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ELECTRICAL POLARIZATION OF PHOSPHATIDYLSERINE BILAYER MEMBRANES BY CALCIUM IONS

DAROLD WOBSCHALL[a] AND SHINPEI OHKI[b]

[a]*Departments of Biophysical Sciences and Electrical Engineering and* [b]*Department of Biophysical Sciences, State University of New York at Buffalo, Buffalo, N.Y. 14226 (U.S.A.)*

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SUMMARY

Bilayer membranes formed from a decane solution of phosphatidylserine were polarized by an applied potential. An open circuit voltage pulse following the brief short circuit of the polarized membrane was observed if Ca^{2+} was present in the aqueous phase on both sides of the membrane. This polarization response is proportional to the initial applied voltage and increases with Ca^{2+} concentration until a maximum response of 11 % is obtained at 0.5 mM. Two possible mechanisms, voltage-dependent Ca^{2+} binding and border conduction are discussed.

INTRODUCTION

The apparent polarization of phosphatidylserine bilayer membranes formed by the Rudin–Mueller technique was determined as a function of Ca^{2+} concentration with the objective of clarifying the role of Ca^{2+} in excitable membranes. Preferential Ca^{2+} binding to one side of membranes containing acidic phospholipids such as phosphatidylserine is thought to be responsible for the shift in the current–voltage curve of the squid axon[1], the enhanced monovalent ion permeability of lipid vesicles[2], and the potential developed across phosphatidylserine bilayer membranes[3–5]. Ion binding will contribute to membrane electrical polarization or surface charge induced by an applied field if the binding is potential dependent. Such a component of the polarization is expected to be time dependent and therefore may be approached experimentally either by determining the membrane capacitance as a function of frequency[6] or, as reported here, by determining the voltage transient following the removal of a polarizing voltage.

Unfortunately, we have observed that with the Rudin–Mueller technique, the everpresent border (bulk oil phase comprising the Gibbs–Plateau border or torus) may show similar polarization response if the bulk oil phase has an appreciable electrical conductivity. Our study is aimed at obtaining the polarization characteristics experimentally and examining border conduction and voltage-dependent ion binding as possible mechanisms.

EXPERIMENTAL

Bilayer membranes were formed by spreading a solution of phosphatidylserine in decane (25 mg/ml) across a 1.5-mm aperture placed between two chambers containing 0.1 M KCl aqueous solution at 30 °C, without buffer (pH ~ 6.5) but with variable concentrations of $CaCl_2$. The electrical polarization of these membranes was determined by an apparatus shown schematically in Fig. 1. Possible errors produced by electrode polarization are eliminated by the four-electrode technique. The sequence control shown schematically by a three-position switch is an electronic switching circuit designed for this purpose. A fast electronic voltage regulator or voltage clamp (not shown) brings the membrane to zero potential (± 0.2 mV) within 0.3 ms. Membrane resistance and capacitance were determined by an a.c. bridge. More complete circuits have been published elsewhere.

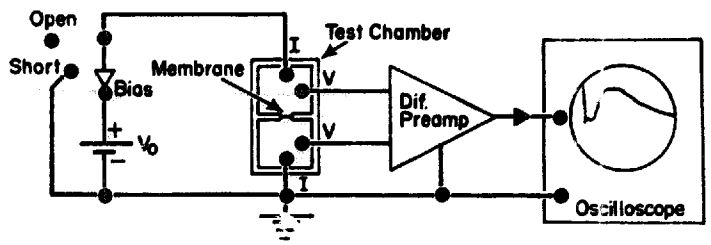

Fig. 1. Membrane polarization apparatus. Initially the membrane is polarized by an external bias voltage. The timing sequency begins by shorting the membrane (electrical equivalent capacitor) briefly as the oscilloscope sweep is triggered. After a few milliseconds the short is removed and the open circuit membrane voltage recorded as a function of time.

Measurement of the bulk conductivity of the membrane forming solution (phosphatidylserine in decane) was made in an apparatus similar to the usual bilayer test chamber except that a thick (1–2 mm) oil drop was entrapped between the two aqueous phases in place of a membrane. Small platinum electrodes were located within the drop so that the voltage drop inside as well as between the two aqueous phases could be measured. In this manner the bulk conductivity and surface conductivity could be separately determined.

RESULTS

Typical polarization responses are shown in Fig. 2. The peak amplitude (V_p) is proportional to the d.c. bias or polarizing voltage (V_0) applied immediately before short circuit and has the same sign. The rising phase deviates appreciably from exponential behavior and has a rate of rise which increases with increasing Ca^{2+} concentration. Rise times are of the order of 10–200 ms. At longer times the voltage decays exponentially with the usual time constant equal to the product of the membrane d.c. resistance and capacitance.

The polarization response, defined here as V_p/V_0, was observed to be nearly zero without Ca^{2+} (if the test chamber was sufficiently clean), but increased with Ca^{2+} concentration until a maximum was reached (see Fig. 3).

The bulk conductivity of the oil has increased approximately linearly with Ca^{2+} concentration (10^{-9} $\Omega^{-1} \cdot cm^{-1} \cdot mmole^{-1}$) although the total membrane con-

ductance remained constant. This behavior can be explained in terms of a high specific resistance at the oil–water interface which is equal to the bilayer specific resistance, that is, $1 \cdot 10^8\ \Omega \cdot cm^2$ and nearly independent of Ca^{2+} concentration[2].

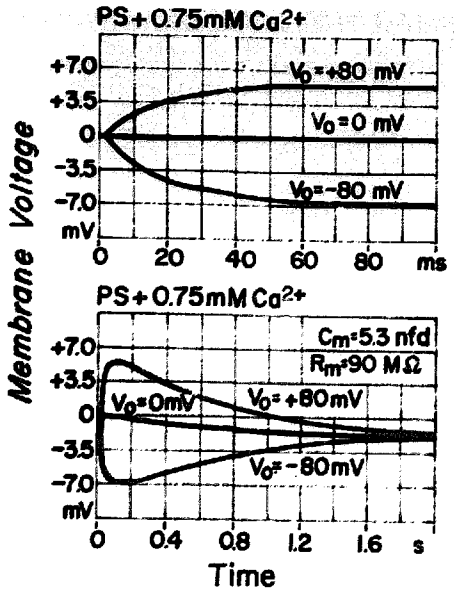

Fig. 2. Membrane polarization response. Tracings of oscilloscope photographs show three responses on two time scales. PS = phosphatidylserine.Typically membranes have a capacitance of 5 nanofarads (nF) and an area of 0.01 cm^2.

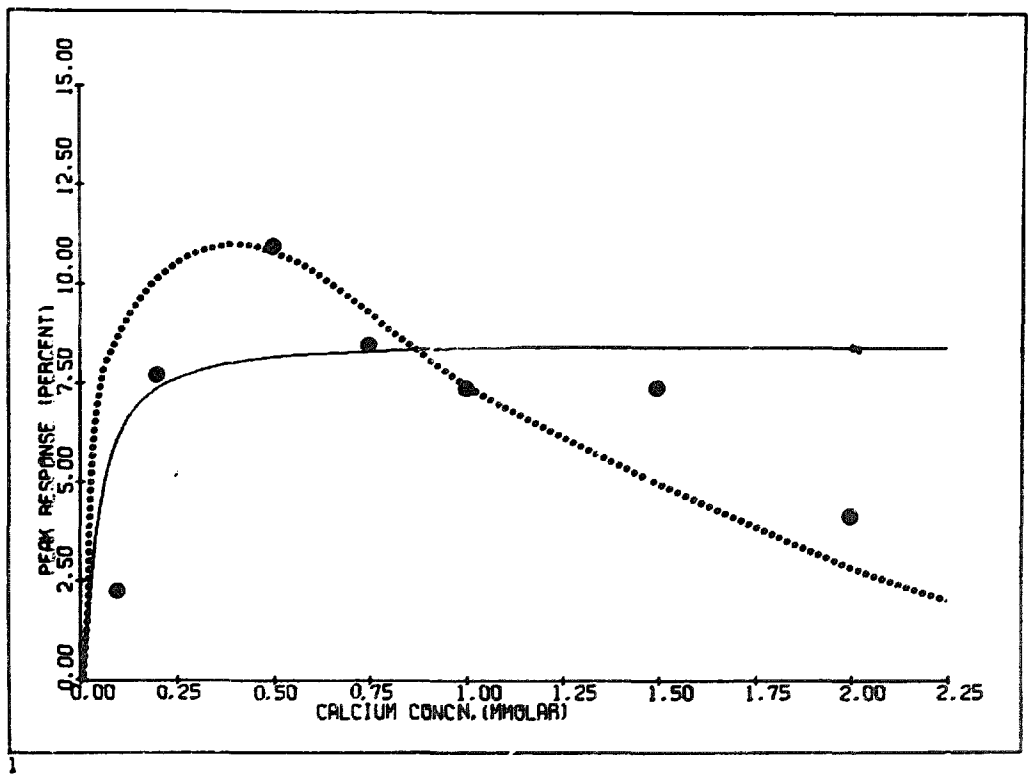

Fig. 3. Polarization response as a function of Ca^{2+} concentration. Points correspond to data similar to that shown in Fig. 2. Peak response is defined as peak membrane voltage divided by applied voltage (V_3) and extrapolated to zero time (infinite time constant). Solid curve is theoretical response predicted by ion binding mechanism. Broken line is theoretical response predicted by border conduction mechanism.

VOLTAGE-DEPENDENT BINDING MODEL

Unlike the strongly voltage-dependent ion binding to mercury–water interfaces[8], it would be expected on the basis of a simple structural picture of a bilayer membrane that the influence on iron binding of a potential applied across a membrane would be rather small because most of the potential drop occurs across the high resistance hydrocarbon interior and not in the polar head region where ion binding occurs.

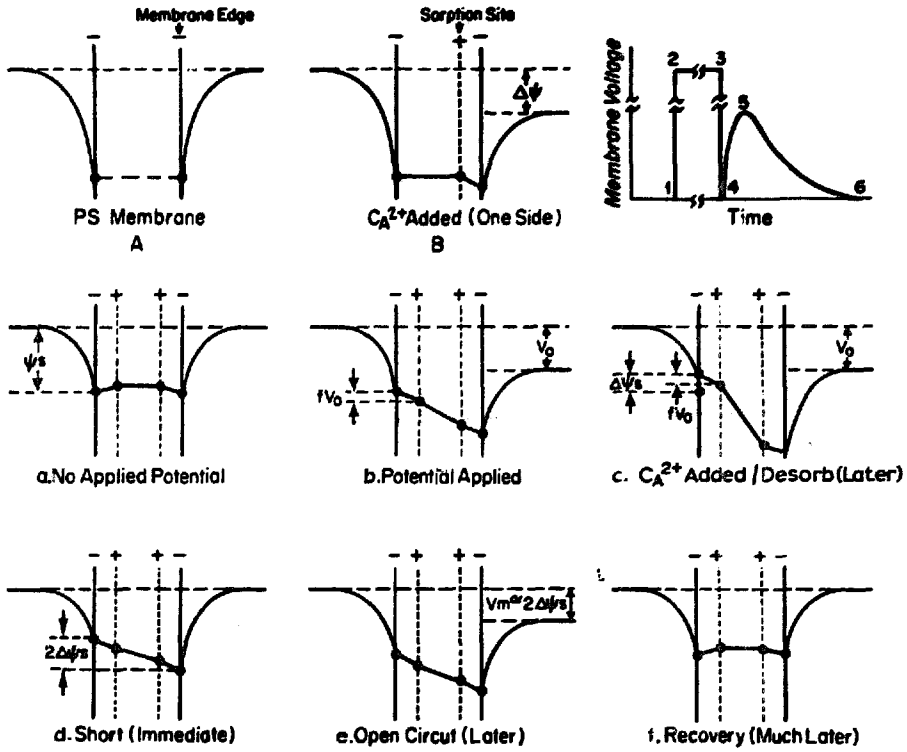

Fig. 4. Voltage-dependent ion binding polarization mechanism. See text. PS = phosphatidylserine.

Fig. 4 illustrates the voltage-dependent ion binding mechanism of membrane polarization and model by which the resultant voltage response can be estimated quantitatively. Without Ca^{2+} (Fig. 4), a phosphatidylserine membrane is negatively charged at neutral pH (about one charge per 65 Å^2) and a potential difference is present between the bulk solution and the interface (ψ). Surface potentials may be calculated reasonably accurately for bilayer membranes from the Guoy–Chapman formulation[8], as demonstrated by McLaughlin *et al.*[9] through a study of the variation of membrane conductivity as a function of surface charge. Since Ca^{2+} as well as K^+ (and to a minor extent, Cl^-) accumulates in the double layer, the more general formulation of Grahame[10,1] was used.

$$(\alpha\sigma)^2 = C_M(e^{-\beta\psi} - 1) + C_D(e^{-2\beta\psi} - 1) + C_A(e^{\beta\psi} - 1) \qquad (1)$$

where

$$\alpha \equiv \frac{F}{N_0}\left(\frac{2\pi}{RTD\varepsilon_0}\right)^{\frac{1}{2}} \text{ and } \beta \equiv \frac{F}{RT}$$

Here σ is the net surface charge, F is the Faraday, N_0 is Avogadro's number, T is the temperature, R is the gas constant, D is the dielectric constant of water, C_M, C_D and C_A are the monovalent (K^+), divalent (Ca^{2+}), and anion (Cl^-) ion concentrations, respectively.

If Ca^{2+} is added to the aqueous phase on one side, it will bind to that side of the membrane and a potential difference will be observed across the membrane when measured, as usual, by the difference in potential between reversible electrodes located on each side and far from the membrane surface. Ohki[3,4] and McLaughlin *et al.*[5] have fitted the observed potential difference for the asymmetric membrane to the Gouy–Chapman equation. Since Mg^{2+} is much less effective at the same concentration, it is apparent that Ca^{2+} is actually bound to phosphatidylserine membranes and not just present in the double layer. Indeed the studies of both Seimiya and Ohki[11] and Ohki and Papahadjopoulos[2] suggest that the Ca^{2+} binding is a rather complex process involving at least two steps, in which the strong Ca^{2+} binding is preceded by a weak Ca^{2+} adsorption at or close to the same site. We, therefore, expect the number bound to be a second order process with respect to bulk Ca^{2+} concentrations (C). An equilibrium (dissociation) constant can then be defined as follows:

$$K_a = \frac{C_S^2(N_S - N)}{N} \tag{2}$$

$$C_S = C\, e^{-2\beta\psi} \tag{3}$$

where C_s is the Ca^{2+} at the surfaces, N_s is the number of fixed (divalent) sites and N is the number of occupied sites per unit area. Eqn 3 accounts for the reduction of ion concentration near the membrane surface because of the surface potential.

Ca^{2+} was present in equal concentrations in the aqueous phases on both sides of the membrane for the polarization data reported here and consequently zero potential between the two sides of the membrane is observed initially (Fig. 4a). Upon the application of external potential (V_c) a small amount of additional Ca^{2+} will bind on one side and be released on the other (Figs 4b, 4c).

An estimate of the time constant for Ca^{2+} diffusion (τ_d) can be made through the relation: $x_d \simeq \tau^2_d/D_{ca}$ where x_d is the diffusion distance and D_{ca} is the diffusion coefficient. Because the solution is unstirred x_d is poorly defined but is roughly equal to distance from the surface within which the number, per unit area, of Ca^{2+} in the bulk phase is comparable to those bound, that is, $x_d N_0 C \simeq N_s$. For $C = 1 \cdot 10^{-6}$ moles/cm^3, $N_s = 1.7 \cdot 10^{-14}$ cm^{-2}, and $D_{ca} = 1 \cdot 10^{-5}$ cm^2/s, τ_d is roughly $1 \cdot 10^{-2}$ s, a value comparable with that observed at oil–water interfaces at these concentrations[8] as well as in order of magnitude in agreement with the data observed here (Fig. 2).

A voltage-dependent binding can only occur if some fraction of the applied voltage (f) appears at the binding site. In the presence of the applied potential in Eqn 3 must be replaced by:

$$\psi' = \psi + fV_0 \tag{4}$$

where ψ is still given by Eqn 1.

The short circuit phase of the polarization measurement (Fig. 4d) is sufficiently long to bring the membrane potential (as measured by the reversible electrodes) to zero, but short enough so that little diffusion of Ca^{2+} to or from the interface takes place. The small internal field produced by the unequal surface change expressed in the open circuit phase (Fig. 4e) as the Ca^{2+} bound to either side of the membrane is equalized in the absence of an applied potential. At this point the peak of the voltage pulse response is reached. It should be noted that the magnitude of the response extrapolated to zero time is equal to the dfference in surface potential produced by the unequal adsorbed Ca^{2+} surface concentration before short circuit. Eventually the membrane potential decays to zero (Fig. 4f) because of finite membrane conductance.

A test of this model is provided by a comparison of its predicted response as a function of Ca^{2+} concentration with the observed response (Fig. 3). Eqns 1–4 were solved simultaneously by an iterative computer program to obtain the theoretical curve shown. The drop in response at high Ca^{2+} concentration occurs because a small change in the equilibrium constant has little effect on the number bound as the binding sites approach complete occupancy. Fit was obtained with $f = 0.13$, $K_a = 10$ (moles/l)^2 and $1/N_s = 90$ Å^2. These parameters fit the asymmetric membrane potential data previously reported[3].

It is possible to give the voltage ratio, f, a simple geometric interpretation if the membrane is thought of as a uniform dielectric slab. Ideally the mobile counter ions cannot penetrate below the membrane surface but the Ca^{2+} can penetrate and are, presumably, bound somewhat below the surface. Since the drop in applied potential with distance is uniform in a uniform dielectric, the ratio of voltage drop at the binding site to the total applied voltage, or f, is also the ratio of binding site depth to the total membrane thickness. If the membrane is approximated by a 70-Å thick uniform dielectric, the binding site is estimated to be about 10 Å below the surface.

Of course the membrane is not uniform, especially near its surface and the potential profile very likely deviates markedly from a straight line. Perhaps the potential profile can be probed by a series of ions which adsorb at different depths or perhaps theoretical approaches will prove successful[12, 13].

BORDER CONDUCTION MECHANISM

Under conditions where the bulk oil phase has an appreciably lower resistance than the membrane resistance (thick or bilayer), it is reasonable to suppose that the high resistance of the border is confined primarily to monolayer at the oil-water interface (Fig. 5a) and that the monolayer acts as a capacitor[14, 15]. An equivalent circuit of the bilayer and border is shown in Fig. 5b. In this sketch the wedge-shaped border has been divided into two sections to suggest a distribution of resistance, and thus time constant, along the border. Time-dependent polarization is exhibited by this network because the border capacitance (C_{b1}, *etc.*) unlike C_M, will only partially discharge upon brief short circuit, and will then transfer part of its charge to C_M during the open circuit phase. The response as calculated by computer (with 20 border subdivisions instead of 2) is shown in Fig. 5c. The similarity to the observed response (Fig. 2), in particular the rise time, should be noted.

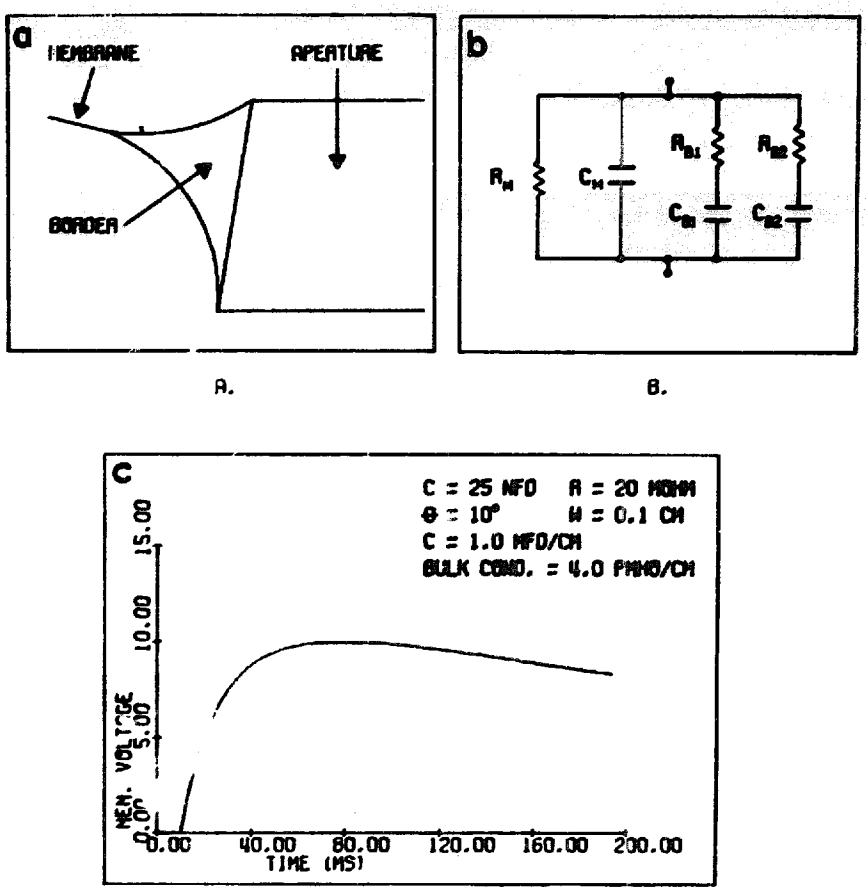

Fig. 5. Border conduction mechanism. a, Membrane-border junction illustrating capacitance at oil–water interface. b, Electrical equivalent circuit of border and membrane. c, Calculated response of equivalent circuit. NFD and MFD are nano- and microfarads, respectively.

If it is assumed that the monolayer capacitance is twice the bilayer capacitance, the relative response (V_p/V_0) due to this mechanism will be, for optimal values of conductivity, approximately equal to the ratio of border to bilayer area. Fit is obtained for an area ratio of about 0.15, a rather high but plausible value (exact value unknown).

An interesting prediction of the model is that the response is a function of bulk conductivity which in the case of phosphatidylserine is proportional to the Ca^{2+} concentration in the aqueous phase. At much higher conductivities than optimum, the border capacitance is mostly discharged during the short circuit phase while at lower conductivities, the membrane capacitance is discharged through R_m. In Fig. 3, the relative response expressed as a function of Ca^{2+} concentration rather than conductance is shown. While the agreement with experiment is satisfactory in view of the simplicity of the model, it should be emphasized that it was obtained by scaling the ordinate through adjustment of the relative border area which is known to only within an order of magnitude.

CONCLUSIONS

The main observation of this study is that phosphatidylserine membranes prepared by the Rudin–Mueller method show an appreciable polarization with a time

constant of 10–100 ms and which is dependent on Ca^{2+} concentration. Both the voltage-dependent binding and artifactial border conductivity mechanisms are consistent with the existing data and we suspect that both may contribute to the observed polarization. Probably the mechanisms can be separated by varying the relative border–bilayer area if the measurement precision can be substantially improved. With further development, we feel polarization determinations may be a useful tool in understanding the relation of membranes structure to electrical properties.

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