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STABILITY OF ASYMMETRICAL PHOSPHOLIPID BILAYERS

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SUMMARY

Instability of a phospholipid bilayer, which has an asymmetrical distribution with respect to Ca^{2+}, pH and electric field in the solution, was investigated experimentally.

It is deduced that the instability of the asymmetrical membrane is due to the difference in surface energy (surface potential) between the opposing sides of the phospholipid bilayer, and that the difference in ionization of one charge per molecule of the membrane between the two sides of the bilayer may produce the instability of the membrane.

It was also observed that an acidic phospholipid membrane (phosphatidyl serine) becomes more unstable for the asymmetrical distribution with respect to the above environment than a neutral charged phospholipid membrane (phosphatidyl choline).

INTRODUCTION

When phospholipid membranes with either neutral or charged polar groups are prepared in a salt solution, the membranes usually have high d.c. resistance, very low permeability to ions, and fairly good stability[1–3]. The membrane thickness has been estimated to be about 50–60 Å by several authors[4–7], and its capacitance is in the range of about 0.3–0.6 $\mu F/cm^2$ (see refs. 1, 3, 8). It is remarkable that such a thin membrane is relatively stable for an electric field of approx. 10^5 V/cm across the membrane in an aqueous solution[2]. There is no doubt that these properties (resistance, stability and dielectric breakdown potential) vary due to the kind of phospholipids and solvents used, and the environmental conditions (such as ion concentration, ion content and pH of the solution[8,9]). As long as the membrane is kept symmetrical with respect to ion concentration, pH or applied electric field on both sides of the membrane, the above general description of phospholipid bilayers is usually obtained[1–3].

However, by changing environmental conditions on one side of the membrane (such as salt concentration, pH) the membrane cannot keep its stability and gradually will show a greater conductance and low dielectric breakdown potential,

and in an extreme state, the membrane breaks into the solution[10,11]. Such asymmetrical phospholipid membranes may be made in many ways. For example, by breaking off a certain polar group of a phospholipid molecule by the addition of some enzymes in the solution on one side of a symmetrical bilayer, we may transform a symmetrical bilayer to an asymmetrical one. By changing the salt content, concentration, or pH in the aqueous solution on one side, an asymmetrical membrane with respect to the surface charge can be prepared[10,11], since the degree of dissociation of the polar groups depends greatly upon the salt content, concentration and pH of the solution[12]. The asymmetrical membrane mentioned above arises from changing only the polar head groups or the degree of dissociation of the polar groups on the surface of the membranes. A few attempts have been made[13] to form another type of asymmetrical membrane by putting two different phospholipid monolayers together in aqueous solution.

Since biological membranes have an asymmetrical distribution of ions between intracellular and extracellular phases[14,15], and the membranes themselves may have an asymmetrical constituent, it is worthwhile to investigate the physical properties of asymmetrical phospholipid membranes which are considered as a fundamental component of biological membranes.

A few studies concerning the properties of asymmetrical phospholipid membranes have been made[10,11]. In this paper, we shall restrict the asymmetry of the membrane to the charge of the polar head groups due to the change of the environmental solution and the applied electrical potential across the membrane. Some experimental evidence concerning the instability of the asymmetrical membrane will be reported here.

MATERIALS AND METHODS

The phospholipids used were chromatographically pure phosphatidyl choline (egg) and phosphatidyl serine (bovine) purchased from Applied Science Laboratories (State College, Pa.) and some prepared by PAPAHADJOPOULOS[16]. These phospholipids were stored in chloroform. The membrane forming solution was prepared in the following way: The solvent was driven out by blowing N_2, or evaporated in a vacuum chamber. The sample was then taken down to complete dryness and about 20 mg of phospholipid was dissolved in 1.0 ml *n*-decane (Fluka, Switzerland). The details of the cell arrangement were described in an earlier paper[9]. All chemicals used were reagent grade (Fisher Scientific). Water was triple distilled, including the process of distillation from $KMnO_4$. Membrane resistance was determined by applying potentials of up to 50 mV across Ag–AgCl electrodes situated in inner and outer compartments, and monitoring the direct current by a sensitive ammeter (610C of Keithley Instruments). The film diameter (black film part) was measured with the aid of a calibrated scale placed across the microscope eyepiece, for every measurement, in order to obtain the specific resistance of the membrane. For the measurement of membrane potential, the electronic circuit used is described schematically in Fig. 1, where A is preamplifier (Unity gain, input impedance, 10^{12} Ω), B is the electrometer 610C of Keithley Instrument and C is the strip chart recorder (Bausch-Lomb, VOM-7). Electrodes were calomel electrodes (London Co., Ohio). Temperature was kept at $23 \pm 1°$.

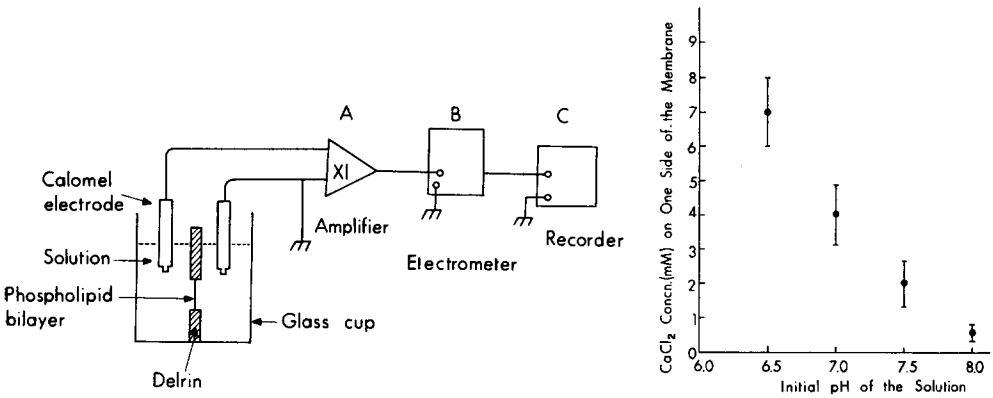

Fig. 1. Schematic diagram of instrumentation for measurements of membrane potential.

Fig. 2. Points of instability of phosphatidyl serine membranes. Closed circles indicate the amount of $CaCl_2$ needed to break the phosphatidyl serine membrane by adding $CaCl_2$ on one side at various pH's.

RESULTS

Asymmetric distribution of Ca^{2+} concentration

In order to obtain an asymmetrical phosphatidyl serine membrane with respect to Ca^{2+} concentration, a small amount of $CaCl_2$ (1 M) was added to the solution on one side of the phosphatidyl serine membrane in 0.1 M NaCl solution. To compensate for the increased volume of the solution on one side, a small amount of 0.1 M NaCl solution was added on the other side of the membrane. In the above case, the electrical resistance of the bilayer was decreased by a factor of more than two. Exceeding a certain concentration of Ca^{2+}, the membrane became unstable and broke. The concentration of $CaCl_2$ necessary to cause breaking of the membrane was highly dependent on the pH. Fig. 2 shows the amounts of $CaCl_2$ necessary to cause this breakage at various pH's. For example, at pH 7.0, 5 mM $CaCl_2$ is needed to break the membrane, at pH 7.5, about 2 mM $CaCl_2$ and at higher pH, even lower concentrations of $CaCl_2$ produced the breakage of the membrane. These experiments were made in the presence of 0.05 mM EDTA in order to remove any higher valent metals (especially Ca^{2+}) either contributed as contaminants of the NaCl reagent or extracted along with phosphatidyl serine fraction from natural sources. When EDTA was not present in the solution, the concentration of Ca^{2+} required for breaking was higher by a factor of more than three. On the other hand, when a phosphatidyl serine membrane was made in the solution of 0.1 M NaCl and 1 mM $CaCl_2$, by injecting $CaCl_2$ on one side, the membrane did not break even at 50 mM $CaCl_2$ concentration.

Also, when equimolar amounts of EDTA (about 1 mM) were added on one side of the membrane, while the pH was kept constant by the addition of 0.1 M NaOH, the membrane became unstable and broke. Addition of equimolar EDTA may inhibit chelation of Ca^{2+} with phospholipid polar groups at the surfaces of the membrane and produce an asymmetrical membrane with respect to Ca^{2+} binding. Therefore, this may create an asymmetrical membrane similar to the above case

of the addition of $CaCl_2$ on one side of the membrane. However, membranes made of phosphatidyl choline in the same salt solution as that for the phosphatidyl serine membrane, did not show any instability with an increase in concentration of Ca^{2+} up to about 40 mM on one side.

Each experimental point shown in Fig. 2 was obtained from the arithmetical mean of more than twenty measurements for each point. There were several scattered values with different samples of the same type of phospholipid. However, all measured values were taken into the arithmetical mean. As shown in Fig. 2, the scattered values from the average were observed more for the case of lower pH (6.5) than for higher pH (8.0).

The membrane potentials resulting from the asymmetrical distribution of $CaCl_2$ concentration were also observed. Fig. 3 shows the membrane potentials with respect to $CaCl_2$ concentration on one side of the phosphatidyl serine and phosphatidyl choline membranes, respectively. For the phosphatidyl serine membrane, increasing membrane potential was observed with the addition of Ca^{2+} on one side of the membrane at pH 7.0 and the membrane potential of about 60 mV was measured with the addition of about 5–6 mM $CaCl_2$ on one side of the membrane. Beyond the above concentration range (5–6 mM $CaCl_2$), the membrane usually became unstable and broke. The membrane potential of more than 60 mV produced across the phosphatidyl serine membrane by the addition of $CaCl_2$ caused breakage of the membrane. For the phosphatidyl choline membrane, as $CaCl_2$ was added on one side of the membrane, a lower membrane potential was observed than that of the phosphatidyl serine membrane and the potential value reached a rather stable plateau after the addition of 1 mM $CaCl_2$ on one side. That is, there was no appreciable increase in the membrane potential with concentrations of more than 1 mM $CaCl_2$. The membrane potential of 25 mV produced for the phosphatidyl choline membrane by the addition of $CaCl_2$ did not cause breakage of the membrane. As previously mentioned, the phosphatidyl choline membrane was stable at least up to the addition of 40 mM $CaCl_2$.

These membrane potentials shown in Fig. 3 were the mean values of at least ten measurements for each point, and these potentials were measured after the systems were settled down to an equilibrium state. The membrane potential was

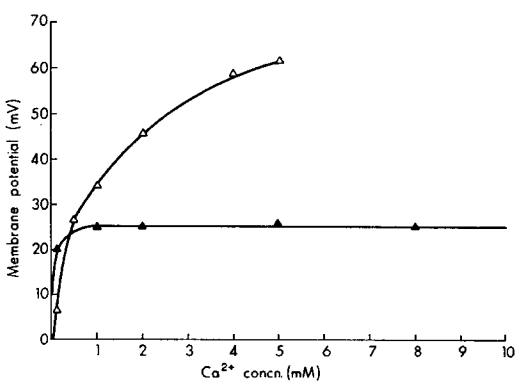

Fig. 3. The membrane potential with respect to $CaCl_2$ concentration on one side of phosphatidyl serine (△) and phosphatidyl choline (▲) membranes prepared in 0.1 M NaCl at pH 7.0.

gradually developed by stirring after the addition of small amounts of $CaCl_2$ (1 M) on one side of the membrane. By a normal stirring (it was made sure that the solution was completely mixed by 2 min stirring in our system), the membrane potential produced reached a plateau value (stationary state) after 2 or 3 min. This stationary state lasted at least an hour as long as the membrane did not break.

Asymmetric distribution with respect to H^+

If phosphatidyl serine membranes are formed at a certain pH and the pH of the solution on one side of the membrane is subsequently changed, the altered pH which produces membrane instability depends upon the initial pH of the solution. Fig. 4 illustrates the results indicating the instability of phosphatidyl serine membranes under asymmetric distribution of H^+. Each experimental value was the arithmetical mean of more than ten measurements for each case.

Figs. 4a represent experiments in the absence of Ca^{2+}. If a phosphatidyl serine membrane was formed in 0.1 M NaCl solution at pH 3.0, it reached the point of instability (breaking) when the outside pH was increased to 6.0 (Fig. 4A-a). However, when the initial pH was higher (Figs. 4B-a, 4C-a, 4D-a), the outside pH at which the

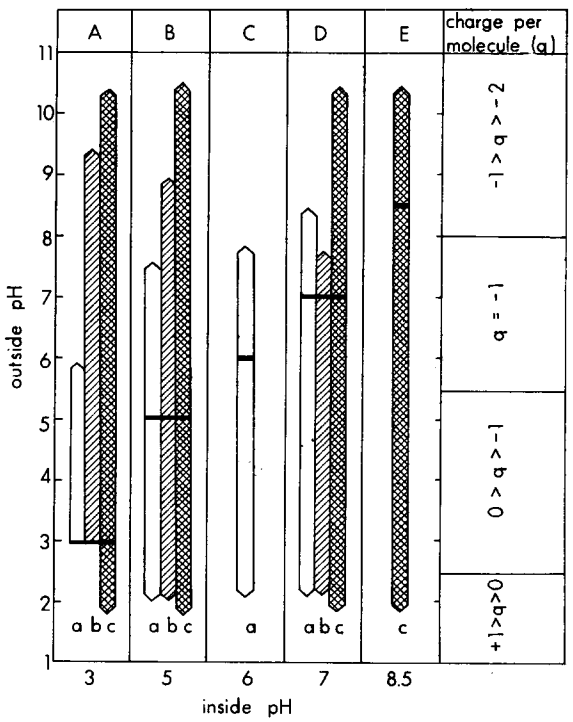

Fig. 4. Stability limits of a phosphatidyl serine membrane with asymmetrical distribution of H^+ and Ca^{2+}. Horizontal bars represent the pH of the solution at which the membrane was prepared. The end point of each vertical bar represent the pH of one side solution at which the membrane broke. Open bars (a) solutions of both sides of the membrane are 0.1 M NaCl and pH was altered by injecting NaOH or HCl. Hatched bars (b) 1 mM $CaCl_2$ was added on one side only. Double hatched bars (c) 1 mM $CaCl_2$ present on both sides of the membrane. The charge per molecule shown on the right of the figure is taken from the titration curves of phosphatidyl serine monolayers (ref. 17).

membrane broke was also higher. At pH 3.0, phosphatidyl serine should be near the isoelectric point (see Fig. 4 (charge per molecule)) and at pH 6.0 each phosphatidyl serine molecule carries one extra negative charge[17]. This difference of one charge per molecule (zero charge on one side of pH 3.0 and one negative charge on the other side of pH 6.0) may be the cause of the instability as shown in Fig. 4A-a. When a phosphatidyl serine membrane was made at pH 7.0, both sides of the membrane have one negative charge per molecule. In this situation, the membrane was fairly stable, because the membrane was symmetrical with respect to the surface charges on both sides of the membrane. However, by increasing the pH on one side of the membrane, the amine groups start losing their protons around pH 8, a fraction of the extra charge per molecule is enough to produce breakage of the membrane. There will be another extra charge per molecule on that side of the membrane. It is thus apparent that one of the factors concerning the instability of the membrane is defined by the difference in ionization of the polar head groups between the two sides of the membrane.

Counterbalance of Ca^{2+} with H^+

The remarkable ability of Ca^{2+} in stabilizing asymmetric membranes is seen in Fig. 4-b. The phosphatidyl serine membranes were formed in 0.1 M NaCl at a certain pH. Then 1 mM $CaCl_2$ was added on one side of the membrane and the pH was increased by adding NaOH while the pH of the solution on the other side of the membrane was kept at the initial pH. Fig. 4A-b shows that when a phosphatidyl serine membrane was formed in 0.1 M NaCl at pH 3 and 1 mM Ca^{2+} was added on one side, the phosphatidyl serine membrane was stable up to pH 9.5 on the one side of the membrane. When $CaCl_2$ was added first on the one side of the phosphatidyl serine membrane, Ca^{2+} would bind with the polar groups and inhibit the dissociation of these groups up to higher pH. This stability of Ca^{2+} at a high pH on Ca^{2+} added side, decreases as initial pH becomes higher. As seen in Figure 4-b, the higher the initial pH, the lower the pH at which the phosphatidyl serine membrane became unstable and broke. In these cases, H^+ seemed not to counterbalance Ca^{2+}. When Ca^{2+} was present on both sides of the phosphatidyl serine membrane, the membrane was very stable and had high d.c. resistance as shown in Fig. 4-c. Such membranes formed at pH 7.0 can be titrated on one side with NaOH up to pH 10.5 (or with HCl down to pH 2.5) before they became unstable. This stability was held irrespective of the initial pH at which the phosphatidylserine membrane was formed (Fig. 4-c).

Asymmetrical potential distribution across the membrane due to an externally applied electric field

When an electrical potential is applied across a membrane, the membrane will be asymmetrical with respect to electrical potential on both sides of the membrane. It has been observed that the membrane resistance was ohmic at least up to an applied voltage of 40 mV, but at higher potentials, the current–voltage curve became convex toward the voltage axis. Above a certain applied voltage, the membrane broke down. This dielectric breakdown voltage of the membrane depended upon the pH of the solution. Observed breakdown potentials of phosphatidyl choline and phosphatidyl serine membranes are shown in Fig. 5 at various pH's of 0.1 M NaCl solution. For the neutral phospholipid membranes such as phosphatidyl choline

membranes, greater dielectric breakdown potentials were observed over wide ranges of pH. However, with acidic phospholipids, such as phosphatidyl serine, lower dielectric breakdown potentials were observed over a narrower range of pH. For example, the breakdown potential of the phosphatidyl choline membrane was 150 mV at pH 7.0 whereas that of the phosphatidyl serine membrane was 100 mV at the same pH. Generally speaking, at a pH near the isoelectric points of phospholipids, the dielectric breakdown potential was the largest. Toward lower and higher pH's from the isoelectric point, the breakdown potential with respect to pH was lower for each case (phosphatidyl choline and phosphatidyl serine). Each value shown in Fig. 5 is the average value of measurements taken more than 20 times for each case. Although there were some scattered values from the average, almost all values (90 %) were within 10 % of the average value. The relation between breakdown potential and pH was the similar behavior as for the membrane resistance with various pH's described in the previous paper[9].

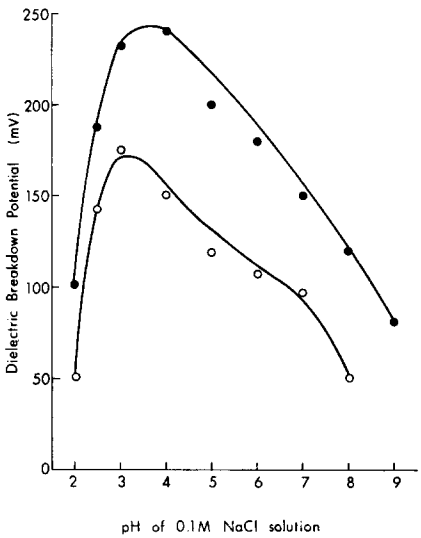

Fig. 5. Dielectric breakdown potentials of phosphatidyl choline and phosphatidyl serine in 0.1 M NaCl at various pH's. ●, phosphatidyl choline membranes; ○, phosphatidyl serine membranes.

DISCUSSION

As shown in Fig. 2, the addition of small amounts of $CaCl_2$ (1 M) on one side of the membrane at various pH's, caused the breakage of the membrane. The mechanism of this instability may be considered as follows:

Since Ca^{2+} binds strongly with acidic phospholipid polar groups, they should chelate with the negatively charged polar groups of phospholipids on the surface of the membrane and tend to cancel their negative charges[11]. Since the ionization of the polar group depends on the pH of the solution, amounts of $CaCl_2$ needed to cancel the ionized charge of the polar groups would be different at various pH's. Early studies show that each molecule of phosphatidyl serine carries one net negative

charge at pH 6–8, more than one negative charge at pH above 8 and less than one negative charge to neutral, between pH 6–3[17]. Therefore, the addition of a small amount of $CaCl_2$ on one side of the phosphatidyl serine membrane at a certain pH would create a charge asymmetry between the opposing surfaces of the bilayer.

For example, as stated, a phosphatidyl serine molecule has one net negative charge at pH 7.4. If Ca^{2+} chelates with the charged polar groups on one side of the membrane and cancel the charge, there will be one charge difference per molecule (zero *vs.* one) between the opposing surfaces of the membrane. If this is the case this one net charge difference per molecule may cause the instability of the membrane. This interpretation corresponds to the result of the membrane instability obtained by changing pH on one side of the membrane. All four cases (Figs. 4A-a, 4B-a, 4C-a, 4D-a) show that the membrane became unstable and broke with a certain pH difference between two sides of the membrane so as to produce the one charge difference in ionization per molecule between the surfaces of the membrane.

The instability effect due to Ca^{2+} is in general agreement with the observation that indicated an increase in permeability of phosphatidyl serine vesicles at 1 mM $CaCl_2$ concentration[12,18]. Since a phosphatidyl choline molecule does not have a net charge at neutral pH, and a phosphatidyl choline membrane does not show the instability with concentration of Ca^{2+} up to at least 40 mM on one side of the membrane, it is concluded that the binding of Ca^{2+} with the polar groups of a phosphatidyl choline molecule, which has neutral charge, is not strong enough to break the membrane as it does when bound to the polar groups of the phosphatidyl serine molecule.

It seems reasonable to suggest that the instability of phosphatidyl serine membranes described is due to the difference in surface energy (surface potential) between the opposing sides of the bilayer. Calculation[19] of energy differences of the surfaces resulting from asymmetrical distribution of charges suggest that, under these conditions, molecules or clusters of molecules will invert from one side to the other. In doing so, they would increase the permeability of the membrane. Under extreme conditions the membrane reaches a breaking point. The membrane potential produced for the asymmetrical phosphatidyl serine membrane with about 5 mM $CaCl_2$ on one side of the membrane in 0.1 M NaCl at pH 7.0 was observed at about 60 mV. Above this concentration of $CaCl_2$ the membrane broke.

The mechanism of the dielectric breakdown for phospholipid bilayer membranes is difficult to analyse only with the present experimental results, because many factors may be involved in the mechanism. However, it is interesting to note that the relation between breakdown potentials and pH's showed a similar tendency as for the membrane resistance with various pH values[9]. Also, the neutral phospholipid (such as phosphatidyl choline) bilayer membrane has greater dielectric breakdown potentials over wide ranges of pH's than the phosphatidyl serine bilayer. The phosphatidyl choline bilayer containing cholesterol (wt./wt. = 1/1), has greater dielectric breakdown potential than the pure phospholipid bilayer, and a phosphatidic acid bilayer has lower dielectric breakdown potential over a narrow range of pH than the phosphatidyl serine bilayer. It seems that the dielectric breakdown phenomenon of the bilayer membrane is related to the interior structure as well as the surface properties of the membrane (such as orientation of polar groups or electrostatic interaction due to dissociation of the polar groups of the lipids). In order to elucidate the mechanism of the breakdown phenomena, more experiments are needed. Some of the proper-

ties of the axon membrane in relation to Ca^{2+} and pH[15,20-22] suggest that instability (due to asymmetric distribution of Ca^{2+} and pH, and applied electric field) of acidic phospholipid groups in the membrane might be relevant in understanding certain aspects of the nerve excitation process.

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