INTERACTION OF CALCIUM WITH NEGATIVE LIPIDS IN PLANAR BILAYER MEMBRANES

INFLUENCE OF THE SOLVENT

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ABSTRACT The interaction of Ca^{++} with acidic phospholipids in black lipid films and lipid bilayers formed from two monolayers was studied by measuring their physical stability and conductance. It was found that the addition of $CaCl_2$ to only one side of lipid bilayers formed from phosphatidylserine or cardiolipin does not appreciably change these parameters. In contrast, black films are unstable to the asymmetric addition of $CaCl_2$. Therefore, the destabilizing effect of Ca^{++} cannot be attributed to a surface charge difference. The only variation in composition between both bilayer membranes, namely the solvent content of the bilayer, seems to be responsible for the distinctive effect of Ca^{++}. A tentative explanation is presented.

The importance of the calcium ion in membrane biology is widely recognized. It functions, for example, as a modulator of membrane phenomena, as a cofactor of membrane enzymes, as a stabilizer in membrane assembly, and as a transmembrane charge-carrier (cf. 1, 2).

Calcium ions interact with the individual membrane constituents, namely, lipid, protein, and carbohydrate (3). Furthermore, water-soluble lipid-protein complexes can be rendered soluble in hydrocarbon solvent upon reaction with Ca^{++} (4). Since the basic structural element of membranes is the lipid bilayer, it is valuable to ask about its interaction with Ca^{++}.

The strong interaction of Ca^{++} with negatively charged lipids is well documented (1, 5, 6). On planar lipid bilayers, however, the only studies available are those of Ohki and Papahadjopoulos with black films (7, 8). They showed that the asymmetric addition of Ca^{++} to phosphatidylserine (PS) black films produces an increase in membrane conductance and at a given concentration, which depends on the pH, induces the breakdown of the film.

The fact that a hydrocarbon solvent is required to form the black films and remains as a constituent after formation (2, 9, 10) raises a question about its influence on the interaction of Ca^{++} with the film. Planar bilayers formed by apposition of two monolayers provide a bilayer structure composed of phospholipids with virtually no solvent

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(2, 10, 11). In addition, this method allows the formation of asymmetric bilayers by adjoining two different monolayers. Using this system, we have previously shown the disruptive effect of Ca^{++} when added to the compartment limited by a monolayer of oleyl phosphate, a negatively charged single-chain amphiphile (12). Here, we describe the distinct response of black films (with solvent) and lipid bilayers (solvent-free) composed of acidic phospholipids to Ca^{++}, and attempt to answer the two following questions: Does the solvent present in black films confer Ca^{++}-sensitivity to the membrane? Does the effect of Ca^{++} on oleyl phosphate bilayers occur in other negatively charged lipids?

Chromatographically pure bovine PS and and bovine cardiolipin (DPG) were purchased from Applied Science Labs, Inc. (State College, Pa.), and glycerolmonoolein (GMO) from Sigma Chemical Co. (St. Louis, Mo.). The hydrocarbon solvents used were from J. T. Baker Chemical Co. (Phillipsburg, N.J.) (*n*-hexane); Matheson Co. (East Rutherford, N.J.) (*n*-decane and *n*-tetradecane); and Phillips Petroleum Co. (Bartlesville, Okla.) (*n*-hexadecane). All other reagents were from the highest purity commercially available. Glass-redistilled water was used throughout.

Black lipid films (13, 14) and lipid bilayers (10, 15) were formed and their electrical properties studied as previously described in detail. The presence of the membrane was monitored by observing the capacitative current induced by a 10-mV voltage pulse

TABLE I

SYMMETRIC MEMBRANES

	Membrane capacity	Number of experiments
	($\mu F/cm^2$)	
Black films: maximum lifetime < 1 min		
PS:decane	0.36 ± 0.03	12
PS:tetradecane	0.42 ± 0.05	9
PS:hexadecane	0.58 ± 0.04	9
DPG:decane	0.41 ± 0.02	11
DPG:hexadecane	0.66 ± 0.03	11
Lipid bilayers: minimum lifetime > 60 min		
PS	0.70 ± 0.03	12
DPG	0.90 ± 0.02	8
PS:hexane*	0.70 ± 0.03	12

Planar bilayer capacity and stability on asymmetric addition of $CaCl_2$. Bilayers were formed in 1 mM KCl unbuffered solutions adjusted to pH 7.5. The pH was checked every 20 min during an experiment and was found to stay within ±0.2 pH units. Black films were formed on apertures with an area of 1 mm^2; lipid bilayers were formed on circular apertures varying in diameter from 0.2 to 0.75 mm without noticeable differences in the reported results. The membranes were maintained unaltered for at least 10 min after formation; thereafter, 10 μl of a concentrated $CaCl_2$ solution were added to one compartment with continuous stirring. In all experiments the final $CaCl_2$ concentration was 5 mM. Control additions of 10 μl of water showed no effect on membrane stability.

*Lipid bilayers were formed by the method of Benz et al. (10).

under voltage clamp conditions (15). All the experiments were performed at room temperature (22 ± 2°C).

The results obtained with symmetric membranes are summarized in Table I. We have reproduced the destabilizing effect of Ca^{++} in PS (7, 8) and DPG black films (upper part, Table I). In contrast, lipid bilayers formed from monolayers of PS or DPG are stable and unresponsive to the asymmetric addition of Ca^{++} (lower part, Table I).

The effect of Ca^{++} was also assayed in asymmetric bilayers formed from monolayers of PS or DPG on one side and GMO on the other (PS/GMO, DPG/GMO). The bilayer lifetime and stability were not altered by additions of $CaCl_2$ to either compartment, as illustrated in Table II. Therefore, hypotheses which attribute the destabilizing effect to a surface charge difference (7, 8) are not plausible.

The only difference in composition between black films and lipid bilayers is the presence of hydrocarbon solvent in the former. If the solvent is determining the sensitivity of black films to Ca^{++}, it should be possible, in principle, to desensitize the black films by reducing their solvent content. Fettiplace et al. (9) reported that the solvent content of black films decreased as the chain length of the hydrocarbon increased. As shown in Table I, black films formed in decane, tetradecane, and hexadecane were all destabilized by asymmetric additions of Ca^{++}. According to Fettiplace et al. (9), the solvent content of hexadecane films is about 17%. This suggests that such a fraction is sufficient to confer the Ca^{++} sensitivity to the system.

How can a given amount of solvent in a lipid bilayer render it sensitive to Ca^{++}? A tentative explanation can be suggested on the basis of the well-known condensing effect of Ca^{++} on negatively charged lipid monolayers (5, 6): the asymmetric addition of Ca^{++} to black films causes the condensation of the monolayer with the consequent formation of discrete domains of solvent clusters devoid of lipid, possibly exposed to the aqueous phase; such an unfavorable configuration eventually leads to film rupture. In contrast, when Ca^{++} is present symmetrically, before the formation of the black

TABLE II
ASYMMETRIC BILAYERS

Composition	Minimum lifetime	Number of experiments
monolayer I/ monolayer II	*min*	
PS/GMO	>60	8
GMO/PS	>60	8
DPG/GMO	>80	11
GMO/DPG	>80	12

Lifetime of asymmetric bilayers under asymmetric addition of $CaCl_2$ (final concentration 10 mM). $CaCl_2$ was added to the monolayer II side. Other experimental conditions as in Table I. Small contaminations with negative lysoderivatives, similar to oleyl phosphate, may yield results different from those reported herein (17).

film, both monolayers are initially condensed, thus excluding the solvent from the interface into the film interior. Lipid bilayers are unresponsive to Ca^{++} because the initially condensed monolayers are only further condensed, but no phase separation occurs since no other component is present at the interface.

It is not clear then why bilayers containing oleyl phosphate break in the presence of Ca^{++} if they have no solvent. We suggest that charge neutralization with Ca^{++} is sufficient to favor the micellization of oleyl phosphate in the bilayer leading to its rupture. This effect may be hindered in bilayers composed of two-chain (PS) or four-chain (DPG) phospholipids due to the intensive hydrophobic anchorage provided by the numerous acyl chains (16). Further studies are required to explore these suggestions.

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