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CALCIUM ASSOCIATION WITH PHOSPHATIDYLSERINE

MODIFICATION BY CHOLESTEROL AND PHOSPHATIDYLCHOLINE IN MONOLAYERS AND BILAYERS

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We have examined the association of Ca^{2+} with phosphatidylserine/cholesterol and phosphatidylserine/dimyristoylphosphatidylcholine mixed monolayers using a surface radiocounting technique. No Ca^{2+} association with pure monolayers of the uncharged molecules was observed. The Ca^{2+}/phosphatidylserine surface ratio was approximately 1:2 in expanded monolayers of the pure anionic lipid and in phosphatidylserine/phosphatidylcholine mixtures. An increase in surface-associated Ca^{2+} to a number ratio of 1:1 was observed in phosphatidylserine/cholesterol films when the mole fraction of cholesterol was raised to 0.5 and above and the phospholipid number density held constant. We interpret these findings as a prevention of intermolecular salt formation by the sterol. Further support is provided by particle electrophoresis

Introduction

Phosphatidylserine (PS) is the major charged component of the phospholipid fraction of mammalian plasma membranes (for review, see Ref. 1 (Rothman and Lenard)). In the red cell membrane it is located primarily on the cytoplasmic aspect [1–3]. By extrapolation of this asymmetry to other cell types, it has been suggested that the interaction of this lipid with ions in the cytosol is central to a variety of physiological processes. In particular, the raised cytoplasmic Ca^{2+} levels predicted during exocytosis [4] have been mooted to trigger the 'membrane fusion reaction' by pathways involving ion association with PS (for review see Ref. 5 (Gingell and Ginsberg)).

Interest in the biological role of Ca^{2+}/PS interactions has prompted studies in model membrane systems. Since the early work of Rojas and Tobias [6] on Ca^{2+} binding to PS monolayers, a vast literature has accumulated (see Ref. 7 (Seimiya and Ohki) for early references). One poorly understood area concerns possible modifications of Ca^{2+} binding to the acidic phospholipid by the presence of uncharged molecules in lipid monolayers and bilayers.

In a recent investigation, Gregory and Mingins (unpublished data) have shown by surface radiocounting techniques that divalent ion adsorption to an oppositely charged monolayer depends strongly on the type and mole fraction of 'spacer' molecules in that monolayer. Their studies were on anionic, zwitterionic and uncharged surfactant molecules. Here, we extend this analysis into the biological domain by examining Ca^{2+} adsorption to PS and its modification by either phosphatidylcholine (PC) or cholesterol. Monolayer mea-

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surements are supplemented by evidence from particle electrophoresis.

Materials and Methods

Lipids. Phosphatidylserine (ex bovine spinal cord, Lipid Products, South Nutfield, Surrey, U.K.), dimyristoylphosphatidylcholine (Applied Sciences Laboratory, State College, PA, U.S.A.) and cholesterol (Field Instruments Co. Ltd., Twickenham, Middlesex, U.K.) were used without further purification. Thin-layer chromatography revealed no lipid class impurities in any of the experimental samples. When a specimen of PS was ashed at 700°C for 4 h, however, an inorganic residue remained (29% of the original weight). This finding was in agreement with an elevated apparent molecular weight (1089) for the lipid, obtained by phosphorus analysis (modification of the method described by Allen [8]). The impurity was suspected to be largely NaCl (Lipid Products, personal communication). At the level detected, its presence does not significantly affect the ionic strength of the aqueous phases.

Phospholipids were stored prior to use at −20°C as solids or in chloroform/methanol solution under nitrogen. Thin-layer chromatography revealed no deterioration of the samples during storage.

Other materials. Suprapur $CaCl_2$ was obtained from Merck (Darmstadt, F.R.G.) and $^{45}Ca^{2+}$ was purchased as the chloride from the Radiochemical Centre (Amersham, U.K.). Demineralised water was distilled from alkaline potassium permanganate in all-glass apparatus. Solvents (chloroform, *n*-heptane and ethanol) were redistilled in glass. All glassware and PTFE components were cleaned routinely in a nitric/hydrofluoric acid mixture followed by thorough rinsing in best distilled water and drying in air [9].

Monolayer measurements. Lipid mixtures were spread to form insoluble monolayers at the air/water interface in a $25 \times 6 \times 1$ cm PTFE trough. The solvent system for the lipids was 90% *n*-heptane/10% ethanol. The subphase contained $^{45}Ca^{2+}$ diluted with non-radioactive $CaCl_2$ to give a final calcium concentration of $1.0 \cdot 10^{-4}$ M. The temperature was 20 ± 1°C and the pH was approx. 6.

Surface-associated radioactivity was measured by a modification of the method of Aniansson [10]. An Actigraph-3 gas flow counting head (operating conditions 98% argon/2% propane at 25 ml/min and 1.2 kV) was held with its window (Mylar film, 6 μm thick) at a distance of approx. 3 mm from the air/water interface. Counts were monitored using an ESI Nuclear ratemeter (Model 5350) whose output drove a pen recorder.

The increase in radiocount rate relative to background following spreading of a monolayer could be converted to surface-associated Ca^{2+} concentration by applying a factor derived from Fig. 1. This calibration curve was obtained by adding small known volumes of the subphase $CaCl_2$ solution to a glass plate of known area which was then dried and counted under the detector head. The geometrical arrangement was the same as that of the trough experiments.

During radiocounting, monolayer surface pressures were monitored by the Wilhelmy plate method with zero contact angle, using a Beckman LM 600 microbalance, and the area was varied by compression.

Particle electrophoresis measurements. PS/cholesterol particles were prepared by drying the phospholipid onto glass from solution in chloroform and adding the required amount of cholesterol dissolved in a small volume of ethanol (0.1–0.3 ml). The mixture was then vortexed for 5 min while adding 2 ml of distilled water or $CaCl_2$ solution (10^{-4} M). Samples were finally heated to 70°C on a steam bath followed by further vortex-

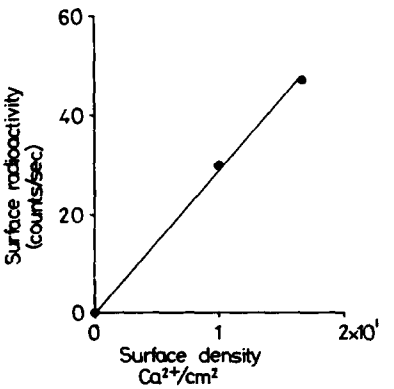

Fig. 1. Typical calibration curve for radiocounting experiments. Points were corrected for background count rate.

ing. Suspensions prepared in this way were diluted before electrophoresis to yield a final lipid concentration of less than 2 mM. They were examined under phase-contrast (Reichert Zetopan microscope) and through crossed polaroids for birefringence. Pure cholesterol dispersions could not be manufactured by the method described. Particles small enough for determination of electrophoretic mobility were, however, obtained by mechanical shaking of a cholesterol/aqueous mixture for 5 min. The larger particles sedimented from the suspension within 15 min. Electrophoretic mobilities were measured using a Rank Mk II apparatus with a four-electrode cylindrical cell (Rank Bros, Bottisham, Cambridge, U.K.). The temperature and pH were the same as in the monolayer experiments.

Results and Discussion

Monolayer data

Fig. 2 shows surface pressure/area isotherms for monolayers of pure PS and mixtures of this lipid with neutral 'spacer' molecules. The pure PS curve is similar to published isotherms (see for examples Ref. 6). Radiocounting results for PS/cholesterol mixtures are illustrated in Fig. 3. At constant number density of the anionic lipid, e.g. $0.5 \cdot 10^{14}$ molecules/cm^2, increasing the mole fraction of cholesterol in the monolayer beyond 0.5 can be seen to raise the ratio of surface-associated Ca^{2+} to PS. This enhancement is not seen in PS/PC mixtures (Fig. 4) and there was no recorded Ca^{2+} association with pure monolayers of the spacer molecules at this bulk divalent ion concentration.

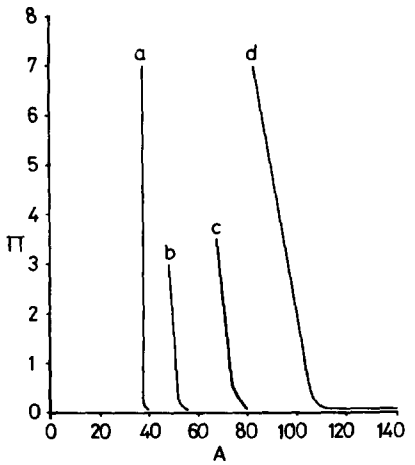

Fig. 2. Surface pressure (π; dyn/cm)/molecular area (A; Å^2/molecule) isotherms for PS/cholesterol monolayers. Subphase contained 10^{-4} M Ca^{2+} at pH 6 and 20°C (these conditions apply to subsequent figures except those involving PC, where experiments were conducted at 25°C). (a) 100% cholesterol; (b) 21% PS/79% cholesterol; (c) 46% PS/54% cholesterol; (d) 100% PS.

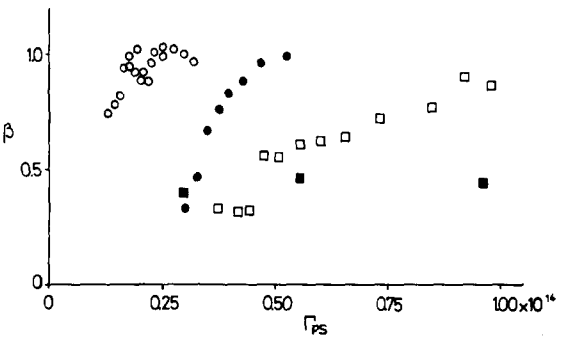

Fig. 3. Radiocounting results for PS/cholesterol mixtures. Surface ratio of Ca^{2+}/PS (β) plotted against the surface density of PS in molecules PS/cm^2 (Γ_{PS}). □, 100% PS; ■, 67% PS/33% cholesterol; ●, 46% PS/54% cholesterol; ○, 21% PS/79% cholesterol.

An attempt was made to measure Ca^{2+} interaction with pure PS films in the presence of 100 mM NaCl. Preliminary results indicate a surface Ca^{2+}/PS ratio of approx. 0.1, with 10^{-4} M bulk Ca^{2+}. The sensitivity of the radiocounting tech-

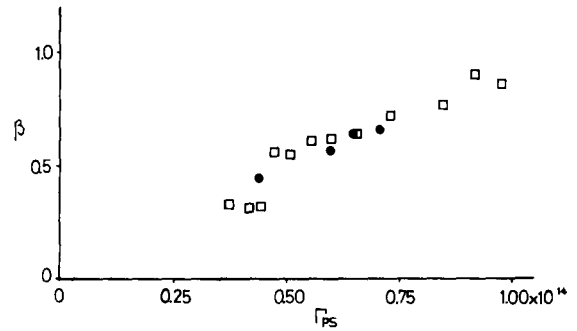

Fig. 4. Radiocounting results for PS/PC mixtures. Axes as in Fig. 3. The 100% PS results in Fig. 3 have been replotted here (□). ●, 25% PS/75% PC. Clearly, no enhancement of surface Ca^{2+} association by PC is seen at a nonionic mole fraction where the cholesterol effect was maximal.

nique did not permit accurate quantitation, however. With 10^{-3} M Ca^{2+} in bulk, the elevated background count rate prevented conclusions being drawn.

Significance of surface-associated Ca^{2+}

A weakness of the radiocounting method is that it measures all the Ca^{2+} at the aqueous surface and therefore fails to distinguish between adsorbed ions and those in the electrical double layer. Hauser et al. [11] have suggested that such a differentiation is itself artificial. They propose a 'constant gradation of binding' away from the interface. The alternative view is to consider adsorbed and double-layer counterions as conceptually distinct. This has prompted a large number of attempts to determine the fraction of surface Ca^{2+} ions tightly bound to lipid monolayers and bilayers under a variety of experimental conditions and to deduce association constants. Some authors have disregarded the double layer problem completely and indeed assume that 'bound' Ca^{2+} is in equilibrium with free ions at the bulk concentration, rather than bulk concentration multiplied by a Boltzmann factor [7,11,12]. The resulting apparent association constants are high (10^6–10^7 M^{-1} for pure PS monolayers) and depend heavily on the ionic environment of the lipid headgroups. More realistic approaches which were intended to yield intrinsic Ca^{2+} binding constants by including consideration of double layer effects [13–15] have produced a wide range of results (10^{-1}–10^4 M^{-1}). This is a reflection of the model-dependence of such methods. One elegant study is that of McLaughlin et al. [13] who measured the conductance of PS bilayers in the presence of nonactin and increasing concentrations of Ca^{2+}. From the conductance data they deduced potential differences across the lipid/water interface and then estimated an appropriate ion binding constant by fitting a theoretical curve to the experimental points. This curve was based on a combination of Gouy-Chapman theory and ion-binding equilibria at surfaces. Despite the logic of the approach, there are a number of drawbacks, including the assumption of constant headgroup packing density with increasing bulk Ca^{2+} concentration. Furthermore, McLaughlin et al. do not consider the possibility that increased Ca^{2+} binding may progressively alter the dipole moment of lipid headgroups. That this may be a serious oversimplification is shown by the fact that interfacial dipole potential differences can regulate bilayer conductance [16].

In this investigation, we have not tried to quantify the ratio of bound/double layer Ca^{2+}. But our results are consistent with significant chemical binding, in view of the raised Ca^{2+}/PS association when cholesterol is present in the monolayer despite the reduction in overall negative surface charge density.

Interpretation of monolayer findings

For a pure PS monolayer at a surface density of $0.5 \cdot 10^{14}$ molecules/cm^2, we find a Ca^{2+}/PS surface ratio of approx. 1 : 2 (Fig. 3). As PS possesses one net negative charge at this pH, this suggests two-point electrostatic attachment of the ions to link pairs of PS molecules, as proposed by Hauser et al. [11] and others. Ca^{2+} association is increased on compression of the film, a result in agreement with that of Hauser et al. [14].

With PC present in the surface, a Ca^{2+}/PS

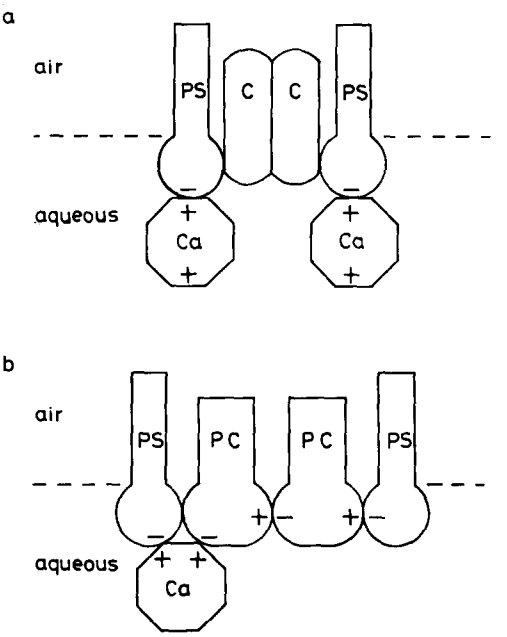

Fig. 5. Proposed mechanisms for the effects of nonionic spacers on Ca^{2+}/PS association. The molecular shapes and charge locations are not intended to be realistic. (a) PS/cholesterol (C) mixtures: the spacer prevents intermolecular salt formation beteen PS molecules. (b) PS/PC mixtures: see text for details.

ratio of 1 : 2 is still seen at $0.5 \cdot 10^{14}$ molecules/cm^2 PS surface density (Fig. 4). But PS/cholesterol films at the same number density of the anionic lipid show an increased ratio of 1 : 1 (Fig. 3). Since there is no Ca^{2+} association with pure cholesterol monolayers we interpret these results in terms of prevention of intermolecular salt formation (where Ca^{2+} bridges between two PS molecules) by the nonionic spacer (Fig. 5). We envisage cholesterol molecules surrounding a PS headgroup to which Ca^{2+} is bound, thereby impeding approach to another phospholipid molecule. This interpretation is favoured over others where the cholesterol is affecting Ca^{2+} binding either directly through effects on the ionisation of both anionic groups on the PS or through increasing the local surface densities of PS through its phase separation. This is because a similar increase in Ca^{2+} binding is seen in a related system of mono-alkyl chain surfactants with only one anionic group available (Gregory, D.P. and Mingins, J., unpublished data). Here, an alkyl sulphonate replaced PS, octadecanol was used by analogy with cholesterol and a sulphobetaine was the zwitterion analogous to PC.

The PS/PC results may be interpreted in the same framework by assuming that Ca^{2+} binding to the acidic lipid permits bridging to the phosphate moiety of a vicinal PC headgroup via the ion's other valency. This bridging does not occur in pure PC monolayers at the Ca^{2+} concentration used. It may however occur in mixed PS/PC monolayers where the strong Ca^{2+}/PS interaction sterically favours binding to PC via the remaining Ca^{2+} valency. A consequence of such bridging would be the unveiling of the positively charged quaternary ammonium of the PC zwitterion. This would enable it to associate directly or indirectly with the negative charge on an 'unoccupied' PS headgroup. This postulated mechanism would give a 1 : 2 Ca^{2+}/PS ratio. Thus no increase in Ca^{2+} binding, compared to pure PS, is seen (Fig. 5). An alternative mechanism for the lack of enhanced binding by PC is that Ca^{2+} may effect a phase separation in PS/PC films, similar to that observed in ESR studies [17]. In this case 1 : 2 binding of Ca^{2+} to PS is envisaged, with the anionic molecules located in clusters, separated from the PC to which no Ca^{2+} is bound.

One anomalous result is that the Ca^{2+} association found in PS/cholesterol films at low nonionic mole fraction is less than that seen with pure PS (Fig. 3). This finding is mirrored in the alkyl sulphonate/octadecanol system (Gregory, D.P. and Mingins, J., unpublished data) and there is no ready explanation. It is conceivable that the stoichiometry of the PS/cholesterol complex at the surface is a continuous function of cholesterol mole fraction. One could then postulate that the complex at high nonionic mole fraction impedes intermolecular salt formation as described, whereas that at low mole fraction permits bridging but reduces the overall Ca^{2+}/PS binding constant. This difficulty in interpretation highlights our ignorance of surface phase relations in these systems and hence their thermodynamic stability. Recent investigations have stressed the risk of metastability in lipid mixtures both in bulk [18] and at surfaces [19]. This risk was reduced here by following the behaviour of monolayers which were for the most part expanded (cf. Fig. 2), over a time-course of hours. Studies in a related system (PS/cholesteryl acetate, [20]) suggest that phase separation in the presence of Ca^{2+} only occurs at high surface pressures. We cannot eliminate the possible existence of metastable surface phases in our study without performing a rigorous thermodynamic analysis on adsorbed films, as described by Tajima and Gershfeld [19] for PC/cholesterol mixtures. But this does not necessarily diminish the biological interest of our results.

Electrophoretic studies

The implication from surface radioactivity measurements that PS may carry a net positive charge in mixed films with cholesterol following Ca^{2+} binding prompted an examination of the electrophoretic mobility of PS/cholesterol particles. Phase-contrast microscopy revealed that these particles were spherical. They were assumed to be multilamellar vesicles but no birefringence was observed through crossed polaroids. This was possibly a consequence of the increased interlamellar spacing predicted in dilute electrolyte. There was no significant variation in particle size with cholesterol mole fraction. It is conceivable that incorporation was incomplete for PS/cholesterol mixtures of high nonionic mole fraction, in view of

the reported instability of PC/cholesterol combinations containing more than 50% sterol [18,21]. That the nonionic spacer was incorporated into the particles is clear from the reduction in electrophoretic mobility observed in distilled water from $-5.4\ \mu/s$ per V per cm ($\zeta = -76$ mV) for pure PS to $-3.0\ \mu/s$ per V per cm ($\zeta = -42$ mV) for 25% PS/75% cholesterol particles. Electrophoretic velocities (v) in the presence of 10^{-4} M Ca^{2+} were converted to electrokinetic potentials (ζ) using the Helmholtz-Von Smoluchowski equation:

$$\zeta = \frac{4\pi\eta v}{E\epsilon}$$

where η and ϵ are respectively the viscosity and dielectric constant of the solution in the electrical double layer adjacent to the surface (taken to be equal to bulk values) and E is the applied field strength. The ionic strength and particle size were such that this equation could be used satisfactorily without applying Henry's correction (Overbeek [22]). Results are presented in Fig. 6 for ζ potential against mole fraction of cholesterol in the presence of 10^{-4} M Ca^{2+}.

There are interpretative difficulties associated with particle electrophoresis. These include uncertainty about molecular areas in the bilayers (which does not arise in monolayer studies) and inaccuracy in estimating surface charge densities from ζ potentials [23], particularly at high surface potentials [24,25]. The origin of the ζ potential in dispersions of 'uncharged' molecules, e.g. cholesterol (Fig. 6) also remains unclear, though this is seen in a variety of systems (hexadecane [26], octadecanol [27]). Clearly, however, Ca^{2+} affected the mobility of PS/cholesterol particles. This effect was apparent at 10^{-4} M bulk ionic concentration when the total lipid concentration was in the millimolar range. No depletion of bulk Ca^{2+} was detected in the experimental solutions using a Ca^{2+} electrode. Thus the majority of the lipid was presumably sequestered in the inner leaflets of multilamellar vesicles and not available for Ca^{2+} binding.

We attempted to quantify the cholesterol effect by taking the ζ potential for pure cholesterol and scaling its contribution to the mixtures by simply multiplying by the relevant cholesterol mole fraction. Subtracting this calculated ζ potential from the experimentally measured potential for each point gave a value for the PS contribution to each mixture. (This approach assumes a linearised Poisson-Boltzmann equation, and that ζ may be equated with the Gouy potential). Results are shown in Fig. 7. At high PS mole fraction (approx. 70%) there is a positive deviation from a monotonically decaying ζ potential curve with increasing cholesterol mole fraction, at low PS mole fraction (approx. 20%), there is a negative deviation. This result is exactly in accord with the surface radioactivity findings. When there is little cholesterol pre-

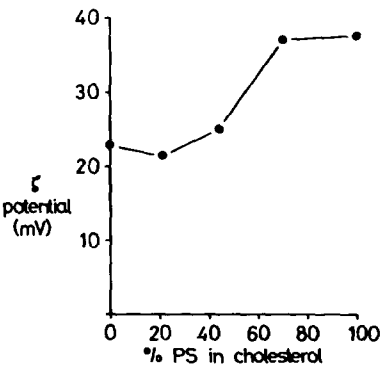

Fig. 6. Electrophoretic ζ-potential as a function of mol % PS in cholesterol, in the presence of 10^{-4} M Ca^{2+}. Potentials all negative.

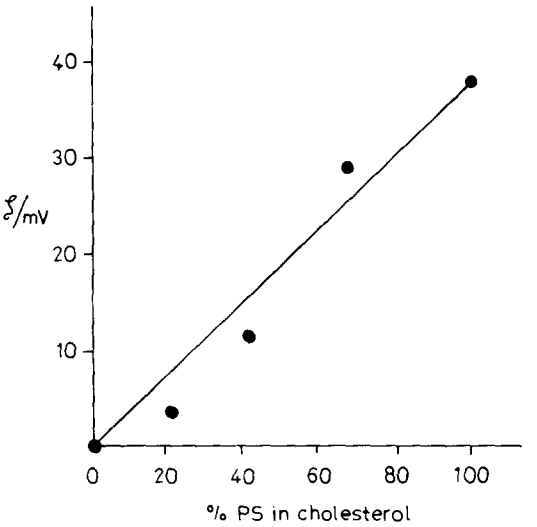

Fig. 7. Calculated ζ-potential contribution from PS as a function of mol% PS in cholesterol, in the presence of 10^{-4} M Ca^{2+}. Potentials all negative. Solid line shows 'expected' values from simple scaling of PS ζ potential. Standard error of the mean for all experimental points 2 mV.

sent the Ca^{2+} binding to PS is reduced leaving a greater residual surface charge density, whereas when cholesterol is the major component, Ca^{2+} binding is increased and the surface charge density is reduced.

It can be seen that the PS potential in no case becomes positive. This seems at variance with the monolayer findings. A possible explanation is that Ca^{2+} binding to PS prevents ionization of the phospholipid's amino group [5,28]. Thus, in PS/cholesterol mixed films, each Ca^{2+} occupied phospholipid headgroup would have no net charge, the two Ca valencies being exactly balanced by the phosphate and carboxyl moieties of PS.

Biological relevance

It is encouraging that effects originally seen in surfactant systems (Gregory, D.P. and Mingins, J., unpublished data) are obtained in mixtures of lipids that are major components of biological membranes, particularly as the critical PS number density is comparable to that expected on the inner monolayer of plasma membranes [1]. Rojas et al. [29] did not observe this effect of cholesterol as their experiments were conducted using more compressed monolayers. While we ascribe no specific function to the present findings, a possible role in the modulation of surface charge during membrane contact phenomena is apparent. The major drawback of our experiments is that they were performed in dilute media rather than physiological concentrations of monovalent salt. Some early studies [30] suggest that there is significant Ca^{2+} association with PS surfaces in the presence of 100 mM NaCl. The limitations of the present experiments did not permit accurate quantitation of the extent to which Na^{+} competes with Ca^{2+}.

This information might be obtainable from longer counting time experiments or studies of Ca^{2+} displacement from surfaces by Na^{+} although a surface Ca^{2+}/PS ratio of approx. 0.1 was indicated with 10^{-4} M bulk Ca^{2+}.

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References

1 Rothman, J.E. and Lenard, J. (1977) Science 195, 743–753
2 Bretscher, M.S. (1972) Nature New Biol. 236, 11–12
3 Gordesky, S.E. and Marinetti, G.V. (1973) Biochem. Biophys. Res. Commun. 50, 1027–1031
4 Douglas, W.W. (1968) Br. J. Pharmacol. 34, 451–474
5 Gingell, D. and Ginsberg, L. (1978) in Cell Surface Reviews (Poste, G. and Nicolson, G.L., eds.), Vol. 5, pp. 791–833, North - Holland, Amsterdam
6 Rojas, E. and Tobias, J.M. (1965) Biochim. Biophys. Acta 94, 394–404
7 Seimiya, T. and Ohki, S. (1973) Biochim. Biophys. Acta 298, 546–561
8 Allen, R.J.L. (1940) Biochem. J. 34, 858–865
9 Taylor, J.A.G. and Mingins, J. (1975) J. Chem. Soc. (Faraday Trans. 1) 71, 1161–1171
10 Aniansson, G. (1951) J. Phys. (Colloid) Chem. 55, 1286–1299
11 Hauser, H., Chapman, D. and Dawson, R.M.C. (1969) Biochim. Biophys. Acta 183, 320–333
12 Hauser, H. and Phillips, M.C. (1973) in Proc. 6th Int. Congr. on Surface-Active Substances, Vol. 2, pp. 371–380, Carl Hanser Verlag, Munich
13 McLaughlin, S.G.A., Szabo, G. and Eisenman, G. (1971) J. Gen. Physiol. 58, 667–687
14 Hauser, H., Darke, A. and Phillips, M.C. (1976) Eur. J. Biochem. 62, 335–344
15 Newton, C., Pangborn, W., Nir, S. and Papahadjopoulos, D. (1978) Biochim. Biophys. Acta 506, 281–287
16 Hladky, S.B. and Haydon, D.A. (1973) Biochim. Biophys. Acta 318, 464–468
17 Ohnishi, S.-i. and Ito, T. (1974) Biochemistry 13, 881–887
18 Gershfeld, N.L. (1978) Biophys. J. 22, 469–488
19 Tajima, K. and Gershfeld, N.L. (1978) Biophys. J. 22, 489–500
20 Handa, T. and Nakagakai, M. (1979) Colloid Polymer Sci. 257, 374–381
21 Lecuyer, H. and Dervichian, D.G. (1969) J. Mol. Biol. 45, 39–57
22 Overbeek, J.T.G. (1952) in Colloid Science (Kruyt, H.R., ed.), Vol. 1, pp. 194–244, Elsevier, Amsterdam
23 Aveyard, R. and Haydon, D.A. (1973) An Introduction to the Principles of Surface Chemistry, Cambridge University Press, Cambridge
24 MacDonald, R.C. and Bangham, A.D. (1972) J. Membrane Biol. 7, 29–53
25 Carrol, B.J. and Haydon, D.A. (1975) J. Chem. Soc. (Faraday Trans. I) 71, 361–377
26 Todd, I. and Gingell, D. (1980) J. Cell Sci. 41, 125–133
27 Hollingshead, S., Johnson, G.A. and Pethica, B.A. (1965) Trans. Faraday Soc. 61, 577–582
28 Papahadjopoulos, D. (1968) Biochim. Biophys. Acta 163, 240–254
29 Rojas, E., Lettvin, J.Y. and Pickard, W.F. (1966) Nature 209, 886–887
30 Joos, R.W. and Carr, C.W. (1967) Proc. Soc. Exp. Biol. Med. 124, 1268–1272