Electrostatics of phosphoinositide bilayer membranes

Theoretical and experimental results

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ABSTRACT We made fluorescence, electron paramagnetic resonance (EPR), electrophoretic mobility, and ionizing electrode measurements to study the effect of the monovalent lipid phosphatidylinositol (PI) and the trivalent lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) on the electrostatic potential adjacent to bilayer membranes. When the membranes were formed from mixtures of PI and the zwitterionic lipid phosphatidylcholine (PC), the Gouy-Chapman-Stern (GCS) theory described adequately the dependence of potential on distance (0, 1, 2 nm) from the membrane, mole % negative lipid, and [KCl]. Furthermore, all EPR and fluorescence probes reported identical surface potentials with a PC/PI membrane. With PC/PIP_2 membranes, however, the anionic (coion) probes reported less negative potentials than the cationic (counterion) probes; the deviations from the GCS theory were greater for the coions than the counterions. Discreteness-of-charge theories based on the Poisson-Boltzmann equation incorrectly predict that deviations from the GCS theory should be greater for counterions than for coions. We discuss a consistent statistical mechanical theory that takes into account three effects ignored in the GCS theory: the finite size of the ions in the double layer, the electrical interaction between pairs of ions (correlation effects), and the mobile discrete nature of the surface charges. This theory correctly predicts that deviations from GCS theory should be negligible for monovalent lipids, significant for trivalent lipids, and greater for coions than for counterions.

INTRODUCTION

We are interested in the electrostatic properties of the phosphoinositides for two reasons. First, these lipids are important biologically. Second, membranes containing these lipids are useful for testing theories of the electrostatic diffuse double layer.

The phosphoinositides are important biologically because they are the source of two intracellular second messengers. The binding of several different neurotransmitters, hormones, and growth factors to receptors on the outer surface of a plasma membrane activates a specific phospholipase C on the inner surface (Berridge, 1984, 1987; Berridge and Irvine, 1984, 1989; Nishizuka, 1984, 1986, 1988; Hokin, 1985; Sekar and Hokin, 1986), a process mediated by a G protein (Litosh and Fain, 1986; Williamson, 1986; Cockcroft, 1987). This lipase catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), which is preferentially located on the inner monolayer of the plasma membrane, into inositol trisphosphate and diacylglycerol. The inositol trisphosphate molecules diffuse through the cytoplasm to the endoplasmic reticulum (Berridge and Irvine, 1984; Berridge, 1987) or calcisome (Volpe et al., 1988), where they induce the release of stored calcium. The diacylglycerol molecules remain in the plasma membrane and activate protein kinase C (Nishizuka, 1984, 1986), a process that also requires calcium and phosphatidylserine (PS) (Takai et al., 1979; Kaibuchi et al., 1981; Hannun et al., 1985; Hannun and Bell, 1986; Azhar et al., 1987), and involves the translocation of protein kinase C from the cytoplasm to the membrane.

We discuss elsewhere how electrostatic potentials might affect this second messenger system (McLaughlin, 1989). For example, the ability of calcium ions to bind to the inner surface of plasma membranes from sea urchin eggs and induce exocytosis depends on the surface potential of the membrane (McLaughlin and Whitaker, 1988). The interaction of positively charged regions on protein kinase C (Bazzi and Nelsestuen, 1987*a–c*) and its substrates (Newton and Koshland, 1989) with the plasma membrane also depends on the electrostatic potential produced by negative lipids in the membrane.

Which negative lipids are mainly responsible for producing the negative surface potential? PS is the major charged lipid in most mammalian plasma membranes. For example, PS comprises ~15% of the phospholipids in the human erythrocyte and there is good evidence that all the PS is located on the inner or cytoplasmic monolayer of

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this membrane (Op den Kamp, 1979; Ferell and Huestis, 1984; Bishop and Bell, 1988; Middelkoop et al., 1988). The distribution of lipids in other membranes is not as well established. Nevertheless, phosphatidylinositol (PI), which is the major negative lipid in the plasma membrane of sea urchin eggs, is probably also located on the cytoplasmic surface of the plasma membrane (Schmell and Lennarz, 1974).

How does one describe the electrostatic potential produced by monovalent negative lipids such as PS and PI? Specifically, how does the potential depend on distance from the surface of the membrane, salt concentration, and fraction of negative lipid in the membrane? Evidence from a number of different laboratories demonstrates that the Gouy-Chapman-Stern theory can adequately describe the potential produced by PS or phosphatidylglycerol (PG) in a bilayer membrane (McLaughlin, 1977, 1989; Cevc and Marsh, 1987). Here we extend these measurements to membranes containing the monovalent anionic lipid PI. Specifically, we measure the potential as a function of distance from the membrane using fluorescent probes located 0 and 1 nm from the surface, as described elsewhere (Winiski et al., 1988). We also report measurements on membranes containing PI or PS with new fluorescent probes located 2 nm from the surface. We use quenching measurements with fluorescent probes located at the surface to investigate how the potential depends on salt concentration in the aqueous phase and mole % PI in a membrane. We can summarize our results very simply: PI is electrostatically indistinguishable from PS or PG, and all our results can be described by the simplest form of the Gouy-Chapman-Stern theory.

In this theory the charge is assumed to be uniformly smeared over the surface. Measurements of the electrophoretic mobility (zeta potential) of a vesicle and of the surface potential above a monolayer give estimates of the average potential adjacent to a membrane. The Gouy-Chapman-Stern theory can describe these measurements. More surprisingly it can also describe the adsorption of ions to charged membranes containing PS or PG (Winiski et al., 1986; Hartsel and Cafiso, 1986). The simplest discreteness-of-charge theories (Nelson and McQuarrie, 1975) predict deviations from the Gouy-Chapman-Stern theory (Winiski et al., 1986). If one follows Nelson and McQuarrie (1975) and uses the linearized Poisson-Boltzmann equation (Debye-Hückel theory) to calculate the potential due to a single charged lipid, it is easy to show that the average of the potential over a surface containing many charged lipids is equal to the potential calculated from the Gouy-Chapman or smeared charge theory (Mathias et al., 1988). However, the adsorption of an ion depends not on the potential but on the exponent of the potential via the Boltzmann relation. As we discuss in more detail elsewhere (see Materials and Methods and Winiski et al., 1986), the average of the exponent of the potential over the surface of a membrane containing charged lipids is not equal to the exponent of the average potential. Thus, the Nelson-McQuarrie treatment predicts significant discreteness-of-charge effects, but none were observed with membranes containing monovalent lipids PS or PG (Winiski et al., 1986; Hartsel and Cafiso, 1986). Discreteness-of-charge theories predict that deviations from the Gouy-Chapman-Stern theory should be larger for trivalent than for monovalent lipids. Thus, we report here measurements with the monovalent lipid PI and with the trivalent lipid PIP_2 using techniques similar to those used previously to investigate PS and PG membranes (Winiski et al., 1986; Hartsel and Cafiso, 1986). In brief, we studied the effect of PI and PIP_2 on the adsorption of coions and counterions to the membrane.

We characterized a new fluorescent probe, a positive analogue of the negative probe TNS used previously (Winiski et al., 1986). Thus we have available a pair of positive and negative fluorescence probes and a pair of positive and negative spin label (EPR) probes characterized previously (Hartsel and Cafiso, 1986). Our results with both the fluorescence and EPR probes indicate that the electrostatic properties of membranes containing PIP_2, in contrast to membranes containing monovalent lipids, cannot be described by the Gouy-Chapman-Stern theory. We wanted to investigate theoretically the reasons for these deviations and to understand why no discreteness-of-charge effects were observed with monovalent lipids.

The Gouy-Chapman method is a consistent mean-field theory, where the interaction between the pairs of ions is replaced by the interaction of an ion in the diffuse double layer with the average electrostatic potential. Despite its success in describing average electrostatic potentials near membranes, the Gouy-Chapman theory is not suitable for studying discreteness-of-charge effects; this theory assumes the surface charges are uniformly smeared over the interface. Nelson and McQuarrie (1975) assumed the surface charges were fixed in a square array and used the linearized Poisson-Boltzmann equation or Debye-Hückel theory to calculate the potential. The Debye-Hückel theory is also a mean-field theory. In discreteness studies, however, ion pair interaction terms between the surface and the solution charges should be retained rather than approximated via the mean potential field. As soon as these explicit interaction terms are included in a theory, the finite size of the ions must also be included; otherwise, the interaction energy at contact becomes infinite. The inclusion of ion size effect is a formidable complication, which cannot be consistently resolved in a simple extension of the Poisson-Boltzmann formalism.

A fully quantitative model of the behavior of electrolytes near surfaces is still not feasible because of the

inherent difficulties in performing calculations that take into account the geometry of water molecules and hydrogen bonding. Nevertheless, the primitive models, where the aqueous solvent is treated as a dielectric continuum, provide a useful indication of where the Gouy-Chapman theory should and should not work. Accurate information about some typical problems within the primitive model is available from Monte Carlo simulations for uniformly charged surfaces, as reviewed elsewhere (Carnie and Torrie, 1984). Although we also treat only a primitive model of an electrolyte solution, we do consider the mobile discrete nature of the surface charges, the finite size of the ions, correlations between ions in the double layer, and image charge effects (Kjellander and Marcelja, 1985, 1986, 1988*a*).

MATERIALS AND METHODS

Materials

Egg phosphatidylcholine (PC), bovine brain PS, egg PG, and brain PI were obtained from Avanti Biochemicals (Birmingham, AL). The triamonium salt of L-α-PIP_2 was obtained from Calbiochem-Behring Corp. (La Jolla, CA). Egg *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE), egg *N*-(1-pyrenesulfonyl)-phosphatidylethanolamine (pyrene-PE), 2-(hexadecylamino)naphtalene-6-sulfonate (HNS), 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS), 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (tempamine), and 8-hydroxypyrene-1,3,6-trisulfonic acid oleate (HPTS-oleate) were obtained from Molecular Probes (Eugene, OR). One sample of 2-*p*-toluidinylnaphthalene-6-[*N*-b-aminoethyl]sulfonamide (TNAES) was a gift from Dr. F. C. Greene, Western Regional Research Laboratory (Berkeley, CA); another sample was synthesized by Molecular Probes. Gramicidin D was obtained from Sigma Chemical Co. (St. Louis, MO), 4-morpholinopropanesulfonic acid (MOPS) from Pharmacia P-L Biochemicals (Piscataway, NJ), neomycin sulfate from Boehringer Mannheim (Indianapolis, IN), and thallium(I) nitrate ($TlNO_3$) from Alfa Products (Danvers, MA). G_{M1} was labeled in the sialic acid residue (Winiski et al., 1988) or in the terminal galactose residue (Appendix 1) with anthraniloyl to measure the potential 1 and 2 nm from the surface of the membrane.

Preparation of unilamellar vesicles

The lipids were mixed in chloroform and evaporated to dryness (Toner et al., 1988). A few milliliters of the appropriate salt solutions were added and the suspension was vortexed to produce multilamellar vesicles, which were taken through five cycles of freezing and thawing, then forced through 0.1-μm polycarbonate filters in an Extruder (Lipex Biomembranes, Inc., Vancouver, BC) to produce unilamellar vesicles of ~100 nm diam (Hope et al., 1985; Mayer et al., 1985).

Fluoresence measurements

In one series of experiments, we measured the effect of water soluble, positively charged, monovalent quenchers (Thallium or tempamine) on fluorophores located 0 nm (NBD-PE, pyrene-PE, or HNS), 1 nm (sialic acid–labeled anthraniloyl-G_{M1}), or 2 nm (galactose-labeled anthraniloyl-G_{M1}) from the surface of membranes. We used the Stern-Volmer equation to calculate the concentration of quencher adjacent to the fluorophore, $[Q]_f$, from the fluorescence measurements

$$(F_0/F) - 1 = K_{sv}[Q]_f$$
$$[Q]_f = [Q] \exp(-e\psi_f/kT), \quad (1)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, and K_{sv} is the Stern-Volmer quenching constant (Lakowicz, 1983). A plot of $(F_0/F) - 1$ vs. $[Q]$ yielded a straight line, where $[Q]$ refers to the concentration of the quencher in the bulk aqueous phase. The ratio of the slopes of these lines for negative and neutral membranes is equal to the Boltzmann factor, $\exp(-e\psi_f/kT)$, when the quencher is a monovalent cation. ψ_f is the difference between the electrostatic potentials the fluorophore experiences in a charged and a neutral membrane. Gramicidin D (~0.5 mol%) in the vesicles allowed Tl to diffuse across the membranes. Thallium and tempamine were added from stock aqueous solutions of $TlNO_3$ or tempamine acetate. We obtained data in the region where the fluorescence quenching (the left-hand side of Eq. 1) varied linearly with the concentration of quencher in the aqueous phase. For 0.1 M salt the quenching was linear for thallium and tempamine up to 5 and 2 mM, respectively, for the negative PI and PS membranes and up to 10 mM for PC membranes. Higher concentrations of these cations change the surface potential. The data presented in Figs. 4 and 5 for quenching experiments represent average values for different probes and quenchers.

We describe elsewhere how the monovalent anion TNS can be used to estimate the electrostatic potential at the surface of phospholipid bilayers (Eisenberg et al., 1979; McDaniel et al., 1984; Winiski et al., 1986; Cafiso et al., 1989). In brief, the amphipathic TNS molecules adsorb to membranes because of "hydrophobic" interactions, the fluorescence they produce increases markedly when they adsorb, and we detect the number of adsorbed TNS molecules by measuring the fluorescence. We also used a monovalent cationic analogue of TNS, TNAES (Greene, 1975), as a fluorescence probe. The structures of TNS and TNAES are illustrated in Fig. 1. These probes were added to aqueous solutions containing extruded unilamellar vesicles and either 0.01 or 0.1 M KCl buffered to pH 7.4 with either 0.5 or 5 mM MOPS at 25°C. The net fluorescence intensity was proportional to the number of probes adsorbed to the membrane. Steady-state fluorescence measurements were made with a Fluorocomp (Spex Ind., Inc., Edison, NJ) fluorometer. Fluorescence lifetime measurements were made with a nanosecond fluorometer system (Model 9200; ORTEC, Oak Ridge, TN). The excitation light was a self-flashing spark gap in continuously flowing nitrogen. The light was filtered through 7-39 and 3-73 filters (Corning Glass Works, Corning, NY).

The simplest discreteness-of-charge theory (Nelson and McQuarrie,

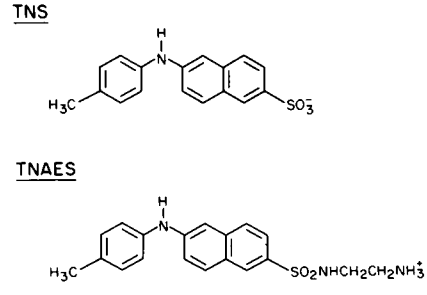


FIGURE 1 The negatively charged probe 2-*p*-toluidinylnaphthalene-6-sulfonate, TNS, and positively charged *N*-(β-aminoethyl)sulfonamido derivative, TNAES, used to estimate surface potentials from fluorescence measurements.

1975) predicts that the ratio of probe molecules adsorbed to a charged surface to those adsorbed to a neutral surface (e.g., a PC bilayer) should be equal to the average of the Boltzmann factor, $\langle \exp(-Ze\psi(0, y, z)/kT) \rangle$, over the surface where Z is the valence of the probe, and $\psi(0, y, z)$ is the potential at any y, z point on the surface of the membrane, which is located at $x = 0$. We can define $(-kT/Ze) \ln \langle\ \rangle$ as the effective potential sensed by the probe. The Nelson-McQuarrie theory, for example, predicts that the magnitude of the effective potential sensed by the counterion should be larger than the magnitude of the effective potential sensed by the coion. Thus, as discussed in more detail in Winiski et al. (1986) for TNS and in Appendix 2 for TNAES, these fluorescent probes (and the matched pair of positive and negative EPR probes described in the next section) can be used to investigate discreteness-of-charge effects.

EPR measurements

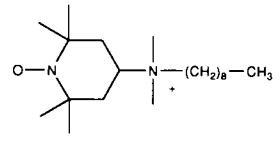
The positively charged alkylammonium nitroxide I (Fig. 2) was synthesized using a procedure described by Castle and Hubbell (1976) and the negatively charged alkylsulfonate probe II (Fig. 2) was synthesized as described by Hartsel and Cafiso (1986).

EPR spectra were obtained using an E-Line Century Series X-band (Varian Associates, Inc., Palo Alto, CA) spectrometer. All spectra were obtained from 100 μl of the membrane sample in quartz flat cells at a label concentration of 20 μM. The modulation field and incident microwave power were 1 G peak-to-peak and 10 mW, respectively. The unilamellar vesicles examined here were formed by extrusion of lipid through 0.08-μm Nucleopore Corp. (Pleasanton, CA) filters using the procedure described above.

We describe elsewhere the procedure for deducing electrostatic surface potentials from the EPR spectra of probes I and II (Hartsel and Cafiso, 1986). In brief, we determine the membrane/aqueous partitioning (λ) of the charged paramagnetic amphiphile from its EPR spectrum. The amplitude of the high-field resonance of the EPR spectrum is a function of the aqueous concentration of spin-label (Castle and Hubbell, 1976). The membrane/aqueous partition coefficient of the probe (λ) is then determined from the amplitude of the high field resonance and the total spin concentration. In the measurements reported here we determined λ at four to eight lipid concentrations. A plot of λ^{-1} vs. V_l/m_ℓ, where V_l/m_ℓ is the total volume per unit mass of lipid in the sample, yielded a straight line with a slope β (Hartsel and Cafiso, 1986). The surface potential was calculated using the following equation:

$$\psi_o = -[kT/(Ze)] \ln (\beta/\beta_o), \qquad (2)$$

I



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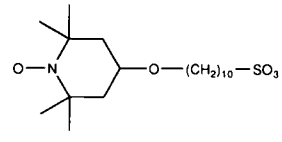

FIGURE 2 The positively charged alkylammonium nitroxide probe I and the negatively charged alkylsulfonate probe II used to estimate surface potentials from EPR measurements.

where β_o is the slope of the binding curve for neutral vesicles formed from egg PC and Z is the valence of the probe.

Theoretical methods

The electrostatic calculations were performed using the primitive model of electrolyte solutions, where ions are represented as charged hard spheres and solvent is represented as a dielectric continuum. (The dielectric constants of the spheres and solvent are identical.) The solutions for the electrostatic potential were obtained from the hypernetted chain approximation applied to the ionic fluid between two planar surfaces, using the method of Kjellander and Marcelja (1985). The hypernetted chain approximation is known to be very accurate for particles interacting via Coulomb interaction (Baus and Hansen, 1980).

Our computational procedure evaluates the properties of a system consisting of an electrolyte solution placed between two impenetrable surfaces. The electrolyte is allowed to equilibrate with an infinite bulk reservoir. In modeling the present system, the two parallel membrane surfaces were set 10 nm apart. In 0.1 M monovalent electrolyte solutions this separation corresponds to 10 Debye lengths and the interaction between the two surfaces is negligible. The surface charges associated with each membrane were all placed in a plane a given distance from the membrane surface, as illustrated in Fig. 3. They were assigned a valence and hard-sphere radius, and were free to move in lateral directions.

Two calculations of the potential as a function of distance from a membrane surface were routinely evaluated. First, the average electrostatic potential at a given distance from a membrane surface was evaluated from Poisson's equation using the average calculated densities of counterions and coions a given distance from the membrane. When the charges associated with the membrane are at the surface, the potential calculated from this procedure agreed very closely with the corresponding potential calculated from the Gouy-Chapman theory, which ignores finite size and correlation effects (results not shown). Second, the average "effective" potentials a distance x from the

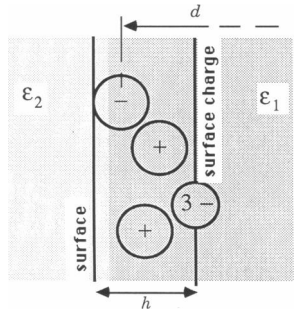

FIGURE 3 A sketch of the model system used for the theoretical calculations. Discrete surface charges are allowed to take any position on a plane located a distance h from the interface. The surface charge groups (*marked* 3−), counterions (*marked* +), and coions (*marked* −) are all modeled as hard spheres moving in a dielectric continuum. The dielectric constant of the membrane interior, ϵ_2, was either the same (no image effects) or smaller than its value in the bulk aqueous solution, $\epsilon_1 = 78.4$. The position of the dielectric boundary could be varied, as indicated by a change in the background shading, but for most of our calculations we assumed it was located at the membrane-solution interface. In presenting the results of the theoretical calculations in Figs. 8–10, the distance from the surface, d, is measured from the point of closest approach of the ion centers to the surface.

membrane were calculated from densities of monovalent cations or anions a distance x from the membrane. In other words, if the calculated density of cations a distance x from the membrane surface was $n(x)$, the average effective potential, $\psi_e(x)$, was defined as:

$$\psi_e(x) = -(kT/e) \ln [n(x)/n(bulk)]. \quad (3)$$

We define the effective potential simply to express the concentrations of the counterions and coions in units of electrostatic potential. Within the framework of the Gouy-Chapman theory (or more general Poisson-Boltzmann equation when the charges are not at the surface), both the effective potentials for the anion and the cation are equal to the average electrostatic potential. In a discreteness-of-charge theory, however, the average density of ions is no longer directly linked to the average electrostatic potential (see section above on Fluorescence Measurements). As a result, the effective potentials estimated from the counterion and coion densities using Eq. 3 are no longer equal. In simpler theories (e.g., Nelson and McQuarrie, 1975), this breakdown follows from averaging of the nonlinear Boltzmann relationship between the local ion density and the local electrostatic potential.

If a theory includes ion–ion correlations and the finite size of ions, the breakdown is more fundamental. Even at the local level, there is no simple relationship between the time-averaged electrostatic potential and the ion density. The local effective potential experienced by any individual ion depends on the spatial distribution of its discrete neighbors. As hard core radii of ions also influence the density, the origin of the effective potential is not purely electrostatic.

RESULTS

Fig. 4 illustrates how the electrostatic potential depends on the distance from the surface of bilayer membranes.

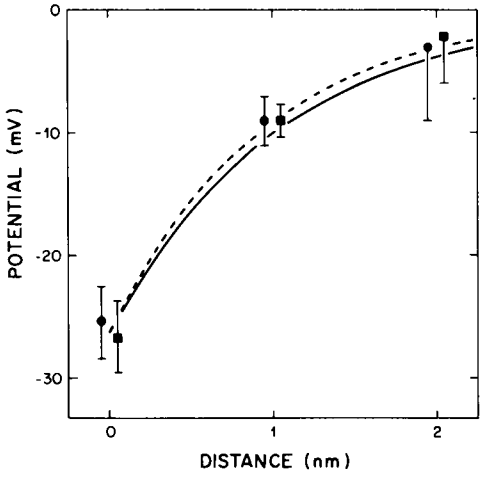

FIGURE 4 The profile of the electrostatic potential in the aqueous phase adjacent to membranes formed from 5:1 PC/PI (*circles*) and 5:1 PC/PS (*squares*). The potentials were determined by quenching the fluorescence of probes located 0, 1, or 2 nm from the surface. The aqueous solutions contained 0.1 M KNO_3, buffered to pH 7.4 at 25°C with 5 mM MOPS. The two curves illustrate the predictions of the linear (Eq. 5, *solid curve*) and nonlinear (Eq. 4, *dashed curve*) Gouy-Chapman theory. The surface potential was chosen to fit the experimental points.

We formed the membranes by mixing zwitterionic lipid, PC, with 17 mol% monovalent anionic lipid, either PI or PS; many biological membranes contain a similar fraction of negative lipids. We obtained six different estimates of the surface potential by quenching the fluorescence of three membrane-bound probes, pyrene-PE, NBD-PE, or HNS, with the cations tempamine or thallium (Winiski et al., 1988; Langner et al., 1988). The circle (PC/PI) and square (PC/PS) at 0 nm distance in Fig. 4 represent the average value (±SD) of these six estimates. Strictly speaking, we measured not the potential but the fluorescence quenching, which is proportional to the concentration of the thallium and tempamine counterions adjacent to the fluorophore; we infer the electrostatic potential from the Boltzmann relation, Eq. 1. We estimated the potential 1 nm from the membrane surface by quenching the fluorescence of anthraniloyl-G_{M1} with thallium and tempamine; in this case we labeled the sialic acid residue of G_{M1}, which is located 1 nm from the membrane surface (McDaniel et al., 1986). We estimated the potential 2 nm from the membrane surface by quenching the fluorescence of galactose-labeled anthraniloyl-G_{M1} with thallium and tempamine; the terminal galactose is located ~2 nm from the membrane surface (McDaniel et al., 1986).

The Gouy-Chapman theory predicts how the potential, $\psi(x)$, should depend on distance, x, from a charged surface (e.g., Aveyard and Haydon, 1973; McLaughlin, 1977) in a monovalent electrolyte solution:

$$\psi(x) = \frac{2kT}{e} \ln \frac{[1 + \alpha \exp(-\kappa x)]}{[1 - \alpha \exp(-\kappa x)]}, \quad (4)$$

where

$$\alpha = \frac{\exp[e\psi(0)/2kT] - 1}{\exp[e\psi(0)/2kT] + 1}.$$

T is the absolute temperature, k is the Boltzmann constant, e is the magnitude of the electronic charge, and $1/\kappa$ is the Debye length. When the magnitude of the surface potential is $< kT/e \simeq 25$ mV, we can approximate the general expression, Eq. 4, by:

$$\psi(x) = \psi(0) \exp(-\kappa x). \quad (5)$$

Fig. 4 shows the agreement between the Gouy-Chapman theory and the experimental data is surprisingly good.

The Gouy-Chapman theory, Eq. 4, predicts the potential should fall more rapidly with distance than illustrated in Fig. 4 if the magnitude of the surface potential is $\gg kT/e \simeq 25$ mV. We tested this prediction by measuring the dependence of potential on distance for PS and PI membranes in 0.1 M salt solutions (see Fig. 5). As discussed in the next paragraph, the curves in Fig. 5 illustrate the predictions of the Gouy-Chapman-Stern theory.

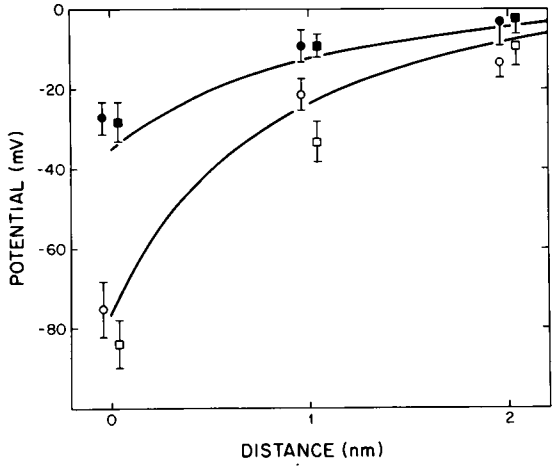

FIGURE 5 The profile of the electrostatic potential in the aqueous phase adjacent to membranes formed from PI (*open circles*) or PS (*open squares*). Solution (0.1 M salt) and techniques as in Fig. 4. The data illustrated in Fig. 4 are reproduced in Fig. 5 for comparison with a different theoretical curve: the closed circles represent 5:1 PC/PI and the closed squares represent 5:1 PC/PS vesicles. The curves are the predictions of the Gouy-Chapman-Stern theory, a combination of Eqs. 4, 6, and 7, with the instrinsic association constant of potassium ions with the negative lipids assumed to be 1 M^{-1} in all cases.

The Gouy-Chapman theory predicts not only how the potential should depend on distance, Eq. 4, but also how the surface potential, $\psi(0)$, should depend on surface charge density, σ, and monovalent salt concentration, c:

$$\sinh [e\psi(0)/2kT] = A\sigma/(c)^{1/2}, \quad (6)$$

where $A = (8N\epsilon_r\epsilon_0 kT)^{-1/2}$, N is Avogadro's number, ϵ_r the dielectric constant, and ϵ_0 the permittivity of free space. Eq. 6 overestimates the magnitude of the surface potential produced by the negative lipids PS, PI, or PG when the membranes are formed in NaCl or KCl solutions if one assumes that the surface charge density, σ, is equal to the surface concentration of negative phospholipid, $\{P\}$: $\sigma = -e\{P\}$ (for references see Eisenberg et al., 1979 or Winiski et al., 1986). The simplest interpretation is that these alkali metal cations bind to the negative lipids. The simplest theoretical approach is to follow Stern and combine a Langmuir adsorption isotherm with the Boltzmann relation:

$$\sigma = -e\{P\}/\{1 + Kc \exp [-e\psi(0)/kT]\}, \quad (7)$$

where K is an intrinsic association constant. Eqs. 4, 6, and 7 were combined to produce the curves illustrated in Fig. 5. The intrinsic association constant of the potassium ions with the negative lipid was assumed to be 1 M^{-1}. This simple Gouy-Chapman-Stern theory can describe qualitatively the experimental data illustrated here and the data obtained in 0.01 M salt.[1]

We now consider the obvious point that the charges on these lipids are not smeared uniformly over the surface of the membrane, as required by the Gouy-Chapman theory. For example, the average distance between the charged PI lipids in a 5:1 PC/PI membrane is ~2 nm, twice the Debye length of $1/\kappa$ = 1 nm in a 0.1 M salt solution. Simple extensions of the theory that take into account the discrete nature of the surface charges (Nelson and McQuarrie, 1975) predict that counterions and coions should sense different "effective" surface potentials (see Eq. 3) when they adsorb to the surface (Winiski et al., 1986).

Fig. 6 *A* illustrates how the adsorption of anionic (TNS) and cationic (TNAES) probes depends on the mol% PS in PC bilayer membranes. The electrostatic potential produced by the monovalent anionic PS molecules increases the concentration of cationic probe and decreases the concentration of anionic probe in the aqueous phase near the membrane surface. The number of probe molecules adsorbed to the membrane is proportional to the concentration in the aqueous phase immediately adjacent to the membrane surface. The fluorescence is proportional to the number of adsorbed probe molecules. It is apparent from Fig. 6 *A* that PS has a symmetrical effect on the adsorption of the positive and negative probes. We applied the same approach to PC/PI membranes and again observed a symmetrical effect of the negative lipid on the fluorescence produced by the positive and negative probes (Fig. 6 *B*). Moreover, both PS and PI produce the same change in the fluorescence. The curves in Fig. 6, *A* and *B* illustrate the predictions of the Gouy-Chapman-Stern theory, Eqs. 6 and 7, with the intrinsic association constant of potassium ions with the negative lipids equal to 1 M^{-1}. The same theory and association constant were used to describe the data obtained from quenching measurements that are illustrated in Fig. 5, as well as the data obtained from zeta potential, conductance, ^{31}P NMR, and EPR measurements on PC/PS vesicles in NaCl solutions (Winiski et al., 1986; Hartsel and Cafiso, 1986). The data obtained with the TNS and TNAES probes can be adequately described by this theory and there is no need to invoke discreteness-of-

[1]The theory predicts that the dependence of potential on distance should be a function of salt concentration: the Debye length, $1/\kappa$, is ~1 nm in 0.1 M salt and 3 nm in 0.01 M salt. We tested this prediction by measuring the dependence of potential on distance for PS and PI membranes in 0.01 M KCl. The results at 0 and 1 nm distance from the membrane for PI and PS are very similar to those reported previously for PS and PG in 0.01 M NaCl (Winiski et al., 1988). The results at 2 nm distance (−43 ± 7 mV for PS; −37 ± 7 mV for PI) agree qualitatively with the predictions of the Gouy-Chapman-Stern theory (−50 mV if K in Eq. 7 is 1 M^{-1}).

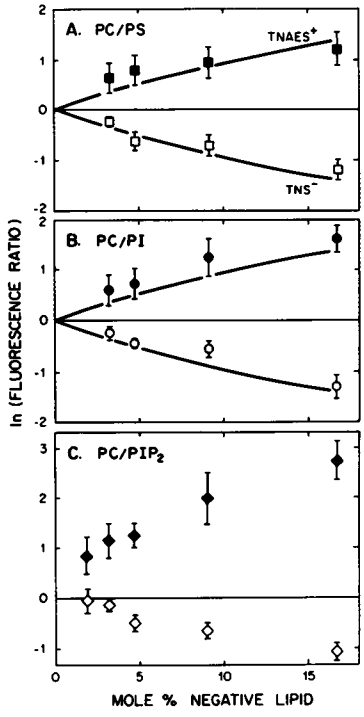

FIGURE 6 The natural logarithm of the normalized fluorescence due to the anionic probe TNS (*open symbols*) or the cationic probe TNAES (*filled symbols*) plotted as a function of the mole % negative lipid in the unilamellar PC vesicles. The negative lipids were (*A*), PS; (*B*), PI; (*C*), PIP_2. The fluorescence is normalized to the fluorescence produced by the same concentration of probes exposed to the same concentration of PC vesicles. The aqueous solutions contained 0.1 M KCl buffered to pH 7.4 with 5 mM MOPS at 25°C. The potential sensed by these probes can be obtained from the Boltzmann relation by multiplying the numbers on the ordinate by $-kT/e \simeq -25$ mV for the cation TNAES and by $+kT/e \simeq +25$ mV for the anion TNS. The curves in Fig. 6, *A* and *B* illustrate the prediction of the Gouy-Chapman-Stern theory if we assume the intrinsic association constant of potassium ions with the negative lipids is 1 M^{-1}.

charge effects to account for the results in Fig. 6, *A* and *B*.

We also used the TNS and TNAES probes to study membranes formed from mixtures of PC and PIP_2 (Fig. 6 *C*). PIP_2 has three negative charges at pH 7 in 0.1 M KCl (Toner et al., 1988). PIP_2 has a larger effect on the cationic probe, TNAES, than on the anionic probe, TNS. (We also observed an asymmetry in 0.01 M KCl; TNS sensed a potential of -3 mV and TNAES a potential of -31 mV for a 50:1 PC/PIP_2 membrane.) The potential sensed by the positively charged TNAES probe in the experiments illustrated in Fig. 6 *C* agrees with the potential determined from independent zeta and surface potential measurements. For example, the zeta potential of a 5:1 PC/PIP_2 bilayer vesicle formed in 0.1 M KCl is ~ -60 mV and the surface potential measured with an ionizing electrode above a 5:1 PC/PIP_2 monolayer (relative to a PC monolayer) is ~ -50 mV (Gabev et al., 1989). The surface potential sensed by the TNAES probe for a membrane with 17 mol % PIP_2 is ~ -65 mV, whereas the TNS probe senses only ~ -25 mV for membranes of this composition (Fig. 6 *C*). Thus it is the adsorption of coions to membranes containing the trivalent PIP_2 molecule that is anomalous. To check that the results illustrated in Fig. 6 *C* were not due to an artifact involving the fluorescent probes, we made similar measurements with the matched pair of EPR probes illustrated in Fig. 2. Symmetrical results, similar to those illustrated in Fig. 6 *A*, were reported elsewhere for PC/PS membranes using these EPR probes (Hartsel and Cafiso, 1986). Fig. 7 illustrates that the cationic EPR probe, I, senses a more negative potential than the anionic EPR probe, II, when the membrane contains PIP_2. (This asymmetry is also observed at lower ionic strengths. For example, in 0.01 M NaCl at 5 mol % PIP_2 probe I measures a potential of -70 mV and probe II a potential of -35 mV.) Thus, similar results are obtained with both fluorescence (Fig. 6 *C*) and EPR (Fig. 7) probes.

We wanted to know if the asymmetries seen in Figs. 6 *C* and 7 were due to a charge distribution that was unique to PIP_2 or were due to a general electrostatic phenomena. We looked for and observed similar asymmetries with membranes formed with different polyvalent fixed charges.[2]

We can summarize succinctly all the experimental results we obtained with monovalent lipids. The Gouy-Chapman theory (Eqs. 4 and 6), as modified by Stern to include ion adsorption (Eq. 7), can describe how the electrostatic potential depends on mole % negative lipid, salt concentration, and distance from membranes formed from a mixture of a zwitterionic lipid with the monovalent anionic lipids PS, PI, or PG (see McLaughlin, 1977 and

[2]For example, we measured the surface potential of membranes formed from a mixture of egg PC and 5 mol % HPTS-oleate, a polyvalent anionic lipid analogue. In 0.1 M monovalent salt, the cationic EPR probe (I, Fig. 2) reported a potential of -52 mV, whereas the anionic EPR probe reported a potential of only -27 mV. We could not easily interpret measurements made with our fluorescence probes on these membranes because HPTS-oleate is itself fluorescent. However, we used TNS and TNAES to investigate membranes that contained polyvalent cationic groups. These membranes were formed by exposing PC/PI membranes to neomycin, which has +4.5 charges at pH 7. Neomycin does not bind to PC, but it does bind to PI (McLaughlin and Whitaker, 1988). Neomycin affects neither the TNS nor TNAES fluorescence of PC membranes, a control that illustrates the drug does not affect the probes. However, exposure of 5:1 PC/PI vesicles to 10 mM neomycin, a concentration that changes the zeta potential from -30 mV to -5 mV (McLaughlin and Whitaker, 1988), increases the TNS fluorescence 30% but decreases the TNAES fluorescence by only 3%. Thus, the asymmetrical responses seen in Figs. 6 *C* and 7 with the polyvalent anion PIP_2 can also be observed with other polyvalent anions or when some of the fixed charges are polyvalent cations.

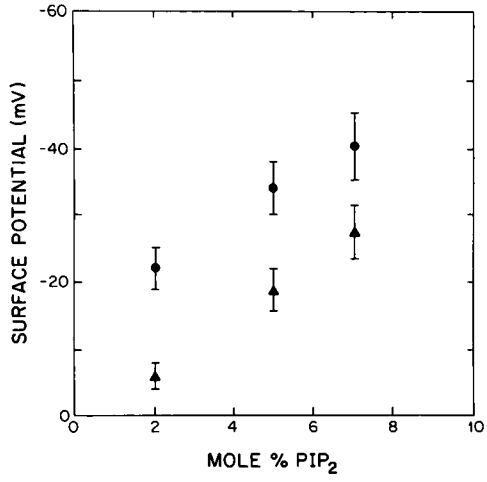

FIGURE 7 Surface potentials measured using the alkylammonium probe I (*circles*) or the alkylsulfonate probe II (*triangles*) as a function of the mol% PIP_2 incorporated into egg PC vesicles. The unilamellar vesicles were formed in 0.1 M NaCl buffered to pH 7.2 with 5 mM MOPS. The potentials were calculated using Eq. 2.

1989 for references to other work on these phospholipid membranes). Furthermore, no discreteness-of-charge effects are observed when one studies the adsorption of monovalent anions or cations to these membranes (Hartsel and Cafiso, 1986; Winiski et al., 1986; Fig. 6, *A* and *B*).

Why does the simple theory work so well for membranes formed with monovalent lipids? After all, the surface charges are not smeared uniformly over the surface; in the experiments illustrated in Fig. 6 they are further apart than the Debye length. Furthermore, the ions in the aqueous phase are not point charges, they must interact with each other electrostatically (correlation effects), and image charge effects should be considered. Finally, we would like to understand why the deviations from the smeared charge theory observed with the polyvalent lipid PIP_2 involve coions rather than counterions because simple discreteness-of-charge theories predict opposite effects.

We attempted to answer these questions by examining the solutions for the structure of the primitive model monovalent electrolytes near surfaces bearing mobile discrete charged groups. Fig. 3 illustrates the geometry of the model system. We have evaluated the counterion and the coion density profiles and the average electrostatic potential near a surface for a number of surface configurations, surface charge densities, and electrolyte concentrations. To present the evaluated counter- and coion profiles, we use the Boltzmann relation to calculate an effective electrostatic potential sensed by the ions (see Eq. 3).

Fig. 8 shows the effective potentials obtained from the counterion and the coion densities near a surface as the valence of the surface charges is increased from 1 to 3. The two effective potentials are almost equal when the surface charges are monovalent. For trivalent surface charges, the effective potential at the membrane surface evaluated from the coion density is less negative than the effective potential evaluated from the counterion density. A similar effect was observed when the surface charge density was doubled to 0.064 Cm^{-2}. Fig. 9 illustrates the effect of varying the distance between the surface charge groups and the membrane. There is less difference between the counterion and coion profiles when the

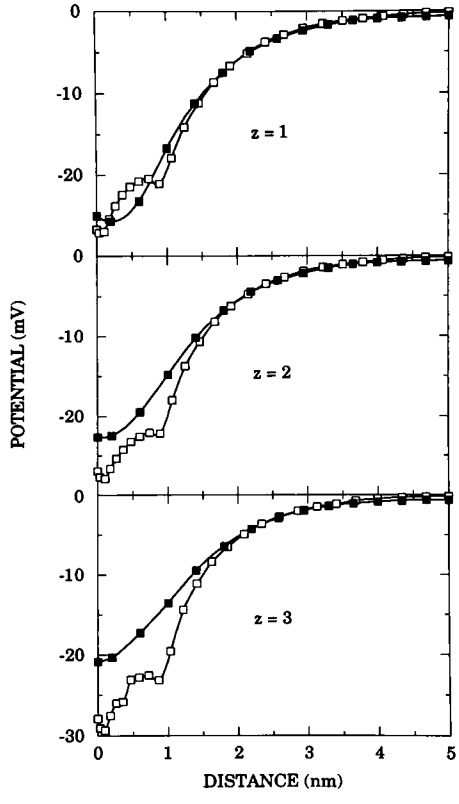

FIGURE 8 The effective potentials calculated from the predicted counter- and coion concentrations by applying Eq. 3, the Boltzmann relation, when discrete surface charge groups were placed at $d = 0.5$ nm and the salt concentration is 0.1 M. As illustrated in Fig. 3, d is measured from the point of closest approach of the counter- and coion centers to the surface. The radii of the counter- and coiions was 0.23 nm, hence the distance of the surface charges from the interface was $h = 0.73$ nm (Fig. 3). The surface groups were modeled as hard spheres of radii 0.2 nm. When the valence of the surface charges is increased from $z = 1$ to $z = 3$ while the surface charge density (0.032 Cm^{-2} or one elementary charge per 5 nm^2) is maintained a constant, the effective potentials evaluated from the coion (*filled symbols*) and counterion density (*open symbols*) become progressively separated. In this and the following figure, there are no dielectric images: $\epsilon_1 = \epsilon_2$ in Fig. 3. The curves in this figure were drawn through the calculated points merely to guide the eye.

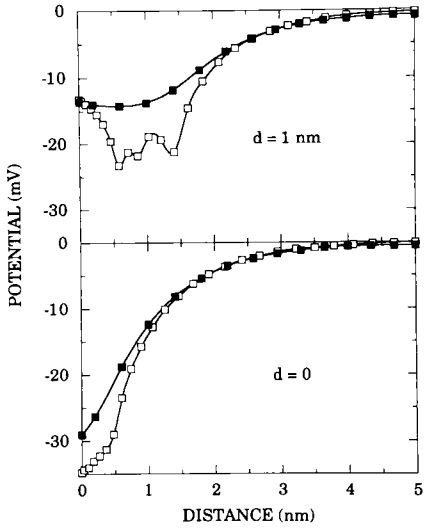

FIGURE 9 Effect of changing the distance between the trivalent surface charge groups and the surface. The surface charges were placed at $d = 1$ nm and $d = 0$ nm, and the effective potentials were calculated from the coion (*filled symbols*) and counterion density (*open symbols*). In this and the following figure, the surface charge density is identical to the value used in Fig. 8.

charges are at the membrane surface (Fig. 9, *bottom*) than when they are 0.5 nm from the surface (Fig. 8, *bottom*).

We have also evaluated the average electrostatic potential for the examples discussed above. In each case the average electrostatic potential is correctly described by the simplest form of the Poisson-Boltzmann equation (which reduces to the Gouy-Chapman theory when the charges are at the surface), the deviation being only a few millivolts overall shift to smaller values. To relate these theoretical calculations to the experiments, we note that this average potential is the potential that should be sensed by an ionizing electrode above a monolayer or by electrophoretic mobility measurements (zeta potential). The effective potentials illustrated in Figs. 8 and 9 are the potentials that should be sensed by the counterion and coion probes that we used.

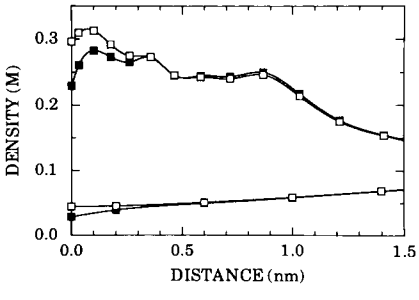

FIGURE 10 Effect of dielectric images on the counterion (*upper curves*) and coion (*lower curves*) density profiles. The dielectric constant of the membrane was lowered from 78.4 (*open symbols*) to 2 (*filled symbols*). Trivalent discrete surface charges were placed at $d = 0.5$ nm, as in Fig. 8. The dielectric boundary was chosen to coincide with the membrane–solution interface.

Fig. 10 illustrates the effect of the dielectric boundary on the ion density profiles. The effect of image charges is rather short-ranged, extending only a few tenths of a nanometer from the boundary. A more realistic choice would be to set the dielectric boundary some 0.5 nm inside the polar head group region. In that case, the image effect on the ion density distribution is too small to show in the figure. Although image charge effects do not significantly affect the average potential adjacent to a membrane, they should have a large effect on the potential adjacent to a single fixed charge on a membrane or protein.[3]

DISCUSSION

Experiment

The experimental results illustrated in Figs. 4, 5, and 6 demonstrate that PI is electrostatically equivalent to other monovalent phospholipids, such as PS. They also demonstrate that the electrostatic potential adjacent to membranes formed from a mixture of the zwitterionic lipid PC and the anionic lipid PI can be described by the Gouy-Chapman-Stern theory.

Consider first the agreement between the theoretically predicted and experimentally observed dependence of

[3]In the linearized Poisson-Boltzmann or Debye-Hückel model used by Nelson and McQuarrie (1975), the dielectric constant of the membrane phase (image charges) has a significant (factor of two) influence on the local electrostatic potential or "micropotential" adjacent to a given fixed charge but it is easy to show that it does not affect the average electrostatic potential at the surface of the membrane, $\langle\psi(0, y, z)\rangle$ (Mathias et al., 1988). Specifically, the potential in the aqueous phase due to a single fixed charge at the interface of a low dielectric membrane is twice the Debye-Hückel expression for the potential adjacent to a charge in the bulk aqueous phase. The equipotential profiles in the aqueous phase have hemispherical symmetry about the fixed interfacial charge. If the dielectric constant of the ion-impermeable membrane phase is raised until it has a value equal to that of the aqueous phase (no image charges), however, the equipotential profiles lose their hemispherical symmetry, and the potential close ($r \ll 1/\kappa$) to the fixed charge falls by a factor of 2 and becomes identical to the Coulomb's law potential close to a charge in the bulk aqueous phase. However, the average potential in the aqueous phase adjacent to the membrane is independent of the dielectric constant of the membrane and is equal to the potential calculated from assuming the charges are smeared uniformly over the surface (i.e., Gouy-Chapman potential). Thus, the Nelson-McQuarrie model and the model we discuss both predict that image charge effects have little influence on the average electrostatic potential adjacent to a membrane. In the Gouy-Chapman theory image charge effects are absent by definition; the situation is analogous to a parallel plate capacitor where the potential between the plates is independent of the dielectric constant of the media outside the plates.

potential on distance illustrated in Figs. 4 and 5. The results we report here (for PI, PC/PI, PS, and PC/PS membranes) and elsewhere (for PG and PC/PG membranes) using fluorescence probes (Winiski et al., 1988; Langner et al., 1988) agree with the results obtained by others using different techniques. Measurements of the force and distance between charged bilayers with a surface force apparatus (Marra and Israelachvili, 1985; Marra, 1986; Pashley et al., 1986) and x-ray diffraction measurements of the distance between charged bilayers upon application of a known force (Loosley-Milman et al., 1982; Evans and Parsegian, 1986) both demonstrate that for separation distances >2 nm the Gouy-Chapman theory, Eq. 4, describes the data very well. Neutron diffraction estimates of the concentration profile of deuteriated tetramethylammonium ions between negatively charged PG lipid bilayers also support the predictions of the Gouy-Chapman theory (Hentschel et al., 1985). Thus, all the available evidence suggests the Gouy-Chapman theory correctly describes the dependence of potential on distance from a bilayer membrane when the surface charges are on the monovalent anionic lipids PS, PG, or PI and the aqueous solution contains only monovalent ions. The theoretical calculations we discuss below help us understand why the simple theory works so well.

The surface potentials produced by mixing either PS or PI with PC are identical (Figs. 4, 5, 6), and no discreteness-of-charge effects are seen with either of these monovalent lipids (Fig. 6; Winiski et al., 1986; Hartsel and Cafiso, 1986). In other words, coions and counterions experience the same effective potentials (Eq. 3) when they adsorb to the membranes (Fig. 6). Furthermore, the dependence of surface potential (Fig. 6) or zeta potential (Winiski et al., 1986) on mole % monovalent anionic lipid can be described by the simplest form of the Gouy-Chapman-Stern theory, which assumes the charges are smeared uniformly over the surface. The intrinsic association constant of sodium or potassium ions with these monovalent lipids is of order 1 M^{-1} (Figs. 5, 6).

In contrast, when the fixed charges are on a polyvalent lipid, such as PIP_2, the effective potential (Eq. 3) sensed by the coion probes (Figs. 6 *C*, 7) is not as negative as either the effective potential sensed by the counterion probes (Figs. 6 *C*, 7) or the zeta potential (Toner et al., 1988; Gabev et al., 1989). Thus the Gouy-Chapman-Stern theory cannot be used to describe the adsorption of coions to the surface of these PC/PIP_2 membranes. The theoretical model we explored can account for this experimental result.

Theory

Ion size and correlation effects are ignored in the Gouy-Chapman theory. In the past decade, several groups performed Monte Carlo simulations of a primitive model electrolyte next to a uniformly charged surface (e.g., van Megen and Snook, 1980; Torrie and Valleau, 1980). These simulations demonstrated that ion size and correlation effects are not very important for surface charge densities characteristic of biological membranes ($\sigma < 1$ electronic charge/nm^2) and salt concentrations typical of biological solutions (<0.2 M), provided the counterions are monovalent. After reviewing these studies, Carnie and Torrie (1984) concluded that "for all its lack of sophistication, the modified Gouy-Chapman theory does as well as far more elaborate theories for a significant range of concentrations and surface charge." The hypernetted chain (HNC) theory gave practically the same results as the Monte Carlo computer "experiments" on this same model. The HNC theoretical results reported here and elsewhere (Kjellander and Marcelja, 1988*b*) extend these calculations to the case where the surface charges are not smeared uniformly over the surface but are discrete mobile entities, as illustrated in Fig. 3.

In HNC results, changing from continuous to discrete monovalent surface charges has only a minimal effect on the calculated average ion density or electrostatic potential. In such cases, the reason for the lack of discreteness effects is the same as the reason for the success of the Gouy-Chapman description of such systems: in situations of weak ion-ion coupling, it is permissible to neglect the discrete structure of neighboring ions, whether they be in the electrolyte or attached to the surface. Replacing discrete surface charges or discrete electrolyte charges by the average electrostatic potential involves the same level of approximation.

The same is not true when the valency of surface charges is higher than one. The effect of correlation in the positions between the surface group and the electrolyte ions is then important, leading to a very strong screening of the multivalent surface group. As a result, coions are not as strongly excluded from the immediate vicinity as would be expected on the basis of the mean-field treatment. In other words, the average coion density is higher than suggested by the average value of the electrostatic potential, and the absolute value of the effective potential obtained from the coion density by using Eq. 3 is smaller (cf. Fig. 9).

A Monte Carlo simulation study of a charged surface screened by 2:1 and 2:2 electrolytes (Torrie and Valleau, 1982) is relevant for qualitative understanding of our results. In their examples, electrostatic coupling was stronger than in the present study, and the coion concentration some two diameters away from the surface was actually higher than the corresponding bulk value.

Theoretical studies by Vorotyntsev and his colleagues (Vorotyntsev and Ivanov, 1985; Vorotyntsev, 1988) provide insight into the statistical mechanics of ions adsorbed to a surface.

Biological importance

The Poisson-Boltzmann equation is widely used to calculate electrostatic potentials adjacent to fixed charges on proteins in solution (Honig et al., 1986), channels in membranes (Jordan, 1987), and lipids in bilayers (McLaughlin, 1977, 1989). Our experimental results and theoretical calculations sanction the continued application of Gouy-Chapman-Stern theory (one dimensional Poisson-Boltzmann equation) to phospholipid bilayer membranes, provided the fixed charges are on monovalent lipids. This is usually the case. For example, the trivalent lipid PIP_2 comprises a minor fraction of the charged lipids in a biological membrane. Most of the charges are on monovalent lipids, such as PS, PG, or PI.

The theoretical results we report here provide an explanation for the curious observation that the Gouy-Chapman-Stern theory, which incorrectly assumes the charges are smeared uniformly over the surface, provides a better description of the adsorption of counter- and coions to bilayer membranes (Winiski et al., 1986; Hartsel and Cafiso, 1986; Fig. 6, this paper) than does a simple discreteness-of-charge theory (Nelson and McQuarrie, 1975). Nelson and McQuarrie (1975) used the three-dimensional Poisson-Boltzmann equation and made all the conventional assumptions in Debye-Hückel theory. The assumption that ions are point charges introduces little error in the smeared charge Gouy-Chapman theory, but does cause the concentration of counterions adjacent to the fixed charges to be grossly overestimated in the simple discrete-charge theory (Nelson and McQuarrie, 1975). The statistical mechanical theory we considered here, which takes into account the finite size of the ions, explains why the Gouy-Chapman theory provides a good description of the experimental results obtained with bilayer membranes containing monovalent phospholipids.

When it is necessary or desirable to consider the discrete nature of the charges, for example when investigating the electrostatic potential produced by charged residues on proteins, the finite size of ions in the aqueous phase should be accounted for in some manner. Groups (e.g., Gilson and Honig, 1988) using the Poisson-Boltzmann equation to calculate potentials produced by charges on proteins are aware of this problem and do account for the finite size of ions in a simple manner that was first used by Stern (Aveyard and Haydon, 1973).

There is another difficulty with calculating the electrostatic potential adjacent to proteins. The dielectric constant of the interior of the protein is unknown, and significantly affects the results of these calculations. Fortunately, our theoretical analysis indicates the dielectric constant of the membrane does not affect the average potential because of the planar geometry of this system (see footnote 3). This allows us to test how well the simple Poisson-Boltzmann equation can describe the potential in the aqueous phase without making assumptions about the dielectric constant of the polar head group region. Thus, the results in Fig. 4, which indicate that the potential profile in the aqueous phase can be described by the Poisson-Boltzmann equation (ignoring the finite size of the ions, correlation effects, and any variation in the dielectric constant of the aqueous phase), provide support for the application of this equation to the more complicated case of charged proteins.

The electrostatic potentials of membranes have been investigated in some detail and the limitations on the use of the Gouy-Chapman-Stern theory are becoming well-defined. The theory cannot be used when the fixed charges are buried within the low dielectric interior of the membrane (McLaughlin, 1977; Andersen et al., 1978; Drain et al., 1989), or when the fixed charges are a significant distance from the surface (Langner et al., 1988). However, in the latter case the Poisson-Boltzmann equation can be used, and a simple extension of the Gouy-Chapman theory for a layer of fixed charges located some distance from the membrane (Langner et al., 1988) provides a good description of the potential profile. When the fixed charges are polyvalent rather than monovalent, more complicated theories, such as the one explored here, must be used to describe the coion concentration next to the surface. Correlation effects are predicted to have a large effect on the force between charged surfaces in the presence of divalent cations. For surface charges typical of biological membranes, the calculated double layer repulsion between the surfaces is about half the corresponding Gouy-Chapman value (Kjellander and Marcelja, 1988*a*). At higher surface charge densities, divalent cations produce an experimentally observed attraction between negatively charged membranes (Marra, 1986), probably because of correlation effects. Although the Gouy-Chapman theory cannot describe this attractive force, it can describe adequately the experimentally observed electrostatic potential and number of adsorbed divalent cations (McLaughlin, 1989; Bloch et al. 1988). An additional complication arises because the electric field produced by the adsorption of cations like calcium to membranes may rotate the polar headgroups out of the plane of the membrane (Seelig et al., 1987; Roux et al., 1989). In spite of these limitations, the Gouy-Chapman theory is even useful for describing the electrostatic potential produced by some proteins in membranes, such as rhodopsin (Tsui et al., 1989).

The technique we used here to measure the dependence of potential on distance from the surface of a bilayer membrane (Fig. 4) could also be used to map the potential profile adjacent to a biological membrane. The labeled gangliosides could be incorporated into these membranes

by transfer from micelles (Masserini and Friere, 1987). Finally, the quenching technique could be used to estimate changes in the electrostatic potential adjacent to fluorescent probes on proteins.

APPENDIX 1

Synthesis of galactose-labeled anthraniloyl G_{M1}

G_{M1} (5 mg) and Triton X 100 were mixed in a 2:1 chloroform/methanol solution, dried under vacuum with nitrogen, then dispersed in a phosphate buffer solution by vortexing. The final concentrations of G_{M1} and detergent were 0.8 and 20 mM. The dispersion was equilibrated at 37°C for 4 h, 150 U of galactose oxidase was added, and the mixture incubated for 6 h with gentle stirring following Ghidoni et al. (1974). The mixture was then passed through a Sep-Pac C_{18} cartridge (Waters Associates, Mitford, MI) three times. The ganglioside was removed with 15 ml of chloroform/methanol (2:1), and the purity checked by thin-layer chromatography (TLC). The solvent was then evaporated from the oxidized G_{M1}. It was exposed to anthraniloyl hydrazine in an acetate buffer for 12 h in the dark, sodium cyanoborohydrate (10 mM) was added, the solution incubated for 1 h, then the labeled G_{M1} purified with the Sep-Pac cartridges as before (Veh et al., 1977; Williams and McCluer, 1980; Winiski et al., 1988). We used preparative TLC with chloroform/methanol/2.5 N ammonium hydroxide (60:40:9 vol/vol/vol) on silica gel 60 plates (EM Science, Cherry Hill, NJ) to purify the lipid.

APPENDIX 2

Calibration of TNAES

The TNAES molecule must satisfy the following criteria to be a good probe of the electrostatic potential at the membrane-solution interface. (*a*) It must adsorb hydrophobically to the membrane, and the intrinsic adsorption coefficient must be independent of the chemical nature of the polar head group of the lipid. (*b*) To distinguish the probe adsorbed to the membrane from those in the aqueous phase, both the adsorption coefficient and the ratio of the quantum yield of the probe adsorbed onto the membrane to the quantum yield of the probe in the aqueous phase must be large. (*c*) The quantum yield of the molecules adsorbed to the membrane must be high because measurements must be made at low surface concentrations of TNAES, where the probe molecules do not themselves perturb the surface potential. (*d*) The fluorescence characteristics of the adsorbed probe (i.e., fluorescence lifetimes and emission spectra) must be independent of both the type of lipids present in the membrane and the electrolyte in the aqueous phase.

TNS satisfies these criteria (Eisenberg et al., 1979) and we show here that TNAES, a positive derivative of TNS (Greene, 1975) also satisfies them. (*a*) We studied the temperature dependence of the binding of TNAES to the membranes by measuring its emission spectra in the temperature range 20–50°C. The fluorescence decreased with an increase in temperature; a plot of the logarithm of the fluorescence vs. the inverse of the absolute temperature was well described by a straight line. The slopes of these plots were similar for all lipid mixtures (data not shown). These results suggest that TNAES adsorbs to these different bilayers by essentially "hydrophobic" forces, as does TNS (Huang and Charlton, 1972; Eisenberg et al., 1979). (*b*) We estimated the binding constant of TNAES to membranes by making electrophoretic mobility measurements. We exposed phosphatidylcholine vesicles to TNAES in 1, 10, and 100 mM KCl solutions. The zeta potential results we obtained were similar to those obtained with TNS (McLaughlin and Harary, 1976; see Fig. 7), but shifted by a factor of five to lower concentrations on the abscissa (data not shown). Thus TNAES binds to PC membrane about factor of 5 more strongly than TNS; the fit of the Gouy-Chapman-Stern theory to the data yields a dissociation constant for TNAES of $\sim 4\ 10^{-5}$ M compared with a value of $2\ 10^{-4}$ M for TNS (McLaughlin and Harary, 1976). Greene (1975) showed that the fluorescence of TNAES in solution depends strongly on the solvent polarity. We observed that fluorescence of TNAES in water was insignificant compared with the fluorescence of TNAES bound to lipid vesicles. (*c*) The electrophoretic mobility experiments on PC membranes show that the amount of TNAES we used in the fluorescent experiments does not produce measurable surface potential on PC bilayers. Specifically, 1 μM TNAES produces <5 mV zeta potential on PC vesicles in 100 mM KCl. Furthermore, the fluorescence produced by TNAES adsorbing to PC, PC/PS, PC/PI, and PC/PIP_2 bilayers depended linearly on the probe concentration under our experimental conditions (data not shown). For bilayers formed with monovalent anionic lipids the TNAES concentration was $<5\ 10^{-7}$ M and for experiments with PIP_2 $<2\ 10^{-8}$ M. This linear dependence of fluorescence on [TNAES] also demonstrates that the adsorption of TNAES ions produce a negligible change in the surface potential. (*d*) The fluorescence is proportional to the number of adsorbed TNAES molecules. We need to know if the fluorescence properties of the adsorbed TNAES molecules are independent of the lipid composition of the bilayer. We measured the lifetimes and corrected spectra of TNAES adsorbed to bilayers formed from different lipids. The shapes of the excitation and the emission spectra of TNAES adsorbed to PC, PC/PS, PC/PI, and PC/PIP_2 vesicles are essentially identical to TNAES spectra obtained in methanol. We also observed no significant difference in the lifetimes of TNAES bound to these lipid mixtures. These experiments suggest that the fluorescence properties of TNAES do not depend on the chemical nature of the lipid bilayer to which it is adsorbed.

We also determined the influence of pH on the fluorescence from TNAES bound to PC membranes. We observed only small (<10%)

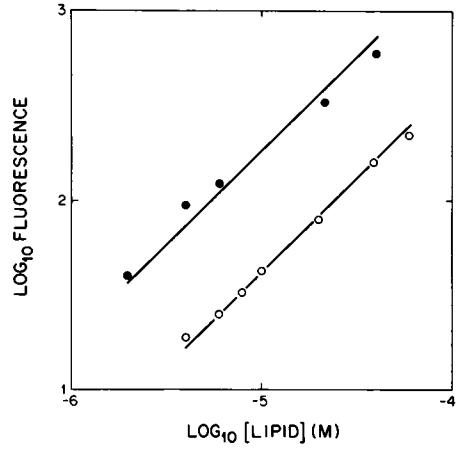

FIGURE 11 The fluorescence (arbitrary units) produced by a unit concentration of the cationic probe TNAES (*filled circles*) or the anionic probe TNS (*open circles*) plotted as a function of the concentration of unilamellar PC vesicles. The lines, which are drawn with a slope of unity, indicate that a linear relationship exists between fluorescence and lipid concentration. The aqueous solutions contained 0.1 M KCl buffered to pH 7.4 with 5 mM MOPS.

changes in fluorescence intensity when we changed the pH from 6 to 9. Above pH 9, the fluorescence intensity falls, ~60% at pH 11. These results are consistent with a pK = 10.5. The shapes of emission spectra do not change from pH 6 to 11.

Fig. 11 shows the net fluorescence of TNS and TNAES adsorbed to PC vesicles in 0.1 M KCl for a range of vesicle concentrations. We measured the fluorescence for different TNS or TNAES concentrations (five or more) in the presence of the same concentration of unilamellar PC vesicles. We then calculated the intensity of the fluorescence per unit concentration of TNS or TNAES and illustrated these values in Fig. 11. As indicated by the lines drawn with a slope of 1, the fluorescence depends linearly on the concentration of vesicles. This demonstrates that there is no significant loss of probe from the aqueous solution because of adsorption onto the vesicles. Moreover, the fluorescence produced by TNAES for a given concentration of PC vesicles is about five times the TNS fluorescence. These data are consistent with the electrophoretic mobility results, which illustrate that TNAES adsorbs to PC vesicles about five times more strongly than TNS.

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REFERENCES

Andersen, O. S., S. Feldberg, S. Nakadomari, S. Levy, and S. McLaughlin. 1978. Electrostatic interactions among hydrophobic ions in lipid bilayer membranes. *Biophys. J.* 21:35–70.

Aveyard, R., and D. A. Haydon. 1973. An Introduction to the Principles of Surface Chemistry. Cambridge University Press, Cambridge. 232 pp.

Azhar, S., J. Butte, and E. Reaven. 1987. Calcium-activated, phospholipid-dependent protein kinases from rat liver: subcellular distribution, purification, and characterization of multiple forms. *Biochemistry.* 26:7047–7057.

Baus, M., and J.-P. Hansen. 1980. Statistical mechanics of simple coulomb systems. *Phys. Reports.* 59:1–94.

Bazzi, M. D., and G. L. Nelsestuen. 1987*a*. Association of protein kinase C with phospholipid vesicles. *Biochemistry.* 26:115–122.

Bazzi, M. D., and G. L. Nelsestuen. 1987*b*. Role of substrate in imparting calcium and phospholipid requirements to protein kinase C activation. *Biochemistry.* 26:1975–1982.

Bazzi, M. D., and G. L. Nelsestuen. 1987*c*. Role of substrate in determining the phospholipid specificity of protein kinase C activation. *Biochemistry.* 26:5002–5008.

Berridge, M. J. 1984. Inositol trisphosphate and diacylglycerol as second messengers. *Biochem. J.* 220:345–360.

Berridge, M. J. 1987. Inositol trisphosphate and diacylglycerol: two interacting second messengers. *Annu. Rev. Biochem.* 56:159–193.

Berridge, M. J., and R. F. Irvine. 1984. Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature (Lond.). 312:315–321.*

Berridge, M. J., and R. F. Irvine. 1989. Inositol phosphates and cell signalling. *Nature (Lond.). 341:197–205.*

Bishop, W. R., and R. M. Bell. 1988. Assembly of phospholipids into cellular membranes: biosynthesis, transmembrane movement and intracellular translocation. *Annu. Rev. Cell Biol.* 4:579–610.

Bloch, J. M., W. B. Yun, X. Yang, M. Ramanathan, P. A. Montano, and C. Capasso. 1988. Adsorption of counterions to a stearate monolayer spread at the water-air interface: a synchrotron x-ray study. *Phys. Rev. Lett.* 61:2941–2944.

Cafiso, D., A. McLaughlin, S. McLaughlin, and A. Winiski. 1989. Measuring electrostatic potentials adjacent to membranes. *Methods Enzymol.* 171:342–364.

Carnie, S. L., and G. M. Torrie. 1984. The statistical mechanics of the electrical double layer. *Adv. Chem. Phys.* 56:141–253.

Castle, J. D., and W. L. Hubbell. 1976. Estimation of membrane surface potential and charge density from the phase equilibrium of a paramagnetic amphiphile. *Biochemistry.* 15:4818–4831.

Cevc, G., and D. Marsh. 1987. Phospholipid Bilayers. Wiley-Interscience, New York. 422 pp.

Cockcroft, S. 1987. Polyphosphoinositide phosphodiesterase: regulation by a novel guanine nucleotide binding protein, G_p. *Trends Biol. Sci.* 12:75–78.

Drain, C. M., B. Christensen, and D. Mauzerall. 1989. Photogating of ionic currents across a lipid bilayer. *Proc. Natl. Acad. Sci. USA.* 86:6959–6962.

Eisenberg, M., T. Gresalfi, T. Riccio, and S. McLaughlin. 1979. Adsorption of monovalent cations to bilayer membranes containing negative phospholipids. *Biochemistry.* 18:5213–5223.

Evans, E. A., and V. A. Parsegian. 1986. Thermal-mechanical fluctuations enhance repulsion between bimolecular layers. *Proc. Natl. Acad. Sci. USA.* 83:7132–7136.

Ferrell, J. E., and W. H. Huestis. 1984. Phosphoinositide metabolism and the morphology of human erythrocytes. *J. Cell Biol.* 98:1992–1998.

Gabev, E., J. Kasianowicz, T. Abbott, and S. McLaughlin. 1989. Binding of neomycin to phosphotidylinositol 4,5-bisphosphate (PIP_2). *Biochim. Biophys. Acta.* 979:105–112.

Ghidoni, R., G. Tettamanti, and V. Zambotti. 1974. As improved procedure for the *in vitro* labelling of ganglioside. *Ital. J. Biochem. (Engl. Ed.)* 23:320–328.

Greene, F. C. 1975. Neutral and cationic sulfonamido derivatives of the fluorescent probe 2-*p*-toluidinylnaphthalene-6-sulfonate. *Biochemistry.* 14:747–753.

Gilson, M. K., and B. H. Honig. 1988. Energetics of charge-charge interaction in proteins. *Proteins.* 3:32–52.

Hannun, Y. A., and R. M. Bell. 1986. Phorbol ester binding and activation of protein kinase C on triton X-100 mixed micelles containing phosphatidylserine. *J. Biol. Chem.* 261:9341–9347.

Hannun, Y. A., C. R. Loomis, and R. M. Bell. 1985. Activation of protein kinase C by triton X-100 mixed micelles containing diacylglycerol and phosphatidylserine. *J. Biol. Chem.* 260:10039–10043.

Hartsel, S. C., and D. S. Cafiso. 1986. A test of the discreteness-of-charge effects in phospholipid vesicles. *Biochemistry.* 25:8214–8219.

Hentschel, M. P., M. Mischel, R. C. Oberthur, and G. Buldt. 1985. Direct observation of the ion distribution between charged lipid membranes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 193:236–238.

Hokin, L. E. 1985. Receptors and phosphoinositide-generated second messengers. *Annu. Rev. Biochem.* 54:205–235.

Honig, B. H., W. L. Hubbell, and R. F. Flewelling. 1986. Electrostatic interactions in membranes and proteins. *Annu. Rev. Biophys. Biophys. Chem.* 15:163–193.

Hope, M. J., M. B. Bally, G. Webb, and P. R. Cullis, 1985. Production of large unilamellar vesicles by a rapid extrusion procedure. *Biochim. Biophys. Acta.* 812:55–65.

Huang, C.-H., and J. P. Charlton. 1972. Interactions of phosphatidylcholine vesicles with 2-*p*-toluidinylnaphthalene-6-sulfonate. *Biochemistry.* 11:735–740.

Jordan, P. C. 1987. How pore mouth charge distributions alter the permeability of transmembrane ionic channels. *Biophys. J.* 51:297–311.

Kaibuchi, K., Y. Takai, and Y. Nishizuka. 1981. Cooperative roles of various membane phospholipids in the activation of calcium-activated, phospholipid-dependent protein kinase. *J. Biol. Chem.* 256:7146–7149.

Kjellander, R., and S. Marcelja. 1985. Inhomogeneous Coulomb fluids with image interactions between planar surfaces. I. *J. Chem. Phys.* 82:2122–2135.

Kjellander, R., and S. Marcelja. 1986. Double layer interaction in the primitive model and the corresponding Poisson-Boltzmann description. *J. Phys. Chem.* 90:1230–1232.

Kjellander, R., and S. Marcelja. 1988*a*. Surface interactions in simple electrolytes. *J. Phys. France.* 49:1009–1015.

Kjellander, R., and S. Marcelja. 1988*b*. Inhomogeneous Coulomb fluids with image interactions between planar surfaces. III. Distribution functions. *J. Chem. Phys.* 88:7138–7146.

Lakowicz, J. R. 1983. Principles of Fluorescence Spectroscopy. Plenum Publishing Corp., New York. 496 pp.

Langner, M., A. Winiski, M. Eisenberg, A. McLaughlin, and S. McLaughlin. 1988. The electrostatic potential adjacent to bilayer membranes containing either charged phospholipids or gangliosides. *In* New Trends in Ganglioside Research. Neurochemical and Neuroregenerative Aspects. R. W. Ledeen, E. L. Hogan, G. Tettamanti, A. J. Yates, and R. K. Yu, editors. Liviana Press, Padova. 121–131.

Litosch, I., and J. N. Fain. 1986. Regulation of phosphoinositide breakdown by guanine nucleotides. *Life Sci.* 39:187–194.

Loosley-Millman, M. E., R. P. Rand, and V. A. Parsegian. 1982. Effects of monovalent ion binding and screening on measured electrostatic forces between charged phospholipid bilayers. *Biophys. J.* 40:221–232.

Marra, J. 1986. Direct measurement of the interaction between phosphatidylglycerol bilayers in aqueous electrolyte solutions. *Biophys. J.* 50:815–825.

Marra, J., and J. N. Israelachvili. 1985. Direct measurements of forces between phosphatidylcholine and phosphatidylethanolamine bilayers in aqueous electrolyte solutions. *Biochemistry.* 24:4608–4618.

Masserini, M., and E. Freire. 1987. Kinetics of ganglioside transfer between liposomal and synaptosomal membranes. *Biochemistry.* 26:237–242.

Mathias, R. T., S. McLaughlin, G. Baldo, and K. Manivannan. 1988. The electrostatic potential due to a single fixed charge at a membrane-solution interface. *Biophys. J.* 53:128*a*. (Abstr.)

Mayer, L. D., M. J. Hope, P. R. Cullis, and A. S. Janoff. 1985. Solute distributions and trapping efficiencies observed in freeze-thawed multilamellar vesicles. *Biochim. Biophys. Acta.* 817:193–196.

McDaniel, R. V., A. McLaughlin, A Winiski, M. Eisenberg, and S. McLaughlin. 1984. Bilayer membranes containing the ganglioside G_{M1}: models for electrostatic potentials adjacent to bilogical membranes. *Biochemistry.* 23:4618–4624.

McDaniel, R. V., K. Sharp, D. Brooks, A. McLaughlin, A. Winiski, D. Cafiso, and S. McLaughlin. 1986. Electrokinetic and electrostatic properties of bilayers containing gangliosides G_{M1}, G_{D1a}, or G_{T1}. *Biophys. J.* 49:741–752.

McLaughlin, S. 1977. Electrostatic potentials at membrane-solution interfaces. *Curr. Top. Membr. Transp.* 9:71–144.

McLaughlin, S. 1989. The electrostatic properties of membranes. *Annu. Rev. Biophys. Biophys. Chem.* 18:113–136.

McLaughlin, S., and H. Harary, 1976. The hydrophobic adsorption of charged molecules to bilayer membranes: a test of the applicability of the Stern equation. *Biochemistry.* 15:1941–1948.

McLaughlin, S., and M. Whitaker. 1988. Cations that alter surface potentials of lipid bilayers increase the calcium requirement for exocytosis in sea urchin eggs. *J. Physiol. (Lond.).* 396:189–204.

Middlekoop, E., B. H. Lubin, E. M. Bevers, J. A. F. Op den Kamp, P. Comfurius, D. T.-Y. Chiu, R. F. A. Zwaal, L. L. M. van Deenen, and B. Roelofsen. 1988. Studies on sickled erythrocytes provide evidence that the asymmetric distribution of phosphatidylserine in the red cell membrane is maintained by both ATP-dependent translocation and interaction with membrane skeletal proteins. *Biochim. Biophys. Acta.* 937:281–288.

Nelson, A. P., and D. A. McQuarrie. 1975. The effect of discrete charges on the electrical properties of a membrane. *J. Theor. Biol.* 55:13–27.

Newton, A. C., and D. E. Koshland, Jr. 1989. Regulation of protein kinase C activity by lipid. *Biophys. J.* 55:209*a*. (Abstr.)

Nishizuka, Y. 1984. The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature (Lond.).* 308:693–698.

Nishizuka, Y. 1986. Studies and perspectives of protein kinase C. *Science* (Wash. DC.). 233:305–312.

Nishizuka, Y. 1988. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature (Lond.).* 334:661–665.

Op den Kamp, J. 1979. Lipid asymmetry in membranes. *Annu. Rev. Biochem.* 48:47–71.

Pashley, R. M., P. M. McGuiggan, B. W. Ninham, J. Brady, and D. F. Evans. 1986. Direct measurements of surface forces between bilayers of double-chained quaternary ammonium acetate and bromide surfactants. *J. Phys. Chem.* 90:1637–1642.

Roux, M., J.-M. Neumann, R. S. Hodges, P. F. Devaux, and M. Bloom. 1989. Conformational changes of phospholipid headgroups induced by a cationic integral membrane peptide as seen by deuterium magnetic resonance. *Biochemistry.* 28:2313–2321.

Schmell, E., and W. J. Lennarz 1974. Phospholipid metabolism in the eggs and embryos of the sea urchin *Arbacia punctulata. Biochemistry.* 13:4114–4121.

Seelig, J., P. M. MacDonald, and P. G. Scherer. 1987. Phospholipid head groups as sensors of electric charge in membranes. *Biochemistry.* 26:7535–7541.

Sekar, M. C., and L. E. Hokin. 1986. The role of phosphoinositides in signal transduction. *J. Membr. Biol.* 89:193–210.

Takai, Y., A. Kishimoto, Y. Iwasa, Y. Kawahara, T. Mori, and Y. Nishizuka. 1979. Calcium-dependent activation of a multifunctional protein kinase by membrane phospholipids. *J. Biol. Chem.* 254:3692–3695.

Toner, M., G. Vaio, A. McLaughlin, and S. McLaughlin. 1988. Adsorption of cations to phosphatidylinositol 4,5-bisphosphate. *Biochemistry.* 27:7435–7443.

Torrie, G. M., and J. P. Valleau. 1980. Electrical double layers. 1. Monte Carlo study of a uniformly charged surface. *J. Chem. Phys.* 73:5807–5816.

Torrie, G. M., and J. P. Valleau. 1982. Electrical double layers. 4. Limitations of the Gouy-Chapman theory. *J. Phys. Chem.* 86:3251–3257.

Tsui, F. C., S. A. Sundberg, and W. Hubbell. 1990. The distribution of

charge on photoreceptor disc membranes and implications for charged lipid asymmetry. *Biophys. J.* 57:85–97.

van Megen, W., and I. Snook. 1980. The grand canonical ensemble Monte Carlo method applied to the electrical double layer. *J. Chem. Phys.* 73:4656–4662.

Veh, R. W., A. P. Corfield, M. Sander, and R. Schauer. 1977. Neuraminic acid-specific modification and tritium labelling of gangliosides. *Biochim. Biophys. Acta.* 486:145–160.

Volpe, P., K.-H. Krause, S. Hashimoto, F. Zorzato, T. Pozzan, J. Meldolesi, and D. P. Lew. 1988. "Calciosome," a cytoplasmic organelle: the inositol 1,4,5-trisphosphate-sensitive calcium store of nonmuscle cells? *Proc. Natl. Acad. Sci. USA.* 85:1091–1095.

Vorotyntsev, M. A. 1988. Solvation and interionic interactions at the metal-electrolyte solution interface. *In* The Chemical Physics of Solvation, Part C. R. R. Dogonodze, E. Kalman, A. A. Kornyshev, and J. Ulstrup, editors. Elsevier Scientific Publishing Co., Amsterdam. 401–431.

Vorotyntsev, M. A., and S. N. Ivanov. 1985. Statistical mechanics of an ion ensemble adsorbed at a metal-insulator interface. *Sov. Phys. JETP.* 61:1028–1032.

Williams, M. A., and R. H. McCluer. 1980. The use of Sep-Pak C_{18} cartridges during the isolation of gangliosides. *J. Neurochem.* 35:266–269.

Williamson, J. R. 1986. Role of inositol lipid breakdown in the generation of intracellular signals. *1985 Blood Pressure Council* (Suppl.) II Hypertension. 8:II-140–II-155.

Winiski, A., A. McLaughlin, R. McDaniel, M. Eisenberg, and S. McLaughlin. 1986. An experimental test of the discreteness-of-charge effect in positive and negative lipid bilayers. *Biochemistry.* 25:8206–8214.

Winiski, A., M. Eisenberg, M. Langner, and S. McLaughlin. 1988. Fluorescent probes of electrostatic potential 1 nm. from the membrane surface. *Biochemistry.* 27:386–392.