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Relaxation spectra of gramicidin dimerization in a lipid bilayer membrane

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The kinetics of formation and dissociation of gramicidin dimers in a lipid bilayer membrane have been studied by pressure-jump and electric field-jump methods. The traditional AC-coupled pressure-jump apparatus has been modified so that a known DC-voltage drop is maintained across a Teflon cell divided by a septum with a hole for membrane formation. From the response of the amplified output voltage after the pressure release, information about the kinetics of channel (dimer) formation is obtained. In addition, using the same apparatus, electric field-jump measurements were performed on the gramicidin/membrane system. In asolectin/7-dehydrocholesterol (5:1) membranes at 25 ± 0.1°C, the best fit to the pressure-jump data gives a dimer dissociation rate constant of 0.5 ± 0.3 s^{-1}. The standard volume change for dimerization determined from the amplitude of the pressure-jump experiments is -66 ± 35 cm^3/mol. Rate data determined by the electric field-jump method are consistent with the pressure-jump values; results obtained with either technique are compatible with other determinations of the kinetics of dimerization on gramicidin/membrane systems.

Introduction

In lipid bilayer membranes, gramicidin A dimerizes, forming a cation-specific channel. The kinetics of channel formation and dissociation have been studied using electric field-jump (E-jump) measurements [1], and fluctuation analysis [2,3]. However, as the increase in electric field not only coupled with the chemical equilibrium, but altered the membrane parameters, gramicidin dimerization was reinvestigated using pressure-jump (P-jump). This technique has the advantage that the perturbing parameter (pressure) only measurably affects the monomer/dimer population. E-jump studies were also performed to determine whether the relaxation times observed in our membrane system were comparable to those of Bamberg and Läuger [1].

The response of the membrane system and its components to a stepwise change in pressure was monitored in a P-jump apparatus developed to study fast reactions in homogeneous solutions [4]. As channel formation increased the conductance of the membrane, conductivity was chosen to monitor the approach to the steady-state population of dimers. Modifications in the traditional P-jump apparatus were required for use on a bilayer system.

Materials and Methods

Materials

All materials were used as purchased. The membrane-forming solution was 1.5% 7-dehydro-

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cholesterol (Sigma Chemical Company) and 6.5% asolectin (w/w) (a gift from C. Miller, Graduate Department of Biochemistry, Brandeis University) in *n*-decane (Fisher Scientific Co.) and was stored at 0°C. A 1 M NaCl (Fisher, Certified Reagent) solution was prepared in glass-distilled water, and was used as the electrolyte in the membrane chambers. A stock solution of $4.44 \cdot 10^{-4}$ M gramicidin in methanol (Fisher, Certified Reagent) was stored at 0°C.

Methods

All glassware was washed with Joy Liquid and hot water two times, rinsed with hot water, rerinsed ten times with glass-distilled water, and air-dried. The membrane cell, soaked in a 50% solution of HCl/ethanol for 15 min, was washed with Joy Liquid and hot water five times, rinsed with hot water, and rinsed more than 100 times with glass-distilled water. The cell was then air-dried for 30 min.

Gramicidin was added to each side of the membrane cell in equal volumes with the methanol concentration kept to less than 0.15%.

Apparatus

The Strehlow and Becker [4] two-cell pressure bomb was modified to accommodate a snap-in cell suitable for membrane formation (Fig. 1). The Teflon membrane cell was machined by the Brandeis University Machine Shop with two press-fit Ag/AgCl electrodes. A 1 mm diameter hole was drilled through the center partition for membrane formation. All seals between adjacent flanges of the pressure bomb were O-ring seals and the pressure chamber was sealed from the atmosphere by a 0.025 inch brass disk (rupture pressure > 100 atm). A Neslab Constant Temperature Bath thermostatted the reaction chamber to 25 ± 0.1°C.

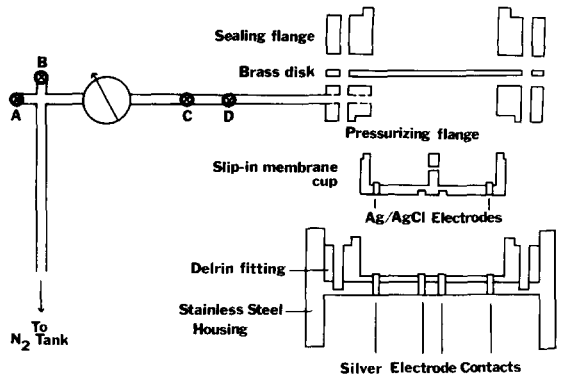

Fig. 1. Membrane cell pressure-jump apparatus. Valves A and C are ten-turn leak valves; valves B and D, ball valves.

Nitrogen gas was regulated through a high-pressure, low-flow gas regulator (Victor Compressed Gas Regulator 541 L) to a final pressure of 40 atm. All connections between the tank and the pressure bomb were made with 0.25 inch i.d. copper tubing. Line pressures were read with a Solfunt 0–1000 lb/in^2 meter. The line was adjusted to the desired pressure and was opened or closed through the use of two leak valves and two ball valves. To achieve the pressure-jump, the leak valves were opened completely, the ball valves were then manually opened, and the pressure release was achieved. Manual manipulation of the valves yielded a response time for pressure release of 0.2 s [5].

Cell resistance between the two Ag/AgCl electrodes, as measured by a voltage clamp, was read as a voltage on a Tektronix Storage Oscilloscope. To ensure electrical contact between the Ag/AgCl electrodes and their contacts, silver grease (Eccoshield VY, Emerson & Cumming) was used to coat the bottom of the electrodes. High-impedance components were used throughout the network to avoid current leakages. When used as a source of constant voltage, this voltage clamp monitored the system's response to a pressure jump. When the voltage was switched from the low to high setting, the network performed an electric field jump. Therefore, E-jump and P-jump measurements could be monitored using the same device.

Experimental procedures

P-Jump. After cleaning the membrane cell, and after electrical warm-up, dabs of silver grease are applied to each electrode contact, and the cell is pressed into the cell cavity.

The area around the membrane hole is then coated with lipid, dried for 15 min, 1 M NaCl is added, and the membrane is painted with a Teflon rod. When a non-leaky, thin membrane has formed, the capacitance of the membrane is mea-

sured. Both Teflon and polyethylene cells were tried. However, consistently stable and thin membranes were obtained using a Teflon support. Therefore, it is used in all experiments with membranes.

When a stable membrane is present, the pressure bomb is sealed by lowering and carefully tightening the pressurizing flange over the bomb housing. The pressure chamber is closed to the line, and the line pressure is taken up to the desired pressure plus three atmospheres to allow for expansion into the pressure chamber. The nitrogen tank is then closed, and the chamber is equilibrated to line pressure by opening the ball valve and the leak valve.

When the reaction chamber and the line are at the same pressure, the ball valve is re-closed. The other ball valve is now opened, releasing the line pressure while the pressure chamber remains pressurized. The leak valve between the first ball valve and the pressure chamber is then opened completely to ensure maximal gas flow. When the oscilloscope trace stabilizes, indicating high-pressure equilibrium, the ball valve is opened, achieving the pressure-jump.

Both before and after the pressure-jump, the voltage magnitude of a polarity switch is recorded. Division by 2 yields the potential of the membrane with respect to electrical zero, allowing initial membrane potentials to be compared with final ones. The determination of the response time of the apparatus to a 40 atm pressure drop was made using a polyethylene cell, 1 M NaCl, and an AC Wheatstone bridge powered with a 3 V peak-to-peak, 100 kHz sine wave.

E-Jump. The P-jump apparatus without the pressurizing flange was used for E-jump measurements. The E-jump up was achieved by switching the voltage clamp from its setting of 20 mV on Low to 200 mV on High. (An E-jump down is accomplished by a switch from High to Low.) Capacitance measurements of the network yield a response time of approx. 10 μs. For the E-jump measurements, the response of the electrical network is instantaneous compared with the chemical response times. As in pressure-jump, the chemical response to the change in electric field is displayed as a voltage on the oscilloscope.

Results and Data treatments

The responses of individual components of the system were examined to eliminate experimental artifacts. Since the P-jump and the voltage clamp method of detection require the application of an electric field, the effects of changing field strength were assessed.

Normally, to assess the effect of an electric field on a membrane, the capacitance as well as the area of the membrane would be measured. However, when using pressure-jump, rounded surfaces must be used to avoid cavitation effects. As it was impossible to 'see' the membrane to obtain area measurements within the physical constraints, capacitance was used to monitor the effect of changing field strength.

A triangular voltage ramp of variable am-

TABLE I

EFFECT OF ELECTRIC FIELD ON MEMBRANE CAPACITANCE

Membrane capacitance is measured in nfarads/membrane. Values for five representative membranes are shown, where lines separate values for different membranes.

30 mV	60 mV	70 mV	100 mV
1.8	1.5	1.4	1.5
1.7	1.7	1.6	1.8
2.0	1.8	2.1	2.2
2.7	2.2	2.6	2.4
2.8	2.5	2.6	2.4
2.2	1.8	2.6	3.1
4.3	3.3	3.8	3.7
3.5	4.0	4.1	3.9
4.7	4.2	4.0	3.9
4.5	3.7	4.1	4.0
6.0	5.3	6.3	5.3
6.0	6.0	6.0	5.5
6.3	5.5	5.7	5.7
6.7	5.7	6.0	5.7
5.0	4.3	5.1	4.6
6.3	4.3	5.3	4.9
5.3	4.7	5.3	4.6
5.3	4.7	5.3	4.9
5.3	4.7	5.0	4.9
4.7	5.2	5.7	5.6
6.7	5.7	6.1	6.0
8.7	5.7	5.7	5.6
6.3	5.3	5.7	5.7

plitude was used to measure the effect of field strength on the lipid bilayer. Several measurements were done on most membranes to determine whether the membrane returned to the same low-voltage capacitive value after being subjected to a higher voltage (peak-to-peak voltages: 30, 50, 60, 70, 100, 200 mV).

We found that the asolectin/7-dehydrocholesterol membranes changed their physical parameters in response to large electric fields. These membranes tended to maintain increases in membrane capacitance rather than returning to their original values (Table I).

Measurements to determine the pressure-dependence of membrane capacitance were also performed. Using a constant voltage, a P-jump was performed as described. Capacitance values remained constant throughout any pressure series (Table II). Some compression of the membrane may have occurred; however, any change was very small (% S.D. ≤ 3.0).

The change in conductivity of 1 M NaCl due to pressure release was measured by an AC Wheatstone bridge and used to determine the time constant of the apparatus. The conductivity change of 1 M NaCl due to pressure was then measured by the voltage clamp/inverting amplifier network. No difference in the output voltage between the pressurized salt and the atmospheric salt was observed. Therefore, the effects of pressure and the accompanying small temperature change on the 1 M NaCl solution were undetectable as measured by a voltage clamp. Similarly, a blank of $1.06 \cdot 10^{-7}$ M gramicidin in 1 M NaCl was subjected to pressure changes. No pressure dependence was found.

TABLE II

PRESSURE DEPENDENCE OF CAPACITANCE

Each column refers to capacitances measured on a new membrane, in nfarads/membrane, 60 mV peak-to-peak applied voltage.

P (lb/in^2)	1	2	3	4	5
15	4.2	4.5	2.7	1.8	3.3
585	4.5	4.7	2.9	2.0	3.7
520	4.6	4.8	2.8	2.0	3.7
465	4.8	4.7	2.8	2.0	3.6
415	4.6	4.8	2.7	2.0	3.7
360	4.5	4.8	2.8	2.0	3.6
310	4.6	4.8	2.8	2.0	3.6
265	4.5	4.7	2.8	2.0	3.5
15	4.7	4.7	2.8	2.0	3.6

In summary, throughout the testing of the separate components of the membrane/gramicidin system, no changes in conductivity were measurable by a voltage clamp. However, changes in electric field applied to membranes caused a measurable change in capacitance. Therefore, any observed change in conductivity was assigned to the dimerization of gramicidin in the lipid bilayer.

Relaxation times of the membrane/gramicidin system were recorded at various concentrations using the E-jump and P-jump techniques. Let A and A_2 be the instantaneous concentrations of gramicidin monomers and dimers, respectively, in the membrane at time t. Then, for the dimerization reaction,

$$2A \underset{k_D}{\overset{k_R}{\rightleftharpoons}} A_2 \tag{1}$$

the relaxation time, τ, is defined by

$$1/\tau = k_D + 4(k_R k_D A_2)^{1/2} \tag{2}$$

or [1]

$$1/\tau = k_D + 4\left(k_R k_D \Lambda_{SS}/\Lambda_{SC} N^0\right)^{1/2} \tag{3}$$

where Λ_{SS} is the steady state conductance, Λ_{SC}, the single channel conductance, and N^0, Avogadro's number. The single channel conductance, Λ_{SC}, has been measured in dioleylphosphatidylcholine membranes and was not found to vary over a range of lipid concentrations. Therefore, a value of $6.0 \cdot 10^{-12}\ \Omega^{-1}$ at 25 mV in 1 M NaCl was used for the single-channel conductance [2]. The steady-state conductance, Λ_{SS}, was calculated from the cell resistance. Variation of the dimer concentration will cause the relaxation time to vary if $k_D \ll 4(k_R k_D \Lambda_{SS}/\Lambda_{SC} N^0)^{1/2}$. A plot of $1/\tau$ vs. $(\Lambda_{SS}/\Lambda_{SC} N^0)^{1/2}$ yields intercept k_D, and slope $4(k_R k_D)^{1/2}$.

For E-jump experiments, relaxation times were measured for different gramicidin/membrane systems using two different applied voltages. In the 'up' experiments, the voltage is switched from 20 mV to 200 mV, giving rate constants at high field

values. Using a jump up from low to high voltage, two relaxation times were consistently observed. One was of the order of 7 s; the other, approx. 0.5 s. To rule out unstirred layer effects for the longer relaxation time, the polarity was switched immediately before the jump. The same two relaxation times were, again, recorded.

After subtracting the contribution of the longer relaxation time, a dissociative rate constant of $1.00 \pm 0.23\ s^{-1}$ was calculated. The data were too scattered to obtain a meaningful associative rate constant.

In the 'down' experiments, the voltage is switched from 200 mV to 20 mV, approximating the zero-field case. Only one relaxation time of approx. 0.5 s was observed. Analysis of the data through linear regression yields $k_R = 9 \cdot 10^{15}\ cm^2 \cdot mol^{-1} \cdot s^{-1}$, $k_D = 0.96 \pm 0.20\ s^{-1}$, and $K = 9 \cdot 10^{15}\ cm^2 \cdot mol^{-1}$. The data scatter only allows determination of K and k_R within a factor of ten. As concentrations are low, relaxation times do not vary appreciably, and yield k_D plus a small number. The high- and low-field values agree very closely, approximating the $1.6\ s^{-1}$ obtained by fluctuation measurements on membranes of approximately the same thickness [2].

The second type of E-field measurement only shows one relaxation time, implying that the longer relaxation time observed in the low- to high-field measurements is related to the change in membrane capacitance observed in control experiments

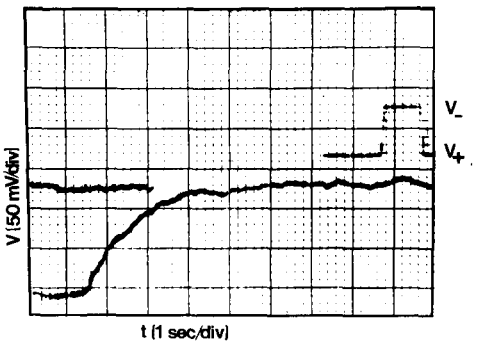

Fig. 2. The voltage response to a pressure-jump from 40 atm to 1 atm with gramicidin in an asolectin/7-dehydrocholesterol membrane. The trace starts at the lowest point at the left. After the trace had 'free-traveled' for 1.4 s, the pressure-jump was initiated. The step up and down in the upper right represents the switch from V_+ to V_- to V_+, and has a magnitude of 1.2 V.

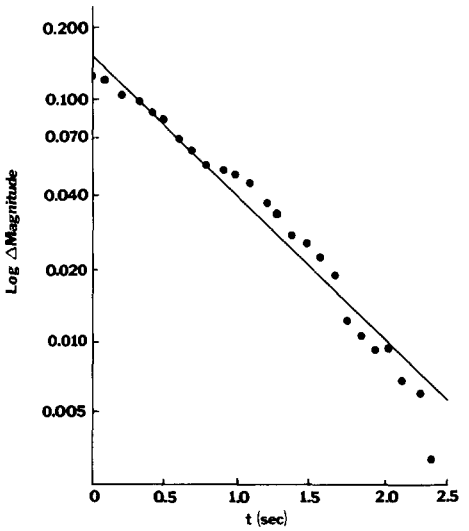

Fig. 3. The plot of log Δ magnitude vs. t for the pressure-jump trace shown in Fig. 2. A relaxation time of 0.76 s was obtained from the linear regression fit to the line.

on varying field strengths. The E-jump 'up' and 'down' experiments were done in pairs with at least 1.5 min between measurements. In the cases where more than one pair of measurements was made on one membrane, if the longer relaxation time is attributed to a slow, irreversible change in membrane size, then after repeated measurements on the same membrane, the longer relaxation time should, ideally, disappear. In repeated experiments on the same membrane, we observed that the longer relaxation time tended towards smaller relaxation amplitudes (unpublished data). As the membrane would not return to its low-field size after the E-jump 'down' – at least on the time scale of these experiments – no second relaxation

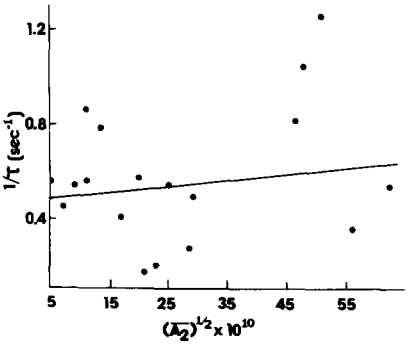

Fig. 4. Pressure-jump from 40 atm to 1 atm. The best fit to the data gives an intercept, k_D, of $0.47\ s^{-1}$. k_R may be calculated from the slope, which gives a value of $2 \cdot 10^{14}\ cm^2 \cdot mol^{-1} \cdot s^{-1}$.

time due to membrane/gramicidin re-equilibration would be expected, and none was observed.

Using a voltage of 20 mV to monitor the response of the system, a typical trace of the system's response to a pressure-jump is shown in Fig. 2. In the P-jump experiment, only one relaxation time is seen (Fig. 3).

A plot of $1/\tau$ vs. $(A_2)^{1/2}$ is shown in Fig. 4. The best fit to the data was obtained with $k_D = 0.47\ s^{-1}$, $k_R = 2 \cdot 10^{14}\ cm^2 \cdot mol^{-1} \cdot s^{-1}$, and $K = 4 \cdot 10^{14}\ cm^2 \cdot mol^{-1}$. This set of rate constants approximates those found by Bamberg and Läuger.

If the equilibrium constant and change in the degree of dissociation, $\Delta\alpha/\alpha$, are known, the molar volume change, ΔV^0, can be calculated using:

$$\frac{\Delta\alpha}{\alpha} = \frac{(1-\alpha)\Delta V^0}{(2-\alpha)}\Delta P \qquad (4)$$

The average value of ΔV^0 for reaction 1 is $-66 \pm 35\ cm^2 \cdot mol^{-1}$. If the dimer has a radius of $8 \cdot 10^{-8}$ cm [6], and is $26 \cdot 10^{-8}$ cm long [7], it occupies a volume of $3.1 \cdot 10^3\ cm^3 \cdot mol^{-1}$; therefore, the measured ΔV^0 corresponds to a 2.1% change in volume due to dimerization.

Discussion

In our measurements, we observed no changes in membrane capacitance in response to changes in pressure. This implied that both the area and the thickness of the membrane were unaltered under our conditions, even after several cycles of pressurization and release. In contrast, a change in electric field caused measurable changes in membrane capacitance, which, over the time course of our measurements, were irreversible. Others have described reversible [8–10] and irreversible [10] changes in membrane capacitance upon the application of an increased electric field, and have speculated that this increased capacitance at high field strength is due to the thinning of the lipid bilayer [10].

As different lipids exhibit very different compressibilities, which is further complicated by the solvent used for membrane formation, measurements of membrane parameters, as well as measurements of properties of peptides interacting with these membranes, are very dependent on membrane composition. Although the change in electric field caused membrane capacitance changes, it was necessary to measure the rate of dimerization of gramicidin in asolectin/7-dehydrocholesterol membranes by the electric-field jump technique to determine whether the rate constants for dimerization were comparable to those previously reported in other membrane systems [1,2]. The stability of the membrane to changes in pressure suggested that this was the optimal method for measurements of the rate constants for gramicidin dimerization in a lipid bilayer membrane.

Although the response time of the P-jump apparatus was the same order of magnitude as the shorter relaxation times measured for the gramicidin/membrane system, the straight line shown in Fig. 3 indicates that the response time does not affect the analysis of the chemical response. If the instrument's response contributed to the measured response to the pressure-jump, a graph of log Δ magnitude vs. time would give a curved line which could be separated into its component responses by a subtraction of exponentials, as is the case for two superimposed chemical response times (see, for instance, Bernasconi [11]).

Table III compares electric field- and pressure-jump rate parameters. Because of the different conditions present, differences between the sets of electric field-jump data are expected. Nevertheless, the two electric field-jump k_D values agree, and our electric field-jump data can be fit by Bamberg and Läuger's k_R [1]. Therefore, values for the rate constants of gramicidin dimerization

TABLE III

COMPARISON OF RATE CONSTANTS

Source	k_R (cm^2/mol per s)	k_D (s^{-1})	S.E. of est.
Bamberg and Läuger [1] E-jump	$2.4 \cdot 10^{14}$	1.6	–
This work, E-jump down	$\approx 10^{15}$	0.96	0.26
This work, E-jump up	–	1.0	–
This work, P-jump	$\approx 10^{14}$	0.47	0.30

in this system using pressure-jump can validly be compared with Bamberg and Läuger's electric field-jump values. The pressure-jump values are in agreement with the electric field-jump values, and confirm the validity of the earlier assignment of the shorter electric field-jump relaxation response to reaction 1.

The fractional change in the degree of dissociation can be calculated from the amplitude of the chemical response. In the two methods, different response amplitudes to the forcing parameter are observed. In pressure-jump, an evaluation of the change in the degree of dissociation, $\Delta\alpha/\alpha$, gives an average of 11%. In aqueous solutions, for the dissociation of two inorganic ions under optimal conditions, a response of 0.8% is calculated, assuming the associated $\Delta V^0 = -10$ cm$^3 \cdot$ mol^{-1} [12]. This difference in amplitude is due to the larger ΔV^0 for reaction 1. Because of the mixed atmosphere (lipid and electrolyte) of the gramicidin, and the surface dimpling caused by the gramicidin dimer in lipid bilayers [2], the volume change in reaction 1 due solely to dimerization is not apparent. However, this large ΔV^0 is in the observed range for biological molecules [13].

The response to electric field-jump is even larger. In the electric field-jump up experiments, the average value of $\Delta\alpha/\alpha$ is $56 \pm 13\%$; for electric field-jump down, $149 \pm 64\%$. If the macroscopic dipole moment, ΔM^0, is calculated using the voltage change from 10 mV to 200 mV for the field strength, a temperature of 25°C, and a $\Delta\alpha/\alpha$ of 100%, a ΔM^0 of $7 \cdot 10^7$ esu-cm/mol is obtained [14], which corresponds to creation of a large dipole where none existed before. However, as would be expected for a symmetric dimer [15,16], Bamberg and Benz [9] found no significant dipole moment for the conducting dimer.

Using solvent-free membranes, Bamberg and Benz [9] still obtained large responses to electric field jumps ($\Delta\alpha/\alpha = 50\%$), suggesting that thinning of the membrane did not account for the response of the gramicidin/membrane system to an increased field. They suggested a direct effect of the electric field on the dimerization process with the qualification that small increases of α at higher voltages may be due to changes of the membrane structure.

Therefore, presumably, the large response to an electric field-jump calculated for the gramicidin/membrane system is partially due to the changes in the membrane measured by the changes in capacitance, and, by inference, not only the area and thickness of the membrane, but the formation of lenses at higher field strengths – a phenomenon described by Requena et al. [17], who noted that compression of a black lipid membrane by an electric field does not occur at constant volumes. White [18] calculated that at 100 mV potential, the equivalent pressure exerted by the electric field on the membrane was 0.05 atm. However, based on his measurements of the voltage-dependence of the molar volume of solvent in the membrane, he calculated that a pressure of 11 atm would need to be applied to account for the changes he observed. He suggested that the membrane consisted of a three-layer 'bilayer' with only a fraction of the hydrophobic portion of the lipid extended into the solvent at any time. White has verified this picture with neutron diffraction studies of the content of solvent in lipid bilayers. This model is then able to explain the changes in molar volume of solvent which White observed with increasing potential [19]. However, in our experiments, we directly tested the effect of a 40 atm pressure-jump on membrane capacitance and found no changes. As the application of pressure affects all parts of the system equally, whereas the application of an electric field causes the greatest pressure at the bilayer, this difference would, perhaps, implicitly explain why the two forces elicit such different responses.

In conclusion, both pressure-jump and electric field-jump measurements are possible on lipid bilayer membrane systems, and give similar rate constants. Compared with electric field-jump, the magnitude of the response for pressure-jump is small. However, this pressure response is of the order of observed relaxational responses using the pressure-jump technique. In contrast, the electric field-jump response is too large to be due solely to the polarizability of the gramicidin channel and is larger than would be expected for dimer formation.

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