) show that when $P = 0$ the membrane will thin to the point where it becomes unstable when

$$\ln \frac{h_c}{h_0} = -\frac{1}{2}, \qquad \frac{h_c}{h_0} = 0.607 \qquad (17)$$

where h_c is the critial thickness at breakdown.

Thus, at this critical point, Eq. 16 with $P = 0$ becomes

$$\frac{\epsilon \epsilon_0}{2Y} \cdot \frac{V_0^2}{h_0^2} = \frac{(.607)^2}{2} = \frac{.368}{2} = 0.184. \qquad (18)$$

The constant "0.368" or "0.37" or "0.3679 or 0.369" appears in many of the publications by Zimmermann et al. including the ones cited here (see Zimmermann, 1982 and 1986 for reviews). Nevertheless, for an incompressible liquid membrane, this particular constant implies that the membrane surface area is capable of an extraordinary amount of expansion. Thus, since $A_h = A_0 h_0$,

$$-\ln\left(\frac{h_c}{h_0}\right) = \ln(1 + \alpha_c) = 0.5 \rightarrow \alpha_c = 0.65.$$

The stability calculation by Crowley (1973) and Zimmermann et al. (1977, 1980) implies a value for α_c that is more than twenty times larger than the value measured by micropipet manipulation of several lipid bilayer membranes.

Because the changes in thickness (and area) are small, the elastic compressibility term in Eq. 16 becomes

$$-\ln\left(\frac{h}{h_0}\right) = \ln(1 + \alpha) \simeq \alpha.$$

Thus, Eq. 16 becomes

$$P + \frac{\epsilon \epsilon_0}{2} \left(\frac{V}{h}\right)^2 = Y\alpha. \qquad (19)$$

Eq. 19 can be compared to Eq. 12 when Eq. 12 is divided by h:

$$\frac{\bar{T}}{h} + \frac{\epsilon \epsilon_0}{2} \left(\frac{V}{h_e}\right)^2 = \frac{K}{h} \alpha. \qquad (20)$$

Thus, for small deformations, the constant "Y" is K/h. From Table 1, the values for Y would vary between 5.1×10^8 dyn/cm^2 and 1.3×10^9 dyn/cm^2 (5.1×10^7 N/m^2 and 1.3×10^8 N/m^2) (c.f., Requena, et al., 1975).

In Eq. 20 we can relate the membrane tension to the turgor pressure using Laplace's equation for an isotropic spherical shell:

$$\bar{T} = \frac{PR_0}{2}. \qquad (21)$$

Thus, Eq. 20 becomes

$$\frac{P}{2} \cdot \frac{R_0}{h} + \frac{\epsilon \epsilon_0}{2} \left(\frac{V}{h_e}\right)^2 = \frac{K}{h} \alpha. \qquad (22)$$

It can be seen by comparing Eq. 22 with Eq. 19 that the turgor pressure is not the correct parameter in Eq. 19. The correct parameter is the turgor pressure times the cell radius divided by twice the membrane thickness. This will scale up the turgor pressure by three orders of magnitude! That this is correct and that our theory embodied in Eq. 14 is correct is shown by the results and correlations given in Figs. 4 and 6.

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