Krystal, M., Li, R., Lyles, D., Pavlakis, G., & Palese, P. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2709–2713.
Lowy, D. R., Rand, E., & Scolnick, E. M. (1978) *J. Virol. 26*, 291–298.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Nakanishi, S., Inoue, A., Kita, T., Nakamura, M., Chang, A. C. Y., Cohen, S. N., & Numa, S. (1979) *Nature (London) 278*, 423–427.
Patzelt, C., Tager, H. S., Carroll, R. J., & Steiner, D. F. (1979) *Nature (London) 282*, 260–266.
Rajavashisth, T. B., Dawson, P. A., Williams, D. L., Shackelford, J. E., Lebherz, H., & Lusis, A. J. (1987) *J. Biol. Chem. 262*, 7058–7065.
Redgrave, T. G., Roberts, D. C. K., & West, C. F. (1975) *Anal. Biochem. 64*, 42–49.
Russell, J. H., & Geller, D. B. (1975) *J. Biol. Chem. 250*, 3409–3413.
Sanger, R., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.
Schmitz, G., Niemann, R., Brennhausen, B., Krause, R., & Assmann, G. (1985a) *EMBO J. 4*, 2773–2779.
Schmitz, G., Robenek, H., Lohmann, U., & Assmann, G. (1985b) *EMBO J. 4*, 613–622.
Shoulders, C. C., Kornblihtt, A. R., Munro, B. S., & Baralle, F. E. (1983) *Nucleic Acids Res. 11*, 2827–2837.
Sliwkowski, M. B., & Windmueller, H. G. (1984) *J. Biol. Chem. 259*, 6459–6465.
Soutar, A., Garner, C., Baker, H. N., Sparrow, J. T., Jackson, R. L., Gotto, A. M., & Smith, L. C. (1975) *Biochemistry 14*, 3057–3064.
Steiner, D. F., Quinn, P. S., Chan, S. J., Marsh, J., & Tager, H. S. (1980) *Ann. N.Y. Acad. Sci. 343*, 1–15.
Suttie, J. W., Hoskins, J. A., Engelke, J., Hopfgartner, A., Ehrlich, H., Bang, N. U., Belagaje, R. M., Schoner, B., & Long, G. L. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 634–637.
Thomas, P. (1983) *Methods Enzymol. 100*, 255–266.
Valls, L. A., Hunter, C. P., Rothman, J. H., & Stevens, T. H. (1987) *Cell (Cambridge, Mass.) 48*, 887–897.
Wise, R. J., Pittman, D. D., Handin, R. I., Kaufman, R. J., & Orkin, S. H. (1988) *Cell (Cambridge, Mass.) 49*, 229–236.
Wu, C. H., Donovan, C. B., & Wu, G. Y. (1986) *J. Biol. Chem. 261*, 10482–10484.
Zannis, V. I. (1986) *Methods Enzymol. 128*, 823–851.
Zannis, V. I., Breslow, J. L., & Katz, A. J. (1980) *J. Biol. Chem. 255*, 8612–8617.
Zannis, V. I., Kurnit, D. M., & Breslow, J. L. (1982) *J. Biol. Chem. 257*, 536–544.
Zannis, V. I., Karathanasis, S. K., Keutmann, H. T., Goldberger, G., & Breslow, J. L. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2574–2578.

# Adsorption of Cations to Phosphatidylinositol 4,5-Bisphosphate†

Maureen Toner,‡ Gerard Vaio,‡ Alan McLaughlin,§ and Stuart McLaughlin*,‡
*Department of Physiology and Biophysics, Health Sciences Center, State University of New York, Stony Brook, New York 11794, and Laboratory of Metabolism and Molecular Biology, NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852*

*Received January 14, 1988; Revised Manuscript Received May 18, 1988*

ABSTRACT: We investigated the binding of physiologically and pharmacologically relevant ions to the phosphoinositides by making $^{31}P$ NMR, electrophoretic mobility, surface potential, and calcium activity measurements. We studied the binding of protons to phosphatidylinositol 4,5-bisphosphate ($PIP_2$) by measuring the effect of pH on the chemical shifts of the $^{31}P$ NMR signals from the two monoester phosphate groups of $PIP_2$. We studied the binding of potassium, calcium, magnesium, spermine, and gentamicin ions to the phosphoinositides by measuring the effect of these cations on the electrophoretic mobility of multilamellar vesicles formed from mixtures of phosphatidylcholine (PC) and either phosphatidylinositol, phosphatidylinositol 4-phosphate, or $PIP_2$; the adsorption of these cations depends on the surface potential of the membrane and can be described qualitatively by combining the Gouy–Chapman theory with Langmuir adsorption isotherms. Monovalent anionic phospholipids, such as phosphatidylserine and phosphatidylinositol, produce a negative electrostatic potential at the cytoplasmic surface of plasma membranes of erythrocytes, platelets, and other cells. When the electrostatic potential at the surface of a PC/$PIP_2$ bilayer membrane is −30 mV and the aqueous phase contains 0.1 M KCl at pH 7.0, $PIP_2$ binds about one hydrogen and one potassium ion and has a net charge of about −3. Our mobility, surface potential, and electrode measurements suggest that a negligible fraction of the $PIP_2$ molecules in a cell bind calcium ions, but a significant fraction may bind magnesium and spermine ions.

A number of hormones, neurotransmitters, chemoattractants, and growth factors bind to receptors in the cell membrane and activate, probably through a G protein (Litosh & Fain, 1986; Williamson, 1986; Cockcroft, 1987), a specific phospholipase C. This enzyme hydrolyzes phosphatidylinositol 4,5-bisphosphate ($PIP_2$)[1] into the second messengers inositol

† This work was supported by National Science Foundation Grant BNS 85-01456 and National Institutes of Health Grant GM 24971.
‡ SUNY at Stony Brook.
§ NIAAA.

[1] Abbreviations: $G_{M1}$, galactosyl-*N*-acetylgalactosaminyl(*N*-acetylneuraminyl)galactosylglucosylceramide; $IP_3$, inositol 1,4,5-trisphosphate; MOPS, 4-morpholinepropanesulfonate; PA, phosphatidic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; $PIP_2$, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine.

0006-2960/88/0427-7435$01.50/0 

trisphosphate ($IP_3$) and diacylglycerol. $IP_3$ diffuses through the cytoplasm and releases Ca ions from a nonmitochondrial source, either the endoplasmic reticulum (Berridge & Irvine, 1984; Sekar & Hokin, 1986; Berridge, 1987) or the calcisome (Volpe et al., 1988), whereas diacylglycerol remains in the plasma membrane and activates a protein kinase C (Nishizuka, 1984, 1986).

$PIP_2$ would have five negative charges if no cations were bound to it, but the hydrogen, potassium, magnesium, calcium, and spermine ions in the cytoplasm could all bind significantly to this lipid. Furthermore, Schacht (1976, 1979) suggested that the nephrotoxic and ototoxic effects of aminoglycoside antibiotics such as gentamicin are related to their ability to bind $PIP_2$.

We wanted to measure the binding of these ions to $PIP_2$ and to other phosphoinositides. The binding of calcium and other small cations to monovalent anionic lipid, such as phosphatidylserine (PS) and phosphatidylglycerol (PG), can be described by equations first used by Stern [e.g., Aveyard and Haydon (1973) and McLaughlin et al. (1981)]. He used the Gouy–Chapman theory to account for the average potential produced by charges at a surface and a Langmuir adsorption isotherm to account for the specific adsorption of ions to the surface.

There are two reasons for suspecting that an adequate description of the adsorption of ions to $PIP_2$ will ultimately require more complicated equations. First, we need to know the electrostatic potential at the phosphate monoester groups in $PIP_2$ to calculate the binding of ions to these groups. When all the charges are at the surface of the membrane, the Gouy–Chapman theory can be used to calculate the potential [e.g., McLaughlin (1977) and Winiski et al. (1986)]. However, the phosphate monoester groups in $PIP_2$ probably extend 0.5–1.0 nm into the aqueous phase. In this case, the more general Poisson–Boltzmann equation must be used to calculate the potential. For example, the Poisson–Boltzmann equation can describe the profile of the potential produced by charges on the ganglioside $G_{M1}$, which are located 1 nm from the surface (McDaniel et al., 1984, 1986; Langner et al., 1988). It can also describe the potential 1 nm from the interface when most of the charges on the membrane are at the surface (Winiski et al., 1988). Second, the binding of cations to polyanionic $PIP_2$ molecules probably depends on the local as well as the average electrostatic potential. For example, the binding of an ion to one monoester phosphate group on $PIP_2$ will affect the binding of ions to the adjacent monoester phosphate in a complicated manner [e.g., Edsall and Wyman (1958) and Tanford (1961)].

We will attempt to describe our data with the Gouy–Chapman–Stern theory, although we acknowledge this theoretical framework provides only a qualitative description of the binding of ions to $PIP_2$ and will require modification as additional molecular information becomes available.

We first studied the binding of hydrogen ions to $PIP_2$ by making $^{31}P$ NMR measurements, which confirmed and extended the observations of van Paridon et al. (1986). We then studied the binding of potassium, magnesium, calcium, spermine, and gentamicin ions to $PIP_2$ by measuring the electrophoretic mobility of PC/$PIP_2$ vesicles. Electrophoretic mobility measurements would not detect the binding of a calcium ion to $PIP_2$ if it displaced two bound monovalent cations (e.g., a potassium and a hydrogen ion). We investigated the possibility that calcium could bind in this "electrically silent" manner to $PIP_2$ by making measurements with a calcium-sensitive electrode.

## Theory

We obtained most of our information about the binding of cations to phosphoinositides by mixing these lipids with phosphatidylcholine (PC), forming multilamellar vesicles, and measuring the electrophoretic mobility of the vesicles, $u$, their velocity in a unit electric field. We calculated the $\zeta$ potential, $\zeta$, from the Helmholtz–Smoluchowski equation (Aveyard & Haydon, 1973; Hunter, 1981)

$$\zeta = u\eta/(\epsilon_r\epsilon_0) \quad (1)$$

where $\epsilon_0$ is the permittivity of free space, $\epsilon_r$ is the dielectric constant, and $\eta$ is the viscosity of the solution. The $\zeta$ potential is the potential at the hydrodynamic plane of shear, which is about 0.2 nm from the surface of a phospholipid vesicle formed from a mixture of PC and either PS or PG in a 0.1 M monovalent salt solution (Eisenberg et al., 1979; Alvarez et al., 1983; Rooney et al., 1983; Winiski et al., 1986).

To calculate the $\zeta$ potential of a PC/$PIP_2$ vesicle from the measured value of the electrophoretic mobility using eq 1, we must assume the multilamellar vesicles are large and relaxation effects are therefore negligible, a reasonable assumption (Wiersema et al., 1966; O'Brien & White, 1978). We must also assume the charges are in a plane at the surface and the surface is smooth, less reasonable assumptions. If the monoester phosphate groups of $PIP_2$ are not at the surface, the vesicle will tend to move more rapidly than predicted by eq 1 for the reason illustrated in Figure 1 of Pasquale et al. (1986). If the inositol sugar group of $PIP_2$ protrudes from the surface and exerts hydrodynamic drag, the vesicle will tend to move less rapidly than predicted by eq 1. There are equations relating $\zeta$ to $u$ that take these phenomenona into account [e.g., Donath and Pastushenko (1979), Levine et al. (1983), and McDaniel et al. (1986)], but we have not used them because we do not know the orientation of the $PIP_2$ head group.

We deduced the intrinsic association constants by fitting the $\zeta$ potential data with a combination of the Gouy–Chapman theory and an adsorption isotherm. Equation 2, which was first derived by Abramson and Muller (1933) and Grahame (1947), relates the surface charge density, $\sigma$, to the electrostatic potential at the surface of the membrane, $\psi_0$

$$\sigma = \pm[2kT\epsilon_r\epsilon_0\sum_i n_i(\infty)\{\exp(-z_ie\psi_0/kT) - 1\}]^{1/2} \quad (2)$$

where $k$ is Boltzmann's constant, $T$ is the temperature, $n_i(\infty)$ is the concentration of ion $i$ in the bulk aqueous phase, $z_i$ is the valence of ion $i$, and $e$ is the magnitude of electronic charge. Aveyard and Haydon (1973) and McLaughlin (1977) give simple derivations of eq 2. We obtained another expression relating $\sigma$ and $\psi_0$ from the adsorption isotherms for mono-, di-, and tetravalent cations (see Appendix). We calculated $\psi_0$ from these two expressions with a bisection method,[2] assuming each lipid occupies an area of 0.7 $nm^2$.

The concentration of an ion of valence $z$ adjacent to a charged membrane, $n(0)$, is different from its concentration in the bulk aqueous phase, $n(\infty)$. The surface concentration is given by the Boltzmann relation:

$$n(0) = n(\infty)\exp(-ze\psi_0/kT) \quad (3)$$

[2] We calculated the theoretical values of $\zeta$ from the theoretical value for $\psi_0$ by integrating numerically the Gouy–Chapman expression for $d\psi/dx$. We assumed $\zeta$ is the potential 0.2 nm from the surface in 0.1 M salt.

The intrinsic association constant for the binding of an ion to a lipid, $K_I$, should be independent of the surface potential. It follows from eq 3 that an apparent association constant, $K_A$, depends on $\psi_0$ according to

$$K_A = K_I \exp(-ze\psi_0/kT) \quad (4)$$

## Materials and Methods

Water from a Millipore Super Q System (Millipore, Bedford, MA) was bidistilled in a Heraeus Bi4 quartz still (Heraeus Schott, Hanau, W. Germany). Tetramethylammonium chloride (TMACl) was purchased from Aldrich Chemical Co. (Milwaukee, WI) or Alfa Products (Danvers, MA) and was recrystallized from ethanol before use. NaCl and KCl were obtained from Allied Fisher Scientific (Springfield, NJ); spermine was obtained from Aldrich and $CaCl_2$ from Sigma Chemical Co. (St. Louis, MO). We measured the conductivity of our stock solutions to check their concentrations. PC (egg), PI (bovine liver), and PA (palmitoyl–oleoyl) were purchased from Avanti Polar Lipids (Birmingham, AL); the sodium salts of PIP (Bovine brain) and $PIP_2$ (bovine brain) were obtained from Sigma; the ammonium salt of $PIP_2$ was obtained from Calbiochem (La Jolla, CA). We did one-dimensional thin-layer chromatography on Analtech (Newark, DE) plates coated with either silica gel H and 1% potassium oxalate (Gonzalez-Sastre & Folch-Pi, 1968) or with silica gel G (Privett et al., 1973) to check the purity of the PI, PIP, and $PIP_2$ lipids and to ensure that sonication did not degrade $PIP_2$.

We formed multilamellar vesicles by the method of Bangham et al. (1974) and made the electrophoretic mobility measurements at 25 °C with a Rank Brothers Mark I machine (Bottisham, Cambridge, U.K.), taking care to focus at the stationary layer (Henry, 1938). We experienced difficulty obtaining reliable measurements with PC/$PIP_2$ vesicles when we used the sodium salt of $PIP_2$. These vesicles were formed by evaporating a chloroform/methanol/water (20:9:1 v/v) solution of $PIP_2$/PC in a round-bottom flask. The standard deviations of the mobility measurements were smaller (see Figure 1) when we used the ammonium salt of $PIP_2$. Both PC and the ammonium salt of $PIP_2$ are soluble in chloroform, and the lipids mix more uniformly when dried from this solvent. We obtained our best results when we used a minimal volume of chloroform and sprayed the mixture under vacuum onto the wall of a round-bottom flask, where it evaporated rapidly. We used a Buchler (Fort Lee, NJ) rotary evaporator with a continuous feed adaptor (Part PF-1030), connected to a Kontes (Vineland, NJ) universal adapter (K-179800) and inlet adapter (K-273410), which was lengthened 1 in. in a flame to protrude into the round-bottom flask.

We measured the effect of calcium and spermine on the $\zeta$ potential of vesicles formed from mixtures of PC and PIP. We do not illustrate these data because they indicate the ions bind less strongly to PIP than to $PIP_2$, and PIP has less biological significance than $PIP_2$.

We made surface potential measurements with an americium 241 ionizing electrode (NRD Inc., Grand Island, NY) positioned with a micromanipulator 2 mm above a monolayer formed from PC or a mixture of PC/$PIP_2$ (17 mol % $PIP_2$). Monolayers were formed on a Teflon Petri dish at room temperature, 22 °C, and were exposed to a nitrogen atmosphere.

We made measurements with a calcium electrode (W.P. Instruments, New Haven, CT) on sonicated PC/$PIP_2$ (16 mol % $PIP_2$) vesicles and on $PIP_2$ micelles in a solution containing 0.1 M KCl and 1 mM MOPS, pH 7.0. The concentration of phospholipid in solution determined by phosphate analysis (Rouser et al., 1970) was between 0.1 and 1.0 mM.

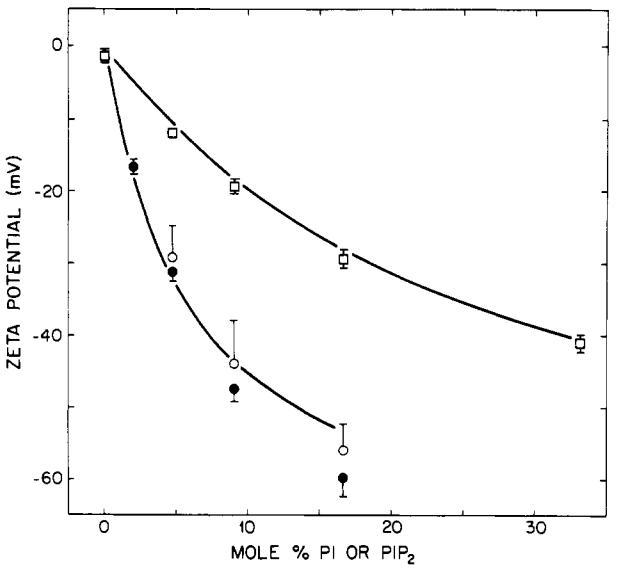


FIGURE 1: $\zeta$ potential of PC/$PIP_2$ (circles) and PC/PI (squares) multilamellar vesicles plotted as a function of the mole percent anionic lipid in the vesicle. The solutions contained 0.1 M KCl buffered to pH 7.0 with 1 mM MOPS. The open circles represent data obtained from vesicles formed by using the sodium salt of $PIP_2$; the closed circles represent data obtained from vesicles formed by using the ammonium salt of $PIP_2$. The vertical bars in all the figures represent the standard deviations when these are larger than the size of the symbols. The curves are the predictions of a combination of the Gouy–Chapman theory with Langmuir adsorption isotherms.

Unilamellar vesicles for the NMR and electrode experiments were formed by drying a chloroform/methanol/water (20:9:1) solution of a PC/$PIP_2$ mixture under a stream of $N_2$ and maintaining it under high vacuum overnight. A 0.1 M KCl, 1 mM MOPS, pH 7.0, solution was added to the lipid, and the mixture was sonicated with a Branson Sonic Power Co. W 185 sonifier (Danbury, CT) under a $N_2$ atmosphere in an ice bath for 30 min. The sonifier was switched on for 5 s and then off for 10 s. Longer duty cycles degraded the $PIP_2$. For the electrode experiments, the sonicated material was centrifuged at 160000$g$ for 80 min at 5 °C, and the upper three-fourths of the supernatant was retained for the measurements (Barenholz et al., 1977). For the NMR experiments the sonicated material was centrifuged for 10 min.

## Results

We obtained $^{31}P$ NMR spectra from PC/$PIP_2$ (10.5 mol % $PIP_2$) vesicles and from the isolated head group of $PIP_2$, glycerophosphoinositol diphosphate, in 0.1 M KCl. The pH titration of the $^{31}P$ NMR signals from the monoester groups of $PIP_2$ and the isolated head group were similar and agreed qualitatively with the results of van Paridon et al. (1986) for $PIP_2$ in micelles. In all cases, the titration curves were less steep in the midrange of the curve than predicted by the Henderson–Hasselbach equation. We can describe the data theoretically if we assume that the binding of a proton to one monoester phosphate group reduces the intrinsic association constant of protons with the adjacent monoester phosphate group by a factor of 20. This interaction may be due to a local electrostatic effect [e.g., Kirkwood and Westheimer (1938), Edsall and Wyman (1958), and Mille (1981)]. For simplicity, we ignore this interaction in our description of the adsorption of other cations to $PIP_2$: We describe the binding of protons to $PIP_2$ with Langmuir (mass action) adsorption isotherms and ignore any interaction other than that produced by the *average* electrostatic potential at the membrane–solution interface. A

detailed description of the interaction of protons with $PIP_2$ will be the subject of a separate paper (A. McLaughlin et al., unpublished results).

We observed half-maximal effects on the chemical shifts at pH 7.0 for one phosphate group and at pH 7.7 for the other phosphate group of $PIP_2$, giving apparent association constants of hydrogen ions with the monoester phosphates of $10^7$ and $5 \times 10^7$ $M^{-1}$. The surface potential of the PC/$PIP_2$ (10.5 mol % $PIP_2$) membranes used for the NMR experiments is about −60 mV (see below), so the intrinsic association constants of hydrogen ions to the monoester phosphates are about $10^6$ and $5 \times 10^6$ $M^{-1}$ (see eq 4).

We measured the electrophoretic mobility of vesicles formed from mixtures of zwitterionic lipid PC and either PI or $PIP_2$ to determine experimentally the charge on $PIP_2$ (Figure 1). When the $\zeta$ potential is small, it is proportional to the surface charge density of the vesicle (McLaughlin, 1977). Specifically, when $\zeta$ is small and only monovalent ions are present, $\zeta \simeq \psi_0$ and eq 2 reduces to $\psi_0 \simeq \sigma/\epsilon_0\epsilon_r\kappa$, where $1/\kappa$ is the Debye length. Thus, when the ratio of the $\zeta$ potentials of the PC/$PIP_2$ and PC/PI vesicles is measured at a given mole percent negative lipid, it should be equal to the ratio of the charge on the two negative lipids. The ratio of the $\zeta$ potentials of PC/$PIP_2$ and PC/PI vesicles[3] comprising 5% negative lipid is 2.6 (Figure 1). Since PI has a charge of −1, the $PIP_2$ in these vesicles has a net charge of charge of approximately −3. We also conclude the $PIP_2$ has a net charge of −3 by using the more exact nonlinear eq 2.

Although our assumptions that the charges on $PIP_2$ are at the surface and that the sugar group of $PIP_2$ exerts no hydrodynamic drag are unlikely to be true, a theoretical analysis indicates these effects might cancel each other [e.g., McDaniel et al. (1986) and Pasquale et al. (1986)]. The assumptions are less unreasonable in a 0.01 than in a 0.1 M KCl solution, where the Debye length is about 3 rather than 1 nm. Thus, we repeated the measurements illustrated in Figure 1 using a 0.01 M KCl, pH 7.0, solution. The electrophoretic mobilities of both the PC/PI and PC/$PIP_2$ vesicles increased, as expected theoretically. The results obtained with PC/PI vesicles are very similar to those obtained in 0.01 M salt with PC/PS and PC/PG vesicles (Winiski et al., 1986). When the $\zeta$ potential is low, the ratio of the potentials of the PC/$PIP_2$ to the PC/PI vesicles in the 0.01 M KCl solution is 3.0, similar to the value in 0.1 M KCl. The results in 0.01 M KCl thus support the simple interpretation of the results obtained in 0.1 M salt.

The curves in Figure 1 are the predictions of the Gouy–Chapman–Stern theory (a combination of the Gouy–Chapman theory, eq 2, the adsorption isotherms described in the Appendix, and the Boltzmann relation, eq 3). The theoretical curve used to describe the PC/PI data is identical with that used to describe PC/PS and PC/PG data by Winiski et al. (1986); the intrinsic association constant of potassium with PI was assumed to be 1 $M^{-1}$. The data in Figure 1 are described adequately by this simple theory.[4] The binding of

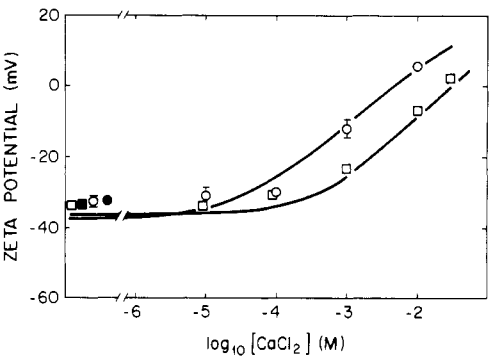


FIGURE 2: Effect of calcium ions on the $\zeta$ potentials of PC/$PIP_2$ (6.4 mol % $PIP_2$, circles) and PC/PI (26 mol % PI, squares) multilamellar vesicles. The solutions, which were buffered to pH 7.0 with 1 mM MOPS, contained 0.1 M NaCl and the indicated concentrations of $CaCl_2$. The filled symbols in Figures 2, 3, 4a, and 4C illustrate the $\zeta$ potentials when the solutions also contained 10 $\mu$M $Na_4EDTA$. The curves illustrate the predictions of the Gouy–Chapman–Stern theory; the intrinsic association constants of calcium ions with PI and $PIP_2$ are 10 and 500 $M^{-1}$.

protons to these monovalent anionic lipids is negligible at physiological pH. The combination of the Langmuir isotherm and the Boltzmann equation indicates that in a 0.1 M KCl solution the net charge on PI in a PC/PI vesicle is $-1/[1 + 0.1 \exp(-e\psi_0/kT)]$, i.e., about −1 when the magnitude of the surface potential is $< kT/e = 25$ mV.

The theoretical curve through the PC/$PIP_2$ data is drawn by assuming that potassium binds with an intrinsic association constant of 1 $M^{-1}$ to each of the phosphate groups of $PIP_2$ and that protons bind with intrinsic association constants of $5 \times 10^6$ and $10^6$ $M^{-1}$ to the monoester phosphates of $PIP_2$ (see Appendix). The curve describes the data reasonably well, and the model suggests that about one proton and one potassium ion are bound to $PIP_2$ when the surface potential is −30 mV and no other cations are present (see Discussion).

We also used a more direct technique to estimate the electrostatic potential. We measured the potential above a monolayer of PC and of PC/$PIP_2$ (17 mol % $PIP_2$) formed at the interface of a 0.1 M KCl, pH 7, solution. The potential above a PC monolayer was 415 ± 8 ($n$ = 12) mV when the lipids occupied an area of 0.66 $nm^2$ and 450 mV when they occupied 0.52 $nm^2$. The latter value agrees with the range of values in the literature for monolayers formed with excess PC (Bangham & Mason, 1979; Reyes et al., 1983; Haydon & Elliot, 1986). The potential above the PC/$PIP_2$ monolayer was 365 ± 12 ($n$ = 5) mV when the average area occupied by each lipid in the monolayer was 0.66 $nm^2$ (Kasianowicz et al., 1988). If the dipole potential is the same in the two monolayers, the surface potential of the PC/$PIP_2$ monolayer is 365 − 415 = −50 mV, a number that agrees with independent estimates from a study of the binding of neomycin to PC/$PIP_2$ monolayers (Kasianowicz et al., 1988). This number is also similar to the $\zeta$ potential of 17 mol % $PIP_2$ vesicles (Figure 1).

Figures 2–4 show the effect of divalent or tetravalent cations on the $\zeta$ potentials of multilamellar vesicles formed from mixtures of PC and either PI or $PIP_2$. The curves illustrate

[3] This disagrees with the experimental results of Hauser and Dawson (1967). They measured the electrophoretic mobility of PC/$PIP_2$ and PC/PI vesicles as a function of mole percent anionic lipid at pH 5.5 in 0.025 M NaCl. At low mole percent anionic lipid, the ratio of the slope of the PC/$PIP_2$ data to the slope of the PC/PI data was 4.9, leading to their conclusion that $PIP_2$ has a charge of −5. We thank Dr. Hauser for discussing with us several possible reasons for the difference between their pioneering results and our results. (Recall that our ratios were 2.6 and 3.0 in 0.1 and 0.01 M monovalent salt solutions at pH 7.0, respectively.) Our NMR results (see above) and those of van Paridon et al. (1986) demonstrate that >1 proton is bound to $PIP_2$ at pH 5.5 and that the conclusion of Hauser and Dawson (1967) is incorrect.

[4] When the mole percent PI > 30%, the theoretical curve for the PC/PI vesicles in Figure 1 continues to become more negative while the experimental values of $\zeta$ level off (not shown). For example, the experimentally observed $\zeta$ potential of PI vesicles is −45 ± 1 mV, while the theoretically predicted value is −58 mV. We do not understand the reason for this discrepancy between theory and experiment, which is not apparent with either PC/PS or PC/PG vesicles (Winiski et al., 1986) but has been observed previously for PC/PI vesicles (Hammond et al., 1984a).

the theoretical predictions of the Gouy–Chapman–Stern theory with the specified values of the intrinsic binding constants. We use the values obtained above for the binding constants of potassium (1 $M^{-1}$) and hydrogen ($5 \times 10^6$ and $10^6$ $M^{-1}$) ions to describe theoretically the data we obtained when multivalent cations are present. Our binding models are highly oversimplified. In the divalent cation–$PIP_2$ binding model we assume that an alkaline earth cation, such as calcium, binds with identical association constants to the diester phosphate, to the unprotonated monoester phosphates, and to the protonated monoester phosphates of $PIP_2$. In the tetravalent cation–$PIP_2$ binding model we assume that spermine and gentamicin bind with identical association constants to forms of $PIP_2$ having 1, 2, 3, 4 or 5 net negative charges.

The data in Figure 2 illustrate that calcium ions are about 10-fold more effective in reducing the $\zeta$ potential of PC/$PIP_2$ than of PC/PI vesicles. We can describe the PC/PI data in Figure 2, as well as the data obtained with 15 mol % PI vesicles (data not shown), by assuming the intrinsic association constant of calcium with PI is 10 $M^{-1}$, about the same value as the intrinsic association constant of calcium with phosphatidylserine (McLaughlin et al., 1981) and phosphatidylglycerol (Lau et al., 1981). We calculated the theoretical curve that describes the PC/$PIP_2$ data in Figure 2 by assuming that the intrinsic association constant of calcium with the phosphate groups of $PIP_2$ is 500 $M^{-1}$. We can also describe the data obtained with vesicles that had either a higher or lower mole percent $PIP_2$ by using the same binding constant (data not shown).

Magnesium ions are about one-third as effective as calcium ions in reducing the $\zeta$ potential of a PC/$PIP_2$ (6.6 mol % $PIP_2$) vesicle in 0.1 M NaCl (data not shown). We can describe these data by assuming the intrinsic association constant of magnesium ions with $PIP_2$ is 100 $M^{-1}$.

Figure 2 illustrates that calcium binds to $PIP_2$ an order of magnitude more strongly than to PI. Do the phosphate groups on $PIP_2$ chelate calcium because of their spatial orientation, or do divalent anionic phosphate groups on lipids simply bind calcium an order of magnitude more strongly than monovalent phosphate groups? To address this question we measured the effect of calcium ions on the electrophoretic mobility of vesicles formed from PC and 1-palmitoyl-2-oleoylphosphatidic acid (10 mol % PA) in 0.1 M KCl at pH 6.5 and at pH 10. A proton is bound to PA at pH 6.5 but not at pH 10. We could describe the data (not shown) with the Gouy–Chapman–Stern theory by assuming the intrinsic association constants of the calcium ions with the divalent and monovalent forms of PA are 1000 and 40 $M^{-1}$, respectively. We also are able to describe the effect of calcium ions on the $\zeta$ potential of PC/$PIP_2$ vesicles using a similar model in which calcium ions bind more strongly to divalent anionic phosphate groups than to monovalant anionic phosphate groups of $PIP_2$. We obtained a satisfactory fit of the PC/$PIP_2$ data in Figure 2 by assuming the intrinsic association constants of calcium ions with the divalent (monoester phosphates) and monovalent (diester phosphate and monoester phosphates bound to an alkali metal cation or proton) phosphates of $PIP_2$ are 1000 and 100 $M^{-1}$, respectively. Thus, it is not necessary to postulate a chelation of the calcium by the two adjacent phosphate groups in $PIP_2$ to account for its ability to bind calcium more strongly than PI.

Figure 2 shows that calcium concentrations lower than $10^{-4}$ M do not affect significantly the $\zeta$ potential of PC/$PIP_2$ vesicles. We obtained results similar to those illustrated in Figure 2 when we measured the effect of calcium on the surface potential of PC/$PIP_2$ monolayers (data not shown). These measurements, however, would not detect "electrically silent" binding of calcium ions. To investigate this possibility, we added calcium chloride to a solution containing $PIP_2$ micelles or PC/$PIP_2$ vesicles and measured the concentration of free calcium ions with an electrode. The ratio of free to bound calcium remained constant for concentrations of free calcium ions between 1 and 10 $\mu$M. We calculate that only 1% of the $PIP_2$ on the outer surface of vesicles and 3% of the $PIP_2$ in micelles are bound to calcium ions at a free calcium concentration of 1 $\mu$M. Thus, electrophoretic mobility, surface potential, and calcium electrode measurements all demonstrate that an insignificant fraction of $PIP_2$ molecules bind calcium ions at calcium concentrations normally found in the cytoplasm.

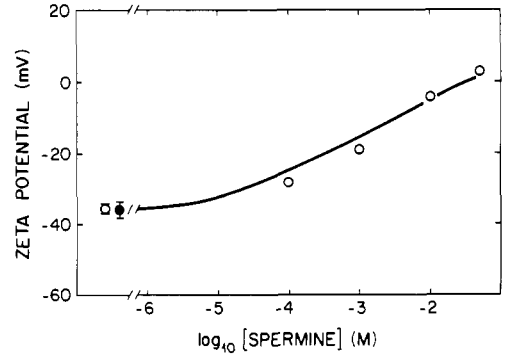


FIGURE 3: Effect of spermine on the $\zeta$ potentials of PC/PI (26 mol % PI) multilamellar vesicles. The solutions, which were buffered to pH 7.0 with 1 mM MOPS, contained 0.1 M NaCl and the indicated concentrations of spermine. The curve illustrates the prediction of the Gouy–Chapman–Stern theory; the intrinsic association constant of spermine with PI is 10 $M^{-1}$.

We studied the binding of spermine and gentamicin to the phosphoinositides. At pH 7.4, gentamicin and spermine have charges of +3.5 and +3.6, respectively (Josepovitz et al., 1982); in our binding model, we assumed that spermine and gentamicin each had a charge of +4.0.

Figure 3 illustrates the effect of spermine on the $\zeta$ potential of PC/PI vesicles. We assumed the intrinsic binding constants of spermine to PI is 10 $M^{-1}$ in calculating the theoretical curve. Spermine binds with a similar association constant to PS (Chung et al., 1985). Gentamicin also binds to both PI and PS with an intrinsic association constant of 10 $M^{-1}$ (Chung et al., 1985).

Figure 4A illustrates the effect of spermine on the $\zeta$ potential of PC/$PIP_2$ vesicles. Comparison of Figures 3 and 4A indicates that spermine reduces the $\zeta$ potential of a PC/$PIP_2$ vesicle an order of magnitude more effectively than of a PC/PI vesicle. We obtained similar results in 0.1 M NaCl (Figure 4A) and in 0.1 M KCl (data not shown). Figure 4C illustrates the effect of gentamicin on the $\zeta$ potential of PC/$PIP_2$ vesicles, which is similar to that of spermine. The data in both parts A and C of Figure 4 can be described qualitatively by the theoretical curves in Figure 4B, which were calculated from a combination of the Gouy–Chapman theory and the adsorption isotherms discussed in the Appendix.

Magnesium and spermine ions are both present in the cytoplasm. Can spermine bind to $PIP_2$ in a bilayer when magnesium ions are present at concentrations normally found in the cytoplasm? Figure 4A illustrates that $10^{-4}$ and $10^{-3}$ M spermine reduce the $\zeta$ potentials of PC/$PIP_2$ vesicles to about −15 and −5 mV in the absence of magnesium ions; $10^{-3}$ M magnesium ions reduce the $\zeta$ potential of a PC/$PIP_2$ vesicle (9 mol % $PIP_2$) in 0.1 M KCl, pH 7, from −47 to −26 mV.

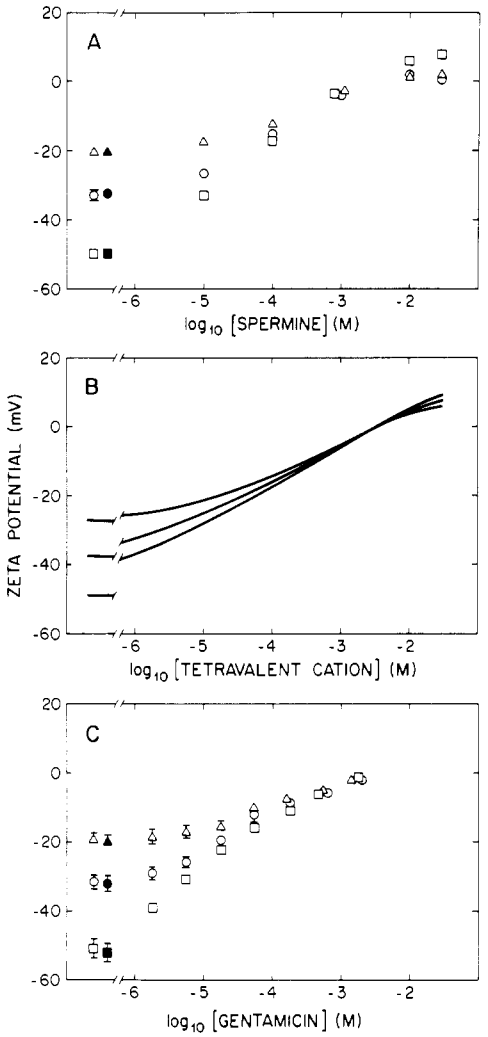


FIGURE 4: (A) Effect of spermine on the $\zeta$ potentials of PC/$PIP_2$ multilamellar vesicles formed with 3.3 (triangles), 6.4 (circles), or 16 (squares) mol % $PIP_2$. The solutions, which were buffered to pH 7.0 with 1 or 10 mM MOPS, contained 0.1 M NaCl and the indicated concentrations of spermine. (B) Theoretical predictions for the effect of tetravalent cations on the $\zeta$ potentials of PC/$PIP_2$ vesicles; the intrinsic association constant of the tetravalent cation with $PIP_2$ is 500 $M^{-1}$. (C) Effect of gentamicin on the $\zeta$ potentials of PC/$PIP_2$ multilamellar vesicles formed from 3.3 (triangles), 6.4 (circles), or 16 (squares) mol % $PIP_2$. The solutions, which were buffered to pH 7.4 with 1 mM MOPS, contained 0.1 M NaCl and the indicated concentrations of gentamicin.

Subsequent additions of $10^{-4}$ and $10^{-3}$ M spermine reduce the $\zeta$ potential of the vesicles to −14 and −6 mV, values similar to those obtained in the absence of magnesium ions. Thus, spermine and other polyvalent cations like neomycin (McLaughlin & Whitaker, 1988), can bind to $PIP_2$ even when magnesium ions are present. We did not attempt to formulate adsorption isotherms for the simultaneous binding of hydrogen, potassium, magnesium, and spermine ions to $PIP_2$.

The theoretical model we used to analyze the electrophoretic mobility data assumes the charges are at the membrane surface and the headgroups do not protrude from the surface and exert hydrodynamic drag. The finite size of the $PIP_2$ head group could complicate the interpretation of the data. The Gouy–Chapman theory assumes the ions in the diffuse double layer are point charges (Carnie & McLaughlin, 1983). This assumption is inaccurate in the case of spermine and gentamicin because these molecules are the same size as the Debye length in a 0.1 M monovalent salt solution, about 1 nm. We made some electrophoresis measurements in 0.01 M TMACl, where the Debye length is about 3 nm and the above assumptions are more realistic. We used TMA because it binds less strongly to negative lipids than sodium or potassium ions (Eisenberg et al., 1979), which simplifies analysis of the data. The values for the intrinsic association constants of calcium, magnesium, or spermine ions with $PIP_2$ in 0.01 M TMACl were about 3-fold greater than the values measured in 0.1 M NaCl or KCl. Similar discrepancies were noted previously with other lipids (McLaughlin et al., 1981; Lau et al., 1981) when the binding of divalent cations was studied. Although we do no understand why the association constants appear to increase when the ionic strength decreases, the available data suggest the finite sizes of spermine, gentamicin, and $PIP_2$ do not invalidate the simple model used here.

Table I: Number of Cations Bound to $PIP_2$ in a Bilayer Membrane Exposed to a 0.1 M KCl, pH 7, Solution

| | | no. of cations bound per $PIP_2$ molecule | |
|---|---|---|---|
| cation | concn (M) | $\psi_0$ = −30 mV | $\psi_0$ = −60 mV |
| hydrogen | $10^{-7}$ | 0.9 | 1.3 |
| potassium | $10^{-1}$ | 0.7 | 1.5 |
| calcium | $10^{-6}$ | 0.01 | 0.08 |
| magnesium | $10^{-4}$ | 0.2 | 1.0 |
| magnesium | $10^{-3}$ | 1.3 | 2.5 |

## Discussion

The available evidence suggests that $PIP_2$ is located in the inner monolayer of plasma membranes (Mauco et al., 1987), and we are interested in the extent to which cations normally found in the cytoplasm adsorb to this lipid. The apparent association constants and the number of cations bound to $PIP_2$ depend on the surface potential (eq 4); the potential at the cytoplasmic surface of many plasma membranes is negative. For example, the anionic lipid PS comprises 30% of the phospholipids in the inner monolayer of human erythrocyte membranes (Rothman & Lenard, 1977; Op den Kamp, 1979; Etemadi, 1980; Van Deenen, 1981; Ferrell & Huestis, 1984); double-layer theory predicts the surface potential should be between −30 and −60 mV, and experiments demonstrate the potential is negative (Lin et al., 1983).

We now calculate the number of hydrogen and potassium ions bound to $PIP_2$ using the Gouy–Chapman theory and the binding model described in the Appendix. For all the calculations presented in Table I, we assume $PIP_2$ is present in trace amounts (e.g., 1.4% for human erythrocytes; Ferrell & Huestis, 1984) and the surface potential of either −30 or −60 mV is produced by monovalent anionic lipids such as PS or PI. We assume the ion listed in the first column of the table is the only cation present besides potassium and hydrogen ions. We also assume the intrinsic association constants of protons with the two monoester phosphate groups are $5 \times 10^6$ and $10^6$ $M^{-1}$, and the intrinsic association constant of potassium ions to each of the phosphate groups in $PIP_2$ is 1 $M^{-1}$. The first two lines in Table I illustrate that about one proton and one potassium ion are bound to $PIP_2$ when the surface potential is between −30 and −60 mV and no other ions are present.

Of course, divalent cations are present in the cytoplasm. The third line in Table I illustrates that a negligible fraction of $PIP_2$ molecules bind calcium ions when these ions are present at a concentration of $10^{-6}$ M, an overestimate of the concentration found in the cytoplasm of a quiescent cell. However, a significant fraction of $PIP_2$ molecules do bind magnesium ions when this cation is present at concentrations between $10^{-4}$ and $10^{-3}$ M (Velosa et al., 1973; Gupta & Gupta, 1987). For example, our model predicts each $PIP_2$ molecule binds 1.3

magnesium ions, 0.9 hydrogen ion, and 0.4 potassium ion when the surface potential is −30 mV and magnesium ions are present at a concentration of $10^{-3}$ M in the bulk aqueous phase. We assumed the intrinsic association constants of calcium and magnesium ions with $PIP_2$ are 500 and 100 $M^{-1}$ for these calculations. Our value for the intrinsic association constant of calcium with $PIP_2$ in $PC/PIP_2$ bilayers ($5 \times 10^2$ $M^{-1}$, see Figure 2) agrees with Buckley and Hawthorne's (1972) value for the apparent association constant of calcium with $PIP_2$ in swine erythrocyte membranes ($4 \times 10^4$ $M^{-1}$) if the electrostatic potential of these erythrocyte membranes is −60 mV (see eq 4), a reasonable estimate of the potential for their experimental conditions (20 mM Tris).

Hendrickson and co-workers (Hendrickson & Fullington, 1965; Hendrickson & Reinertsen, 1969) deduced stability constants for the binding of calcium and magnesium ions to $PIP_2$ from pH titration curves that are higher than the values reported here. They also deduced stability constants for the binding of calcium to PS that are higher than values subsequently obtained from other measurements [e.g., McLaughlin et al. (1981) and Bentz et al. (1985)]. The reasons for these differences are not clear. Our electrophoretic mobility measurements of the interaction of calcium ions with vesicles formed from a mixture of egg PC and PI (Figure 2) agree qualitatively with the previous electrophoretic mobility measurements of Hammond et al. (1984b) on DPPC/PI vesicles. They observed a stronger interaction, but calcium ions bind more strongly to PC in a gel than in a liquid-crystalline state (Lau et al., 1980).

Au et al. (1986) also made electrophoretic mobility measurements to study the interaction of gentamicin with $PC/PIP_2$ vesicles. Our results agree qualitatively with their measurements. We both observed charge reversal at a gentamicin concentration of 0.001 M. However, the $\zeta$ potential of their 4:1 $PC/PIP_2$ vesicles in the absence of gentamicin (−25 mV) was much less negative that the $\zeta$ potentials of our vesicles (see Figure 1).

Finally, we consider the interaction of spermine with $PIP_2$. The free concentration of spermine in the cytoplasm is not known with any certainty. However, the total concentration can approach the millimolar level (Cohen, 1971). Even if the free concentration of spermine is only $10^{-5}$ M, a significant fraction of $PIP_2$ molecules (about 0.4 at a surface potential of −30 mV) will bind spermine. The biological significance of this interaction is unclear, but the concentration of polyamines increases in rapidly growing cells (Heby, 1981; Tabor & Tabor, 1984), and spermine can affect the interaction of a variety of enzymes with membranes (Moruzzi et al., 1987).

The numbers in Table I are probably qualitatively correct, even if our theoretical model is oversimplified. Better models should take into account local electrostatic interactions between ions that bind to $PIP_2$ and should be able to explain the NMR results discussed above. They should also take into account the possibility that the monoester groups are a significant distance from the surface[5] and do not experience the full surface potential produced by charges located at the membrane–solution interface (e.g., on lipids such as PS or PI). Finally, they should take into account the possibility that potassium, magnesium, and other cations bind more strongly to divalent phosphomonoester than to monovalent (protonated) phosphomonoester groups.

In conclusion, we offer a last caveat: The results summarized in Table I may not be applicable to all the $PIP_2$ molecules in biological membranes. A simple electrostatic calculation suggests that $PIP_2$ should adsorb strongly to all proteins that have several positive charges in the vicinity of the membrane–solution interface. More importantly, Anderson and Marchesi (1985) have demonstrated experimentally that $PIP_2$ binds strongly to glycophorin, and there is some evidence for separate metabolic pools of $PIP_2$ in human erythrocytes (King et al., 1987). If a fraction of the $PIP_2$ in biological membranes is bound to proteins, its ability to interact with other ions may be significantly different from that reported here.

## ACKNOWLEDGMENTS

We thank Dr. T. Thompson for valuable discussions about forming multilamellar vesicles from mixtures of lipids.

## APPENDIX: A SIMPLE MODEL FOR THE BINDING OF CATIONS TO $PIP_2$

*Monovalent Cations.* $P_1$ and $P_2$ represent the monoester phosphates, and $P_3$ represents the diester phosphate in $PIP_2$. $HP_1$ and $HP_2$ denote monoester phosphates to which a hydrogen ion is bound. $KP_1$, $KP_2$, $KP_3$, $KHP_1$, and $KHP_2$ denote phosphates to which a potassium (or sodium) ion is bound. For simplicity we assume the binding of a cation to one phosphate group does not affect the binding of cations to other phosphate groups, except through effects on the average charge density and surface potential (see eq 4). We also assume potassium ions bind equally well to $P_1$, $P_2$, $P_3$, and to the protonated monoester phosphates $HP_1$ and $HP_2$. We write seven Langmuir isotherms to describe binding of hydrogen and potassium ions to the phosphate groups of $PIP_2$. Braces represent surface concentrations. For example

$$\{HP_1\} = K_1[H]_0\{P_1\} \quad (1A)$$

$$\{HP_2\} = K_2[H]_0\{P_2\} \quad (2A)$$

$$\{KP_1\} = K_3[K]_0\{P_1\} \quad (3A)$$

We assume hydrogen and potassium ions do not bind to PC. Thus, when hydrogen and potassium ions are the only cations present, the surface charge density of a $PC/PIP_2$ membrane is the sum of the surface charge densities of the three phosphate groups on $PIP_2$. This surface charge density can be expressed through the seven isotherms in terms of the surface concentration of $PIP_2$, the intrinsic association constants $K_1$–$K_3$ (in $M^{-1}$ units), and the concentrations of hydrogen and potassium ions in the aqueous phase at the membrane–solution interface, $[H]_0$ and $[K]_0$ (in M units). These concentrations are related to the bulk aqueous concentrations by the Boltzmann relation (see eq 3). Since eq 2 is also an expression for the surface charge density, we have two equations in two unknowns, $\sigma$ and $\psi_0$, which we solve numerically. The predictions are illustrated in Figure 1. We follow a similar procedure when divalent or tetravalent cations are present.

*Divalent Cations.* We used the same model to describe the binding of divalent cations to PC/PI vesicles (Figure 2) that McLaughlin et al. (1981) used to describe the binding of divalent cations to PC/PS vesicles. In both this model and

[5] As measured by a fluorescent probe technique (Winiski et al. 1987), the electrostatic potential 1 nm from the surface of a $PC/PIP_2$ vesicle formed in 0.1 M KCl is about equal to the potential at the surface (Langner & McLaughlin, 1988). In contrast, the potential 1 nm from the surface of a PS, PG, PC/PI, or PC/PS vesicle is less negative than the surface potential, whereas the potential 1 nm from the surface of a $PC/G_{M1}$ vesicle is more negative than the surface potential (Winiski et al., 1988; Langner et al., 1988). The charges on PS, PI, and PG are at the surface, whereas the charge on the ganglioside is located 1 nm from the surface. The simplest interpretation of these $PC/PIP_2$ results is that the monoester phosphates of $PIP_2$ are 0.5–1 nm from the surface.

the one for PC/$PIP_2$ vesicles, we assume the intrinsic association constants for the binding of calcium and magnesium ions to PC are 3 and 1.8 $M^{-1}$ (McLaughlin et al., 1981). We assume that calcium (or magnesium) ions bind independently to the three phosphate groups on $PIP_2$ and that potassium and calcium ions do not bind simultaneously to the same phosphate moiety.

*Tetravalent Cations.* Since neither spermine nor gentamicin bind to PC (Chung et al., 1985), we need consider only the interaction of these cations with $PIP_2$. Since we do not know how spermine (or gentamicin) interacts with the individual phosphate groups of $PIP_2$ and we consider it likely that spermine binds to more than one phosphate group on $PIP_2$, we specify no details of the molecular interaction. We assume that spermine binds with the same intrinsic association constants to the forms of $PIP_2$ that have 1, 2, 3, 4, or 5 net negative charges, that sodium (or potassium) ions bind to each of these forms of $PIP_2$ with an intrinsic association constant of 1 $M^{-1}$, and that hydrogen ions bind to the pentavalent and tetravalent forms of $PIP_2$ with intrinsic association constants of $5 \times 10^6$ $M^{-1}$ and $1 \times 10^6$ $M^{-1}$, respectively.

## REFERENCES


Abramson, H. A., & Muller, H. (1933) *Cold Spring Harbor Symp. Quant. Biol. 1*, 29–33.

Alvarez, O., Brodwick, M., Latorre, R., McLaughlin, A., McLaughlin, S., & Szabo, G. (1983) *Biophys. J. 44*, 333–342.

Anderson, R. A., & Marchesi, V. T. (1985) *Nature (London) 318*, 295–298.

Au, S., Weiner, N., & Schacht, J. (1986) *Antimicrob. Agents Chemother. 30*, 395–397.

Aveyard, R., & Haydon, D. A. (1973) *An Introduction to the Principles of Surface Chemistry*, Cambridge University, Cambridge, U.K.

Bangham, A. D., & Mason, W. (1979) *Br. J. Pharmacol. 66*, 259–265.

Bangham, A. D., Hill, M. W., & Miller, N. G. A. (1974) *Methods Membr. Biol. 1*, 1–68.

Barenholz, Y, Gibbes, D., Litman, B. J., Goll, J., Thompson, T. E., & Carlson, F. D. (1977) *Biochemistry 16*, 2806–2810.

Bentz, J., Duzgunes, N., & Nir, S. (1985) *Biochemistry 24*, 1064–1072.

Berridge, M. J. (1987) *Annu. Rev. Biochem. 56*, 159–193.

Berridge, M. J., & Irvine, R. F. (1984) *Nature (London) 312*, 315–321.

Buckley, J. T., & Hawthorne, J. N. (1972) *J. Biol. Chem. 247*, 7218–7223.

Carnie, S., & McLaughlin, S. (1983) *Biophys. J. 44*, 325–332.

Chung, L., Kaloyanides, G., McDaniel, R., McLaughlin, A., & McLaughlin, S. (1985) *Biochemistry 24*, 442–452.

Cockcroft, S. (1987) *Trends Biochem. Sci. (Pers. Ed.) 12*, 75–78.

Cohen, S. S. (1971) *Introduction to Polyamines*, Prentice-Hall, Englewood Cliffs, NJ.

Donath, E., & Pastushenko, V. (1979) *J. Electroanal. Chem. 104*, 543–554.

Edsall, J. T., & Wyman, J. (1958) *Biophysical Chemistry*, Vol. 1, Academic, New York.

Eisenberg, M., Gresalfi, T., Riccio, T., & McLaughlin, S. (1979) *Biochemistry 18*, 5213–5223.

Etemadi, A. (1980) *Biochim. Biophys. Acta 604*, 423–475.

Ferrell, J. E., Jr., & Huestis, W. H. (1984) *J. Cell Biol. 98*, 1992–1998.

Gonzalez-Sastre, F., & Folch-Pi, J. (1968) *J. Lipid Res. 9*, 532–533.

Grahame, D. C. (1947) *Chem. Rev. 41*, 441–507.

Gupta, R. K., & Gupta, P., (1987) *$^{31}P$ NMR Spectroscopy of Cells and Organisms* (Gupta, R. K., Ed.) Vol. II CRC, Boca Raton, FL.

Hammond, K., Reboiras, M., Lyle, I., & Jones, M. (1984a) *Biochim. Biophys. Acta 774*, 19–25.

Hammond, K., Reboiras, M., Lyle, I., & Jones, M. (1984b) *Colloids Surf. 10*, 143–153.

Hauser, H., & Dawson, R. M. C. (1967) *Eur. J. Biochem. 1*, 61–69.

Haydon, D. A., & Elliott, J. R. (1986) *Biochim. Biophys. Acta 867*, 337–340.

Heby, O. (1981) *Differentiation (Berlin) 19*, 1–20.

Hendrickson, H. S., & Fullington, J. G. (1965) *Biochemistry 4*, 1599–1605.

Hendrickson, H. S., & Reinertsen, J. L. (1969) *Biochemistry 8*, 4855–4858.

Henry, D. C. (1938) *J. Chem. Soc.*, 997–999.

Hunter, R. J. (1981) *Zeta Potential in Colloid Science*, Academic, New York.

Josepovitz, C., Pastoriza-Munoz, E., Timmerman, D., Scott, M., Feldman, S., & Kaloyanides, G. J. (1982) *J. Pharmacol. Exp. Ther. 223*, 314–321.

Kasianowicz, J., Gabev, E., & McLaughlin, S. (1988) *Biophys. J. 53*, 517a.

King, C. E., Stephens, L. R., Hawkins, P. T., Guy, G. R., & Michell, R. H. (1987) *Biochem. J. 244*, 209–217.

Kirkwood, J. G., & Westheimer, F. H. (1938) *J. Chem. Phys. 6*, 506–517.

Langner, M., & McLaughlin, S. (1988) *Biophys. J. 53*, 128a.

Langner, M., Winiski, A., Eisenberg, M., McLaughlin, A., & McLaughlin, S. (1988) in *New Trends in Ganglioside Research: Neurochemical and Neuroregenerative Aspects* (Ledeen, R., Hogan, E., Tettamanti, G., & Yates, A., Eds.) Liviana, Padova, Italy (in press).

Lau, A., McLaughlin, A., MacDonald, R., & McLaughlin, S. (1980) *Adv. Chem. Ser. No. 188.*

Lau, A., McLaughlin, A., & McLaughlin, S. (1981) *Biochim. Biophys. Acta 645*, 279–292.

Levine, S., Levine, M., Sharp, K. A., & Brooks, D. E. (1983) *Biophys. J. 42*, 127–135.

Lin, G. S. B., Macey, R. I., & Mehlhorn, R. J. (1983) *Biochim. Biophys. Acta 732*, 683–690.

Litosch, I., & Fain, J. (1986) *Life Sci. 39*, 187–194.

Mauco, G., Dajeans, P., Chap, H., & Douste-Blazy, L. (1987) *Biochem. J. 244*, 757–761.

McDaniel, R. V., McLaughlin, A., Winiski, A. P., Eisenberg, M., & McLaughlin, S. (1984) *Biochemistry 23*, 4618–4624.

McDaniel, R. V., Sharp, V., Brooks, D., McLaughlin, A. C., Winiski, A. P., Cafiso, D., & McLaughlin, S. (1986) *Biophys. J. 49*, 741–752.

McLaughlin, S. (1977) *Curr. Top. Membr. Transp. 9*, 71–144.

McLaughlin, S., & Whitaker, M. (1988) *J. Physiol. 396*, 189–204.

McLaughlin, S., Mulrine, N., Gresalfi, T., Vaio, G., & McLaughlin, A. (1981) *J. Gen. Physiol. 77*, 445–473.

Mille, M. (1981) *J. Colloid Interface Sci. 81*, 169–179.

Moruzzi, M., Barbiroli, B., Monti, M. G., Tadolini, B., Hakim, G., & Mezzetti, G., (1987) *Biochem. J. 247*, 175–180.

Nishizuka, Y. (1984) *Nature (London) 308*, 693–698.

Nishizuka, Y. (1986) *Science (Washington, D.C.) 233*, 305–312.

O'Brien, R. W., & White, L. R. (1978) *J. Chem. Soc., Faraday Trans. 2, 74*, 1607–1626.
Op den Kamp, J. A. F. (1979) *Annu. Rev. Biochem. 48*, 47–71.
Pasquale, L., Winiski, A., Oliva, C., Vaio, G., & McLaughlin, S. (1986) *J. Gen. Physiol. 88*, 697–718.
Privett, O. S., et al. (1973) in *Quantitative Thin Layer Chromatography* (Touchstone, J. C., Ed.) p 59, Wiley, New York.
Reyes, J., Greco, F., Motais, R., & Latorre, R. (1983) *J. Membr. Biol. 72*, 93–103.
Rooney, E. K., East, J. M., Jones, O. T., McWhirter, J., Simmonds, A. C., & Lee, A. G. (1983) *Biochim. Biophys. Acta 728*, 159–170.
Rothman, J. E., & Lenard, J. (1977) *Science (Washington, D.C.) 195*, 743–753.
Rouser, G., Fleischer, S., & Yamamoto, A. (1970) *Lipids 5*, 494–496.
Schacht, J. (1976) *J. Neurochem. 27*, 1119–1124.
Schacht, J. (1979) *Arch. Oto-Rhino-Laryngol. 224*, 129–134.
Sekar, M. C., & Hokin, L. E. (1986) *J. Membr. Biol. 89*, 193–210.
Tabor, C. W., & Tabor, H. (1984) *Annu. Rev. Biochem. 53*, 749–790.
Tanford, C. (1961) *Physical Chemistry of Macromolecules*, Wiley, New York.
van Deenen, L. L. M. (1981) *FEBS Lett. 123*, 3–15.
van Paridon, P. A., de Kruijff, B., Ouwerkerk, R., & Wirtz, K. W. A. (1986) *Biochim. Biophys. Acta 877*, 216–219.
Veloso, D., Guynn, R. W., Oskarsson, M., & Veech, R. L., (1973) *J. Biol. Chem. 248*, 4811–4819.
Volpe, P., Krause, K., Hashimoto, S., Zorzato, F., Pozzan, T., Meldolesi, J., & Lew, D. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1091–1095.
Wiersema, P. H., Loeb, A. L., & Overbeek, J. Th. G. (1966) *J. Colloid Interface Sci. 22*, 78–99.
Williamson, J. R. (1986) *Hypertension (Dallas) 8* (Suppl. II), II-140–II-156.
Winiski, A. P., McLaughlin, A. C., McDaniel, R. V., Eisenberg, M., & McLaughlin, S. (1986) *Biochemistry 25*, 8206–8214.
Winiski, A. P., Eisenberg, M., Langner, M., & McLaughlin, S. (1988) *Biochemistry 27*, 386–392.

# Estimation of the Equilibrium Lateral Pressure in Liposomes of 1-Palmitoyl-2-[10-(pyren-1-yl)-10-ketodecanoyl]-*sn*-glycero-3-phosphocholine and the Effect of Phospholipid Phase Transition

Rauno Konttila, Ilkka Salonen, Jorma A. Virtanen, and Paavo K. J. Kinnunen*
*Langmuir-Blodgett Section, Department of Membrane Physics, KSV Research Laboratories, Helsinki, Finland*
*Received December 8, 1987; Revised Manuscript Received May 5, 1988*

ABSTRACT: A phosphatidylcholine analogue, 1-palmitoyl-2-[10-(pyren-1-yl)-10-ketodecanoyl]-*sn*-glycero-3-phosphocholine (k-PPDPC), which contains a chromophore covalently attached to one of the acyl chains, was characterized spectroscopically as liposomes and monolayers on an air/water interface. Differential scanning calorimetry of k-PPDPC liposomes showed two endothermic transitions with peaks at 13.7 and 23.8 °C, having enthalpies of 0.6 and 1.7 kcal/mol, respectively. Liposomes of k-PPDPC also exhibit thermotropic changes in the absorption spectra so that the ratio of the peaks at 289 and 356 nm changes from 2.15 to 1.60 in the temperature range of 20–26 °C, coinciding with the second calorimetric transition. These bands appear in the reflectance spectra of k-PPDPC monolayers on water and reveal a strong surface pressure dependency. Neglecting any possible effects due to coupling of the two leaflets of liposomal bilayers on the changes in absorption spectra and assuming the spectroscopic changes as a function of lateral pressure in monolayers to be equivalent to those in liposomes, we estimated the equilibrium lateral pressure ($\pi$) of k-PPDPC liposomes to be approximately 39 and 17 mN/m below and above the transition at 23 °C, respectively. The exact nature of the transition of k-PPDPC is uncertain, and thus a direct comparison with phospholipids such as dipalmitoylphosphatidylcholine is ambiquous. However, as far as we know this is the first relatively direct observation of a change in $\pi$ accompanying a phase transition in liposomal membranes.

While it is accepted that the equilibrium lateral pressure ($\pi$)[1] of cell membranes influences a number of important cellular phenomena involving membranes [for references, see Thurén et al. (1986)], it is unfortunate that there is no technique currently available to allow the direct assessment of this parameter. On the basis of thermodynamic calculations, Nagle (1976, 1980) estimated approximately 50 mN/m for $\pi$ in DPPC liposomes. Only a few experimental approaches have been published so far. A value of 20 mN/m was deduced from NMR order parameter calculations for liquid-crystalline bilayers (Marcelja, 1974). Use of phospholipases A with dif-

* Address correspondence to this author at KSV Research Laboratories, POB 128, SF-00381 Helsinki, Finland.

[1] Abbreviations: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSC, differential scanning calorimetry; k-PPDPC, 1-palmitoyl-2-[10-(pyren-1-yl)-10-ketodecanoyl]-*sn*-glycero-3-phosphocholine; NMR, nuclear magnetic resonance; PPDPC, 1-palmitoyl-2-[10-(pyren-1-yl)-decanoyl]-*sn*-glycero-3-phosphocholine; $\pi$, equilibrium lateral pressure.

0006-2960/88/0427-7443$01.50/0 