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SURFACE POTENTIAL OF PHOSPHATIDYLSERINE MONOLAYERS

I. DIVALENT ION BINDING EFFECT

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Summary

Surface potentials of phosphatidylserine monolayers have been measured in the presence of different divalent ion concentrations in order to determine the way in which divalent ions bind to the membrane surface. The association constants for divalent ions (Mg^{2+}, Ca^{2+} and Mn^{2+}) with the phosphatidylserine membrane have been obtained from the experimental data and simple ion binding theory. The order of divalent ion binding to the membrane is $Mn^{2+} > Ca^{2+} > Mg^{2+}$. However, none of the divalent ions used completely neutralized the negative charge of phosphatidylserine even at relatively high concentrations. The amounts of the divalent ions bound depended upon the concentration of the monovalent ions present in the subphase. It is suggested that the amounts of bound ions obtained from the use of radioisotope tracer methods may include a considerable contribution from the excess free ions in the double layer region of the phosphatidylserine membrane.

Introduction

The importance of the surface potential associated with membranes has been pointed out by many authors in connection with ionic permeability through membranes [1,2], membrane stability [3—7], ion adsorption on the membranes [8—12,41], transmembrane potentials [13—17,47,48] etc.

In order to obtain some insight into ionic interaction mechanisms with biological membranes, several authors have investigated the surface potential of both monolayer [20—27] and bilayer membranes [16,28—30,41,42] made of phospholipids relative to various ionic environments.

Bangham and Papahadjopoulos [23] observed the different effects of various divalent cations on the surface potential of acidic phospholipid monolayers and suggested that these differences reflected the specific interaction

of the divalent ions with the acidic phospholipids. Barton [31] determined the degree of divalent cation association with phosphatidylserine membranes by measuring the electrophoretic mobility of phospholipid vesicles. McLaughlin et al. [28] obtained the association constants of various divalent cations by measuring the conductance of phosphatidylserine membranes.

Another way to study ionic interactions with membrane surfaces is the use of radioisotope tracer methods [32]. The adsorption of Ca^{2+} on phospholipid monolayers has been studied with this method by many workers [33–39].

However, there are different interpretations concerning the extent of binding of divalent metal ions to the membrane surfaces obtained by these two methods. For example, the surface potential measurement technique suggests weak binding of Ca^{2+}, while the radioisotope method suggests strong binding of Ca^{2+} on the phosphatidylserine membrane.

To clarify this difference regarding interpretations of ion interaction with the membrane surface, we have measured the surface potential of phospholipid monolayers as a function of various divalent ion concentrations and analyzed the observed experimental results with a simple adsorption theory [28]. Adsorption and screening effects of divalent ions on the phosphatidylserine monolayer are discussed and the advantages and disadvantages of the experiments of surface potential measurements and isotope tracer methods are compared.

Materials and Methods

Materials. Phosphatidylserine was extracted from beef brain and purified according to the method of Rouser et al. [40] with minor modifications. Ultra pure monovalent salts (Brinkman Instruments), Tris-base (hydroxymethyl amino methane, Mann Research Laboratory), and analytical grade reagents (Baker Chemicals) were used in the experiments. The inorganic salts were roasted at about 600° C for 1 h to eliminate contaminants of organic materials. Water was triple distilled, including a process of alkaline $KMnO_4$.

Methods and procedures. Surface potential was measured by use of an Americium air electrode (approx. 3 mm above the air/water interface) and a calomel pH reference electrode. The latter electrode was grounded and the ionizing air electrode was connected to the input of an electrometer (Keithley 610C). The output potential of the electrometer, which was related to "surface potential", was monitored by a strip chart recorder. The entire apparatus was shielded by a Faraday cage.

A lipid monolayer was formed on the aqueous phase in a glass dish (9 cm in diameter) by spreading an aliquot of the hexane-lipid solution from a microsyringe (Hamilton). The concentration of phospholipid was determined by phosphate analysis. All the monolayers used in the experiments had the same area per molecule of 65 $Å^2$.

Before the addition of salt, the aqueous subphase was stirred well and it was ascertained that the monolayer surface potential was not altered by further stirring of the subphase solution. Then, a concentrated salt solution was injected into the subphase of the monolayer by using a microsyringe to alter

the salt concentration of the subphase. The subphase solution was again stirred well for at least 90 s. The homogeneous mixing of the solution, which was attained within 30 s, was checked by use of dyes. Successive additions of salts were made after the surface potential was stabilized at each salt concentration. The final experimental results were corrected for the surface potential changes resulting from the change of aqueous level caused by injection of concentrated salt solutions. All experiments were done at room temperature of 21°C.

Experimental results and analysis

In the first series of experiments, the changes in surface potentials of phosphatidylserine monolayers with various divalent cation (Ca^{2+}, Mg^{2+}, and Mn^{2+}) concentrations were measured in the presence of different concentrations of monovalent salt. Fig. 1 shows the surface potential change $\Delta\Delta V$ of the monolayer with various divalent ion concentrations in the presence of 0.1 M monovalent salt (0.09 M NaCl + 0.01 M Tris buffer solution of pH 7.4). Increasing Ca^{2+} concentrations in the subphase resulted in gradual increases in surface potential. Above 5 mM of Ca^{2+}, the increase in surface potential was at the rate of about 27 mV/10-fold Ca^{2+} concentration difference. The theoretical curve assuming no-ion binding with the monolayer is shown by a solid line with $K = 0$ (where K is the association constant of divalent ions, described in Appendix). With the experimental results shown in Fig. 1, the association constants (K) of these divalent ions with the phosphatidylserine

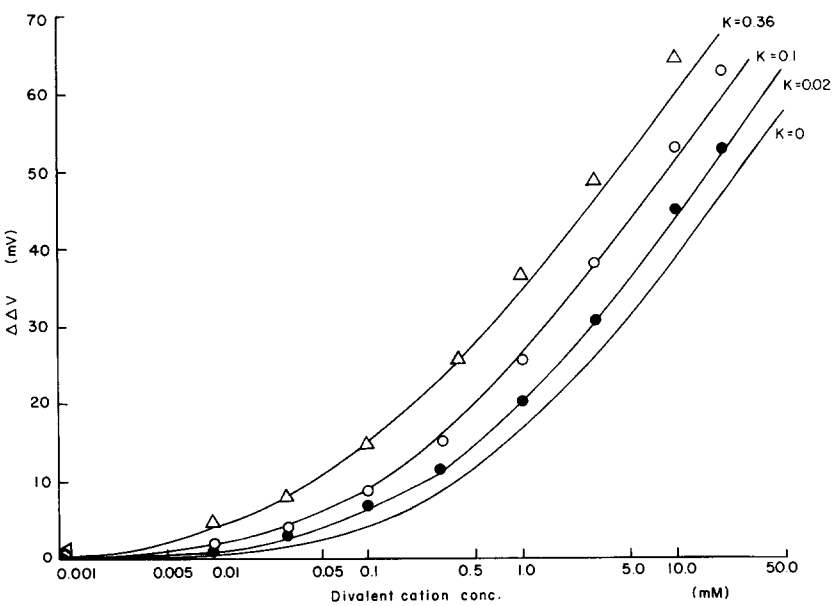

Fig. 1. Surface potential change of phosphatidylserine monolayer (65 Å^2/molecule) with the variation of divalent ion concentration in the presence of 0.09 M NaCl and 0.01 M Tris · HCl at pH 7.4 in the subphase. $\Delta\Delta V = \Delta V$ (with divalent ions) $- \Delta V$ (without divalent ions). Filled circles, Mg^{2+}; open circles, Ca^{2+}; open triangles, Mn^{2+}. The solid lines are the theoretical results for surface double layer potential for the corresponding K values where K is the association constant of divalent ion (see Appendix).

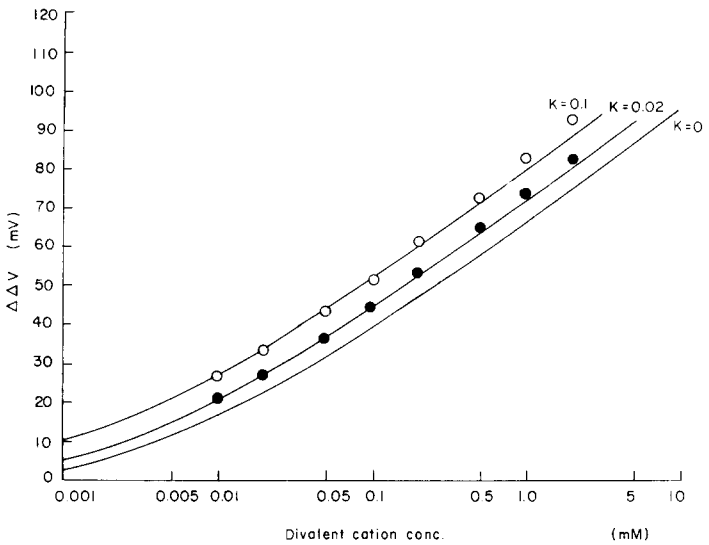

Fig. 2. Surface potential change of phosphatidylserine monolayer with respect to divalent ion concentrations in the presence of 0.009 M NaCl and 0.001 M Tris in the subphase at pH 7.4. Open circles, Ca^{2+}; filled circles, Mg^{2+}. The solid lines are the theoretical results with K = 0.0, 0.02 and 0.1.

monolayer were calculated from the theory proposed by McLaughlin et al. [28]; also see Appendix. The theoretical curves with the values of K = 0.36 for Mn^{2+}, 0.1 for Ca^{2+} and 0.02 for Mg^{2+} fit the experimental data fairly well below 1 mM of divalent ion concentrations. The association constant of 0.1 obtained for Ca^{2+} is in good agreement with that (K = 0.1) obtained by McLaughlin et al. [28], whereas the value (K = 0.02) for Mg^{2+} is quite different from their value (K = 0.1). Above 1 mM of Mg^{2+}, the theoretical curve with K = 0 and the experimental curve are approximately parallel. This indicates [28] that there is no appreciable divalent ion binding with the membrane because the divalent ion concentration near the interface becomes constant at these ionic concentrations. It should be noted, however, that for the cases of Ca^{2+} and Mn^{2+} at concentrations greater than 1 mM, there were deviations of the experimental points from the theoretical values. This tendency was also observed in the earlier studies [23,24].

Fig. 2 shows the surface potential change of the monolayer with divalent cation concentration in 0.009 M NaCl and 0.001 M Tris buffer solutions of pH 7.4. The experimental data for Ca^{2+} and Mg^{2+} fit well the theoretical curves of K = 0.1 and 0.02, respectively, below 0.2 mM divalent ion concentrations. Above 0.2 mM, however, the experimental data for both divalent ions show slight deviations from the theoretical curves.

The changes in surface potential with respect to divalent ion concentrations in the subphase solution without monovalent salts are plotted in Fig. 3. The pH of the solution, which was checked before and after the measurements, was 5.5 ± 0.2. The experimental points for both Ca^{2+} and Mg^{2+} fall approximately on a straight line with a slope of 29 mV per 10-fold divalent ion concentration difference over the range of 0.1 mM—50 mM. The experimental data consistently show a constant difference (approx. 7 mV) in potential

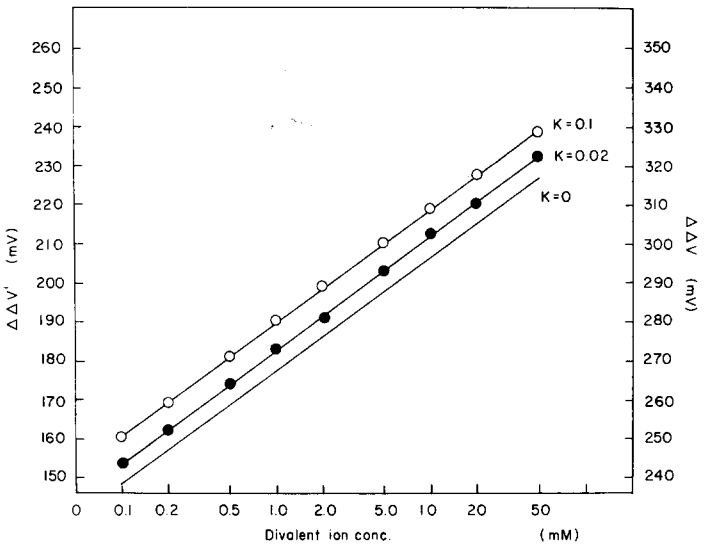

Fig. 3. Surface potential change of phosphatidylserine monolayer with the variation of divalent ion concentration without monovalent salt in the subphase. Open circles, Ca^{2+}; filled circles, Mg^{2+}. The solid lines are the theoretical results for $K = 0.0$, 0.02 and 0.1. The theoretical values of surface potential changes $\Delta\Delta V$ (= ΔV (with divalent ions at pH 5.5) — ΔV (without divalent ions at pH 5.5) are shown on the ordinate scale on the right hand side. The surface potential changes for the experimental as well as theoretical results expressed as $\Delta\Delta V'$ (= ΔV (with divalent ions at pH 5.5) — $\Delta V'$ (without divalent ions but with contaminants equivalent to $3 \cdot 10^{-10}$ M divalent ions at pH 5.5) are shown on the ordinate scale on the left.

between the two cases (Ca^{2+} and Mg^{2+}). The solid lines with $K = 0.0$, $K = 0.02$ and $K = 0.1$, respectively, which have all the same slopes of 29 mV/10-fold difference in divalent ion concentrations, are the theoretical results for the solution of pH 5.5. The difference in potential between the two theoretical lines ($K = 0.02$ and 0.1) is about 7 mV. The theoretical values of surface potential changes should be read by the ordinate scale on the right hand side in Fig. 3. The observed surface potential change for 0.1 mM Ca^{2+} ($\Delta\Delta V = \Delta V(Ca^{2+} = 0.1$ mM) — $\Delta V(Ca^{2+} = 0.0$ mM)) and that for 0.1 mM Mg^{2+} were 160 mV and 153 mV, respectively, which are smaller values than those calculated from the theory. The differences can be accounted for by the presence * of $3 \cdot 10^{-10}$ calcium ion equivalent as contaminants in water. The surface potential changes for the experimental as well as theoretical results expressed as $\Delta\Delta V' = \Delta V$ (with divalent ions) — $\Delta V'$ (without divalent ions but with contaminants equivalent to $3 \cdot 10^{-10}$ M calcium ions) are referred to the ordinate scale on the left side in Fig. 3. The above experimental results suggest that for very low monovalent ion concentrations the divalent cations may be adsorbed mostly at their very low concentrations and show no further substantial binding at the membrane surface with increasing concentration, even though there still seems to be a net negative charge on the membrane surface.

In the second series of experiments, the surface potential changes were measured with various NaCl concentrations in the presence of 0.1 mM divalent salts ($MgCl_2$, $CaCl_2$ and $MnCl_2$) in the subphase (Fig. 4). For all three

* In the case of non-binding divalent ions, the equivalent contaminants would be $8 \cdot 10^{-10}$ M.

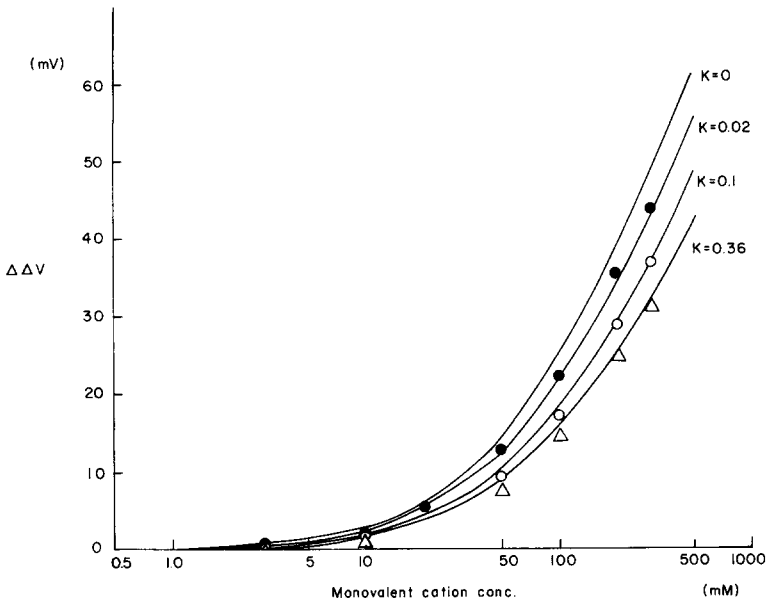

Fig. 4. Surface potential change of phosphatidylserine monolayer with monovalent ion concentrations in the presence of 0.1 mM divalent ions. The subphase always contained 1 mM Tris · HCl, pH 7.4. $\Delta\Delta V = \Delta V$ (with 0.1 mM divalent ions + monovalent salts) $- \Delta V$ (with 0.1 mM divalent ions). Filled circles, Mg^{2+}; open circles, Ca^{2+}; open triangles, Mn^{2+}. The solid lines are the theoretical results of double layer potential with the association constants of K = 0.0, 0.02, 0.1 and 0.36 as indicated in the figures.

cases, there was no appreciable change in surface potential with the change of NaCl concentrations below 1 mM. The potential change developed gradually with an increase in NaCl concentration above 1 mM. Above 200 mM a linear relationship between the change in surface potential and the logarithm of NaCl concentration was obtained. The theoretical curves calculated using the same association constants for individual divalent ions as obtained from the first series of experiments (i.e., K = 0.02 for Mg^{2+}, 0.1 for Ca^{2+} and 0.36 for Mn^{2+}) fit the experimental results farily well (see Fig. 4). It is also seen from this figure that the amount of monovalent ion necessary to cause considerable surface potential change in the presence of divalent ions is about 500—1000 times greater than that of divalent ions. This estimate can also be obtained from an analysis of Fig. 1.

All experimental points presented here are values averaged over at least six individual measurements. The standard mean error for the experimental points is 2.2 mV or less.

Discussion

The association constants of three divalent ions with phosphatidylserine monolayers were in the order of $Mn^{2+} > Ca^{2+} > Mg^{2+}$ which agrees with that obtained from electrophoretic measurements of phosphatidylserine vesicles [31], and also from direct measurements of divalent ion binding to phosphatidylserine molecules [49]. The fact that all the present experimental results obtained in the different monovalent ionic environments are fairly well

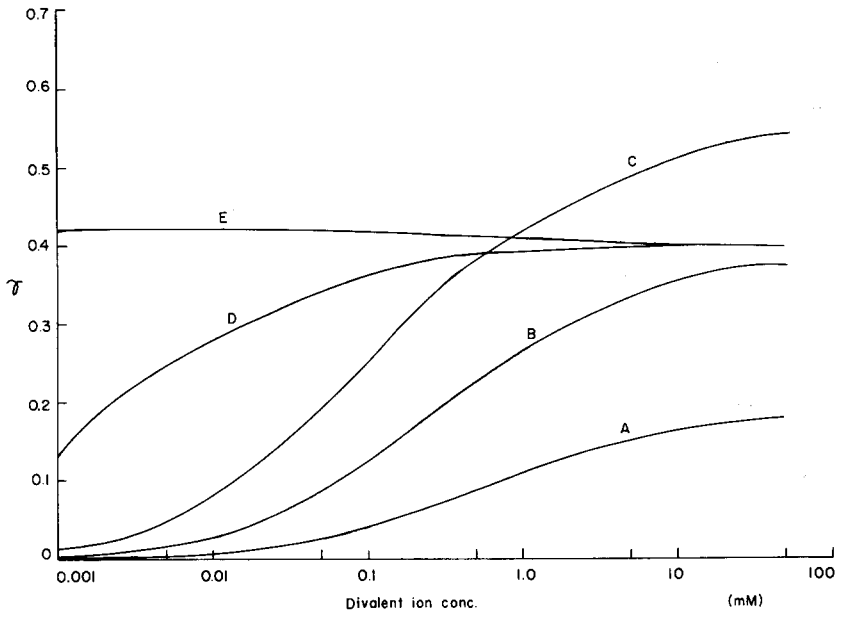

Fig. 5. The theoretical values of ratio (γ) of the total effective surface charge reduction caused by divalent ion binding to the total surface charge without ion binding for the phosphatidylserine monolayer (one net negative charge per molecule and an area of 65 Å^2 per molecule) versus divalent ion concentrations. A, Mg^{2+} in the presence of 100 mM monovalent salt in the subphase for $K = 0.02$; B, Ca^{2+} in the presence of 100 mM monovalent salt in the subphase for $K = 0.1$; C, Mn^{2+} in the presence of 100 mM monovalent salt for $K = 0.36$; D, Ca^{2+} in the presence of 1 mM monovalent salt for $K = 0.1$; E, Ca^{2+} without monovalent cation for $K = 0.1$.

explained by the theory with the same association constant for each divalent cation below a certain divalent ion concentration, indicates that the present simple adsorption theory is sufficient to explain ion binding on the phosphatidylserine membrane in a low concentration range of divalent ions.

The theoretical values of ratios (γ) of the reduction of effective surface charge density, $\sigma^{init} - \sigma$, caused by divalent ion binding to the original surface charge density σ^{init} (with no ion binding) of the phosphatidylserine monolayer (see Appendix), is plotted against divalent ion concentrations (Fig. 5). The calculations were carried out with the corresponding association constants (K = 0.36 for Mn^{2+}, 0.1 for Ca^{2+} and 0.02 for Mg^{2+}) obtained for the divalent cations. According to this simple adsorption theory, it is seen that Mg^{2+} is only slightly bound on the phosphatidylserine monolayer surface in the presence of 100 mM monovalent salt, and the binding tends to show a saturation-like behaviour above 5 mM Mg^{2+} (see Fig. 5, curve A). On the other hand, Mn^{2+} binds fairly extensively to the same membrane and causes a reduction of 40% of the net negative charge of phosphatidylserine at 1 mM Mn^{2+} (Fig. 5, curve C). The binding capacity for Ca^{2+} is between those of Mn^{2+} and Mg^{2+}. The binding seems to approach a plateau level for each divalent ion in the range of relatively high concentrations. These levels are approximately $\gamma = 0.2$ for Mg^{2+}, 0.4 for Ca^{2+} and close to 0.6 for Mn^{2+} (Fig. 5, A, B and C). The reason this saturation-like behaviour occurs is due to the fact that the concentration of free divalent ions at the surface of the membrane becomes independent of the bulk

concentration when the bulk concentration is high enough ($10^{-3} < M^{2+} < 10^{-1}$ M) as shown by the earlier workers [28], and that the concentration of free divalent ions at the surface is a function of the association constants of different divalent ions. However, it should be recalled that the above argument of ion binding is derived from a simple adsorption theory and is limited to the lower limit of the amount of divalent cation binding to the phosphatidylserine membrane. As pointed out earlier, above 1 mM of Ca^{2+} or Mn^{2+} there was a slight deviation of experimental results from the theoretical results with their own association constants (see Fig. 1). Therefore, further refinements of the theory should be made in order to explain satisfactorily the adsorption process at the higher concentrations of divalent ions. (a) There might be more complicated adsorption processes of divalent ions taking place at relatively high divalent ion concentrations. These values of the divalent ion concentrations depend upon the monovalent ion concentrations in the solution. One of these processes would be that by releasing hydrogen ion from its amino group by Ca^{2+} adsorption [43] a phosphatidylserine molecule could effectively possess two net negative charges and thus be able to accommodate one divalent ion per molecule. This possibility could account for the recent observation [39] of the high ratio (0.7) of adsorbed Ca^{2+} to phosphatidylserine molecules at a high Ca^{2+} concentration. (b) Modification of the surface dipole [29,44] or alteration of dielectric constant in the adsorption region caused by the adsorption of divalent ions, without assuming any additional ion adsorption process could also explain the discrepancies between the experimental and theoretical results. These points should be clarified in the future studies.

Although monovalent ions seem not to bind appreciably with the fixed charge groups of the membrane [26], they do influence rather remarkably

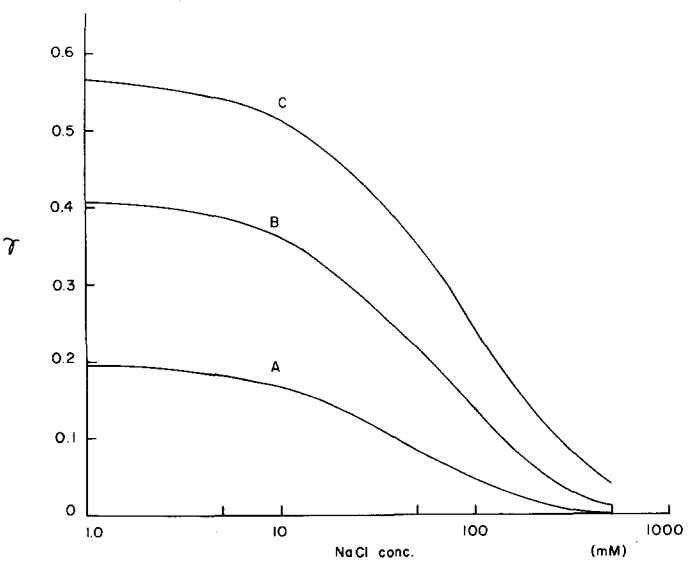

Fig. 6. The theoretical values (γ) with respect to monovalent salt concentration variation in the presence of various divalent ions: Mg^{2+} (A), Ca^{2+} (B) and Mn^{2+} (C) of 0.1 mM. The association constants used are 0.02 for Mg^{2+}, 0.1 for Ca^{2+} and 0.36 for Mn^{2+}.

the adsorption process of divalent ions on the membrane through alteration of the double layer potential (see Fig. 6). The much greater ability of the divalent ion over monovalent ion (500—1000-fold) to influence the surface potential of the phosphatidylserine monolayer is comparable to the ratio of competitive affinities of divalent (Ca^{2+}) to monovalent (Na^+ or K^+) ion adsorption on the same monolayer (obtained by the radioisotope tracer technique [37]).

Here we would like to make some comments concerning ion adsorption studies using radioactive isotope tracers [32—39]. With this technique, the adsorption measurements include not only the ions closely bound with the negatively charged sites of the membrane, but also the excess free ions in the diffuse double layer of the monolayer. This occurs because the half distance of the β-ray emitted by radioactive ^{45}Ca in water is much greater than the effective distance of the double layer of the usual electrolyte solution (more than 10^{-5} M of monovalent salt). It can be calculated that the contribution of Ca^{2+} in the double layer region, to the total "apparent Ca^{2+} adsorption" could be as high as 0.5 of the ratio of bound Ca^{2+}/lipid [46]. Even if the adsorption of divalent ions nearly attains its saturation level, the results in Fig. 5 show that there is still considerable net charge remaining without being neutralized with bound divalent ions. Studies by the radioisotope tracer technique [34—39] to determine the ratio of "adsorbed divalent ions" to phospholipids of the membrane, therefore, may have included both "specifically adsorbed (bound)" ions as well as excess free divalent ions in the double layer (in amounts depending upon the monovalent and divalent ion concentrations, see Figs. 5 and 6).

In ion adsorption studies using monolayer surface potential measurements, there are also many ambiguities concerning contributions of the Stern and Gouy layer potentials to the surface potential. However, the divalent ion adsorption, which has been estimated here using surface potential data, seems to be a lower limit of the actual ion binding. In order to determine the precise quantities of the actual ion binding on the membrane surface, the detailed mechanisms of ion-polar group interaction within the adsorption region of the membrane should be determined in the future.

Appendix

The relation between the diffuse double layer potential and the surface charge density having a uniform distribution is given by the Grahame equation:

$$\sigma = \tfrac{1}{272}\left\{\sum_i \left(C_i \cdot \exp\left(-\frac{Z \cdot F\psi(0)}{RT}\right) - 1\right)\right\}^{1/2} \tag{A1}$$

where σ is the surface charge density in electronic charges per Å^2, and C_i is the ionic concentration of the ith species in the bulk solution in mol/l, Z_i is its valence, $RT/F = 25$ mV at $T = 294$ K and $\psi(0)$ is the surface potential at the membrane interface.

It is assumed that a divalent ion may bind two phosphatidylserine molecules in the membrane, with an association constant K (in l/mol)

$$Ca^{2+} + A_2^{2-} \rightleftharpoons CaA_2, \quad K = \frac{[CaA_2]}{[Ca^{2+}][A^-]/2} \tag{A2}$$

where $[A^-]$ is the surface concentration of phosphatidylserine molecules. Then, according to McLaughlin et al. [28] the charge density, σ, is related to the initial charge density (no-ion binding), σ^{init}, by

$$\sigma = \frac{\sigma^{init}}{1 + K \cdot [Ca^{2+}(0)]} = \frac{\sigma^{init}}{1 + K \cdot [Ca^{2+}] \cdot \exp(-2F\psi(0)/RT)} \quad \text{(A3)}$$

where $\sigma^{init} = 1/65$ in our experiments, $[Ca^{2+}(0)]$ is the concentration of free divalent ions at the surface of the membrane, and $[Ca^{2+}]$ is the Ca^{2+} concentration in the bulk solution. Eqn. A1 together with A3 was solved by using a subroutine for roots of polynomials which is available at SUNYAB Computing Center.

The ratio of the total net charge reduction by the divalent ion adsorption to the total initial net charge of the membrane surface, γ is expressed by

$$\gamma = 1 - \frac{\sigma}{\sigma^{init}} = \frac{K[Ca^{2+}(0)]}{1 + K[Ca^{2+}(0)]}. \quad \text{(A4)}$$

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