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Binding of neomycin to phosphatidylinositol 4,5-bisphosphate (PIP_2)

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Schacht (Schacht, J. (1976) J. Neurochem. 27, 1119–1124) demonstrated that neomycin, an aminoglycoside antibiotic, binds with high affinity to phosphatidylinositol 4,5-bisphosphate (PIP_2). We investigated the binding of neomycin to PIP_2 by making electrophoretic mobility measurements with multilamellar bilayer vesicles and surface potential measurements with monolayers. The bilayers and monolayers were formed from mixtures of PIP_2 and egg phosphatidylcholine (PC) in 0.1 M KCl at pH 7. Neomycin does not bind to PC; 10^{-3} M neomycin affects neither the zeta potential of PC vesicles nor the surface potential of PC monolayers. In contrast, 10^{-6} M neomycin reduces the magnitude of the zeta potential of PC/PIP_2 vesicles (5, 9, and 17 mol% PIP_2) and the surface potential of monolayers (17 mol% PIP_2) to < 50% of their initial values. The electrophoretic mobility results indicate that neomycin forms an electroneutral complex with PIP_2; high concentrations (> 10^{-4} M) of neomycin reduce the zeta potential of the PC/PIP_2 vesicles to zero. We could describe our data with the Gouy-Chapman-Stern theory assuming the intrinsic association constant of the 1:1 neomycin-PIP_2 complex is 10^5 M^{-1}. Neomycin is widely used in cell biology to interfere with the generation of second messengers; we discuss the relevance of our results to these studies. Specifically, 10^{-6} M neomycin binds > 50% of the PIP_2 in a bilayer or monolayer but 10^{-5}–10^{-3} M neomycin is required to affect the turnover of PIP_2 in permeabilized platelets, mast cells, and sea urchin eggs. This result is consistent with a hypothesis that most of the PIP_2 in the inner leaflet of these plasma membranes is not accessible to neomycin because it is associated with proteins.

Introduction

Many investigators have reviewed the evidence that phosphatidylinositol 4,5-bisphosphate (PIP_2), a polyanionic lipid located on the cytoplasmic surface of plasma membranes, is the source of two second messengers [1–14]. Schacht and co-workers have used a number of different techniques to show that neomycin, a polycationic aminoglycoside antibiotic, binds strongly and relatively specifically to PIP_2. For example, neomycin increases the turbidity of an aqueous dispersion of polyphosphoinositides [15], it can be coupled to glass beads and used to purify PIP_2 [16,17], it lowers the phase transition temperature of vesicles formed from a mixture of phosphatidylcholine, PC and PIP_2 [18], it changes the surface pressure of monolayers containing PIP_2 [19–22], and causes release of carboxyfluorescein from PC/PIP_2 vesicles [23].

Neomycin has been used to perturb the turnover of polyphosphoinositides in cells [15,24–34]. Experiments with neomycin on permeabilized cells are particularly informative because the free concentration of drug in the cytoplasm is known. These experiments reveal that different concentrations of neomycin are required to affect PIP_2 turnover in different cells. For example, neomycin is a potent inhibitor of α-thrombin-stimulated polyphosphoinositide-specific phospholipase C, PLC, in isolated platelet membranes; 10^{-5} M neomycin produces a rapid drop to 50% of the control activity followed by a slower rate of decline [35]. However, $0.3 \cdot 10^{-3}$ M neomycin is required to inhibit the PLC in permeabilized mast cells [36] and 10^{-3} M is required in sea urchin egg fragments [37].

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Neomycin can also affect exocytosis [38,39] and other cellular processes [40]. McLaughlin and Whitaker [39] suggested that high concentrations of neomycin ($> 10^{-3}$ M) may inhibit the ability of calcium to induce exocytosis by adsorbing to the membrane and decreasing the magnitude of the negative electrostatic surface potential; neomycin binds to monovalent lipids such as phosphatidylinositol (PI) as well as to PIP_2. Furthermore, neomycin acts as an anion exchanger at high concentrations, binding IP_3 and ATP as well as PIP_2, another effect that complicates its use as a specific tool to study the role of inositol phospholipids in intracellular signalling [41]. Finally, high concentrations of neomycin can induce calcium release and stimulate GTPases in permeabilized platelets [42,44]. Quantitative information about the binding of neomycin to PIP_2 is essential if neomycin is to become a useful tool for cell biologists.

Unfortunately, this information is not available in the literature. One cannot describe satisfactorily the binding of neomycin to monolayers or bilayers containing PIP_2 with a Scatchard analysis or any other analysis that uses a Langmuir adsorption isotherm and assumes both the surface and the drug are electrically neutral. The biological and model membranes used in most studies bear a net negative charge and neomycin has a charge of about +4 at pH 7 (see below). These electrostatic effects can be large.

For example, phosphatidylserine, PS, comprises about 15% of the lipids in a human erythrocyte and is confined to the inner or cytoplasmic monolayer [45–47]. Gouy-Chapman theory predicts and experiments confirm that a bilayer comprised of 30% PS in 0.1 M KCl has a surface potential of about −60 mV [48]. This potential falls in an approximately exponential manner with distance from the surface with a space constant equal to the Debye length, which is about 1 nm in a 0.1 M salt solution [49–51]. If we assume neomycin is a point charge with a valence of +4, the Boltzmann relation predicts the concentration of neomycin in the aqueous phase at the surface of the membrane is four orders of magnitude higher than the concentration in the bulk aqueous phase!

The available experimental evidence suggests the potential is negative at the cytoplasmic surface of the human erythrocyte [52,53]. Cations that are normally present in the cytoplasm, such as magnesium ions, will bind to PS and reduce the magnitude of the surface potential [54], but we anticipate that the potential at the cytoplasmic leaflet of most plasma membranes will be more negative than −15 mV, a potential that should concentrate neomycin at the surface by an order of magnitude. The simplest way to take these electrostatic effects into account is to describe the surface potential with the Gouy-Chapman theory of the diffuse double layer and to describe the adsorption of neomycin to PIP_2 in the membrane by a Langmuir adsorption isotherm. This approach is sometimes referred to as the Gouy-Chapman-Stern theory [55,56]. We wanted to test experimentally if the Gouy-Chapman-Stern theory could be used to describe the binding of neomycin to PIP_2 in bilayers and monolayers. The theory can describe adequately the adsorption of monovalent [48,57] and divalent [54] cations to bilayer membranes and can even describe the adsorption of the polyvalent cations gentamicin and spermine to PIP_2 in membranes [58].

We used two different techniques. We made electrophoretic mobility measurements on multilamellar vesicles and surface potential measurements with an ionizing electrode above a monolayer.

Theory

If we assume the charges on the PC/PIP_2 vesicles are all at the membrane/solution interface and the inositol moieties do not protrude from the surface and exert hydrodynamic drag, we can calculate the value of the zeta potential, ζ, from the measured value of the electrophoretic mobility, u, using the Helmholtz-Smoluchowski equation [55,59–61]:

$$\zeta = \eta u / \varepsilon_0 \varepsilon_r \tag{1}$$

where η is the viscosity, ε_0 is the permittivity of free space, and ε_r is the dielectric constant of the aqueous phase. In fact, the charges on the monoester phosphates of PIP_2 are probably located about 0.5 nm from the surface and the inositol groups probably exert hydrodynamic drag. These effects will act in opposite directions [62] and they may approximately cancel. We do not use a theory that incorporates these effects [63–65] because we do not have direct experimental information about the location of the charges and the orientation of the headgroup.

The zeta potential is the potential at the hydrodynamic plane of shear. For simplicity, we assume the plane of shear is at the surface of the PC/PIP_2 vesicles. Thus, the zeta and surface potentials we calculate from the theoretical model described below are identical.

We measured the electrostatic potential above PC/PIP_2 and PC monolayers with an ionizing electrode. To deduce the potential above the PC/PIP_2 monolayer due to charges, we assume the two monolayers have identical dipole potentials. This assumption was tested experimentally.

We calculated the theoretical curves for the effect of neomycin on the zeta and surface potentials from the simplest possible model. We related the surface potential, ψ_0, to the charge density, σ, with the Gouy-Chapman theory:

$$\sinh(e\psi_0/2kT) = \sigma/(8n\varepsilon_0\varepsilon_r kT)^{1/2} \tag{2}$$

where e is the magnitude of the electronic charge, k is the Boltzmann constant, T is the temperature, and n is the number concentration of monovalent ions in bulk aqueous phase [55,56]. We ignore the screening effect of the neomycin ions, a reasonable assumption under our experimental conditions. Neomycin changes the surface and zeta potentials by binding to PIP_2. We use the following mass action or Langmuir adsorption isotherm:

$$N + PIP_2 \leftrightarrows N - PIP_2 \tag{3}$$

$$K = \{N - PIP_2\}/\{PIP_2\}[N(0)]$$

where K is an intrinsic association constant, the braces denote a surface concentration, and $[N(0)]$ is the concentration of neomycin in the aqueous phase at the surface of the membrane. Our experimental results suggest a neomycin ion of valence z_+ combines with a PIP_2 molecule of valence $-z_-$ to form an electroneutral complex. (Neomycin probably displaces hydrogen and potassium ions bound to PIP_2 to form the neutral complex.) Thus the surface charge density, σ, is the product of the surface concentration of PIP_2 and its valence, $-z_-\{PIP_2\}$.

PIP_2 binds hydrogen and potassium ions, which reduce the net negative charge of PIP_2 from its maximum value of -5 [58]. We choose the valence or net charge on PIP_2 to fit the value of the zeta potential of the PC/PIP_2 vesicles in he absence of neomycin. (A more detailed analysis provides a slightly different and presumably more correct estimate for the valence of PIP_2 [58].) We determined the valence of neomycin by titration experiments with a pH glass electrode. To simplify the analysis, we assume that the charge on neither neomycin ions in the diffuse double layer nor PIP_2 molecules in the membrane are affected by the surface potential of the membrane. In other words, we assume the binding of hydrogen and potassium ions to PIP_2 is not affected by the average surface potential of the membrane.

We calculated the concentration of neomycin at the surface of the membrane, $[N(0)]$, from the Boltzmann relation:

$$[N(0)] = [N(\infty)]\exp(-z_+e\psi_0/kT) \tag{4}$$

We combined Eqns. 2, 3 and 4 to predict the theoretical curves illustrated in the figures.

Materials and Methods

Neomycin sulfate was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN), egg phosphatidylcholine (PC) from Avanti Polar Lipids, Inc. (Birmingham, AL) and the triammonium salt of phosphatidylinositol 4,5-bisphosphate (PIP_2) from Calbiochem (La Jolla, CA). We confirmed that the PC used for the monolayer experiments was uncharged by measuring the electrophoretic mobility of multilamellar PC vesicles. Potassium chloride was roasted in a quartz crucible at 600 °C before use. Chloroform and methanol were spectrophotometric or HPLC grade. Water was purified with a Millipore Super Q System (Bedford, MA) and subsequently bidistilled in a quartz apparatus (Heraeus-Schott, Hanau, F.R.G.).

Electrophoretic mobility measurements. As described in detail elsewhere [54,58] we used a Rank Bros. Mark I machine (Bottisham, Cambridge, U.K.) to measure the effect of neomycin on the electrophoretic mobility of multilamellar PC/PIP_2 vesicles formed in an aqueous solution containing 0.1 M KCl buffered to pH 6, 7, or 8 at 25 °C with 1 mM Mops. The chloroform solution of PC/PIP_2 was evaporated rapidly as described elsewhere [58]. The total concentration of lipid in the 7 ml sample was normally about 0.1 mg/ml, but was reduced to 0.01 mg/ml for the experiments with 10^{-6} M neomycin to prevent loss of antibiotic from the aqueous phase.

Monolayer measurements. We used a 10 cm diameter teflon petri dish (Savillex Corp., Minnetonka, MN) as a monolayer trough. The 100 ml aqueous subphase contained 0.1 M KCl and either 1 mM or 10 mM potassium phosphate buffered to either pH 7.0 or 8.0. We made both gravimetric and phosphate measurements [66] to determine the concentration of lipid in the chloroform solutions; the two techniques gave identical results. The neomycin sulfate stock solution was titrated to the appropriate pH before addition to the subphase and the pH of the subphase was measured at the end of the experiment. The monolayer was spread using a Drummond microdispenser (Broomall, PA). Monolayers were formed from a chloroform solution of either PC or a 5 : 1 mole : mole PC/PIP_2 mixture. We added lipid such that each molecule occupied an average area of 0.66 nm^2, 0.52 nm^2, or we added an excess of lipid. We measured the surface potential with a 20 μCi gold-coated americium-241 ionizing electrode (NRD Inc., Grand Island, NY) positioned 2 mm above the surface with a micromanipulator. A silver-silver chloride electorde (Annex Research, Costa Mesa, CA) maintained the aqueous subphase at ground potential. The potential difference was measured with a Keithley (Cleveland, OH) 616 digital electrometer using a voltage follower. Shifting the electrode horizontally over the surface of the monolayer did not change the potential, which suggests the ionizing radiation did not significantly degrade the lipids. Addition of chloroform without lipid did not significantly change the surface potential. The aqueous subphase was stirred with a teflon-coated magnetic stir bar. A metal shield around the trough reduced the a.c. noise. The apparatus was enclosed with plexiglass to reduce contamination of the monolayer and oxidation of the lipids [67]. Nitrogen was introduced

into the perspex box throughout the experiment and the relative humidity was monitored.

We began an experiment by first measuring the potential above the aqueous subphase. We aspirated the surface until we obtained a stable reading, a procedure that often required several hours. We then spread the monolayer and waited until the potential again reached a stable value, a procedure that required 10–30 min. The noise level was < 2 mV. We added neomycin to the subphase and recorded the new stable potential. At the end of the experiment we aspirated the monolayer and again recorded the potential above the subphase. We also replaced the aqueous subphase and recorded the potential. If the initial and final values of the potential measured in the absence of a lipid monolayer differed by more than 15 mV, we rejected the experiment. The potential usually drifted about 5 mV/h.

Results

Schacht et al. [68] showed that neomycin does not affect the surface pressure of a PC monolayer, which suggests it does not bind to PC. We confirmed this suggestion by showing that 10^{-3} M neomycin affects neither the zeta potential of PC multilamellar vesicles nor the surface potential of PC monolayers (0.1 M KCl at pH 7; data not shown).

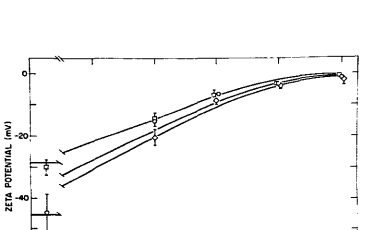

Fig. 1. The effect of neomycin on the zeta potential of PC/PIP_2 multilamellar vesicles. The zeta potential was calculated from the measured value of the electrophoretic mobility using Eqn. 1. The aqueous phase contained 0.1 M KCl buffered to pH 7.0 with 1 mM Mops. The net charge on PIP_2 in the absence of neomycin was chosen to fit the prediction of the Gouy-Chapman theory (Eqn. 2) to the initial value of the zeta potential and was -2, -1.8 and -1.4 for the 20:1 (mol/mol) (squares), 10:1 (circles) and 5:1 (diamonds) vesicles, respectively. The valence of neomycin is 4.5 at pH 7. The curves illustrate the predictions of the Gouy-Chapman-Stern theory, a combination of Eqns. 2, 3, and 4, with the intrinsic association constant for the complex between neomycin and PIP_2 equal to 10^5 M^{-1}. Note the simple theory describes adequately the data obtained at all surface concentrations of PIP_2 and all neomycin concentrations.

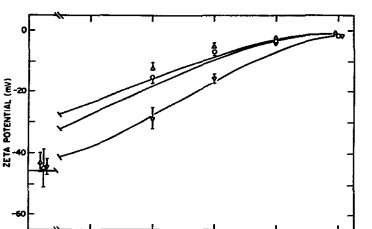

Fig. 2. The effect of neomycin on the zeta potential of 10:1 (mol/mol) PC:PIP_2 vesicles formed in 0.1 M KCl, 1 mM Mops at pH 6.0 (triangles), 7.0 (circles) and 8.0 (inverted triangles). The curves are the predictions of the Gouy-Chapman-Stern theory (Eqns. 2, 3, and 4) with the intrinsic association constant for the complex of neomycin with PIP_2 equal to 10^5 M^{-1}, which describes the pH 6.0 and 7.0 data, and $3 \cdot 10^4$ M^{-1}, which describes the pH 8.0 data. The valence of the PIP_2 molecules in the absence of neomycin was assumed to be -1.8 in all cases and the valence of the neomycin ions, as determined by titration experiments with a glass electrode, was 5.2, 4.5 and 3.0 at pH 6, 7, and 8, respectively.

Fig. 1 illustrates the effect of neomycin on the zeta potential of multilamellar PC/PIP_2 vesicles. Note that 10^{-6} M neomycin has a large effect on the zeta potential. This concentration of neomycin reduces the zeta potential to < 50% its initial value, indicating that > 50% of the PIP_2 molecules in the bilayer have been neutralized by neomycin (see Eqn. 2). High concentrations of neomycin cause the zeta potential to approach a value of zero asymptotically, suggesting neomycin forms an electroneutral complex with PIP_2. The curves in Fig. 1 illustrate the predictions of the Gouy-Chapman-Stern theory, assuming that neomycin forms an electroneutral 1:1 complex with PIP_2 with an intrinsic association constant of 10^5 M^{-1}. The theory is highly oversimplified, but is capable of describing qualitatively the data obtained at the three surface concentrations of PIP_2. This agreement between theory and experiment is consistent with our assumption the antibiotic forms a 1:1 complex with the lipid.

Fig. 2 illustrates that neomycin binds less strongly to PIP_2 at pH 8 than at pH 7 or 6. It binds less strongly at alkaline pH for two reasons. First, neomycin has fewer positive charges at alkaline pH and is concentrated less in the aqueous diffuse double layer (see Eqn. 4). If we ignore any possible binding of chloride ions to neomycin, our titration data (not shown) suggests neomycin has a charge of $z_+ = 5.2$, 4.5, and 3 at pH 6, 7, and 8, respectively; at acid pH it bears a maximum of six positive charges. Second, the intrinsic association constant of neomycin with PIP_2 is lower at pH 8 than 7 or

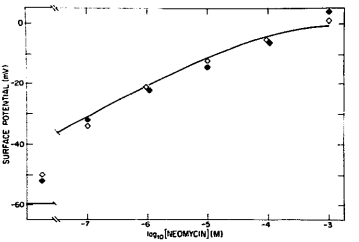

Fig. 3. The effect of neomycin on the surface potential of a 5:1 (mol/mol) PC/PIP_2 monolayer. The aqueous subphase contained 0.1 M KCl, 1 mM phosphate (pH 7.0). The average area/lipid was 0.66 nm^2 (open diamonds) or 0.52 nm^2 (filled diamonds). Identical results were obtained with an average area of 0.52 nm^2 and with excess lipid. The curve is the prediction of the Gouy-Chapman-Stern theory with $K = 10^5\ M^{-1}$; it is identical to the lower curve in Fig. 1. Note that the same theoretical expression can describe adequately both the monolayer and vesicle data.

6; the theoretical curves in Fig. 2 were drawn with association constants of 10^5, 10^5, and $3 \cdot 10^4\ M^{-1}$ at pH 6, 7, and 8, respectively *.

We note in passing that the zeta potential of the PC/PIP_2 vesicles does not depend significantly on pH for $6 < pH < 8$ (Fig. 2). However, ^{31}P-NMR measurements demonstrate that PIP_2 binds about one proton less at pH 8 than at 6 [58,69]. A potassium ion probably replaces the bound proton that comes off PIP_2 when the pH increases from 6 to 8.

Fig. 3 illustrates the effect of neomycin on the surface potential of a PC/PIP_2 monolayer. When the average area occupied by the lipids is 0.66 nm^2, approximately the area occupied by a lipid in a bilayer membrane [70], the difference between the measured surface potential of the lipid/water and air/water surface is V(PC/PIP_2) − $V(H_2O) = \Delta V$(PC/PIP_2) = 365 ± 12 mV (±S.D., $n = 5$) and V(PC) − $V(H_2O) = \Delta V$(PC) = 415 ± 8 mV ($n = 12$). The large positive potential above the PC monolayer must be due to dipoles because PC has no net charge and a PC vesicle formed in a KCl or NaCl solution at pH 7 does not move in an electric field. The molecular origin of this dipole potential is unknown; it could arise from oriented water molecules, from carbonyl oxygens or from some other group. If we assume the dipole potential of the PC/PIP_2 monolayer is the same as the PC monolayer *, the surface potential due to charges is ΔV(PC/PIP_2) − ΔV(PC) = $\Delta\Delta V$ = −50 mV. Fig. 3 illustrates that neomycin reduces the magnitude of this surface potential. Note that 10^{-7} M neomycin has a significant effect on the potential. (We can obtain reliable measurements at low neomycin concentrations using a monolayer because there is no significant loss of neomycin from the aqueous phase onto the monolayer.) The curve is the prediction of the Gouy-Chapman-Stern theory with the same intrinsic association constant used to describe the zeta potential data, $10^5\ M^{-1}$. The theory provides a qualitative description of the data.

We also studied the effect of neomycin on the surface potential of monolayers formed with either excess lipid or with lipids spread to occupy an average area of 0.52 nm^2. We obtained similar results in both cases, an observation consistent with our surface pressure measurements that indicate the minimum area occupied by a lipid in these monolayers is > 0.52 nm^2 (data not shown). The surface potential of these PC monolayers was ΔV(PC) = 450 mV, which agrees with the range of values in the literature [71–73]. The surface potential of these PC/PIP_2 monolayers was ΔV(PC/PIP_2) = 395 mV. Thus, decreasing the surface area/lipid from 0.66 to about 0.52 nm^2 produces a relatively large (35–40 mV) change in ΔV(PC) and ΔV(PC/PIP_2) but a relatively small (5 mV) change in $\Delta\Delta V = -55$ mV. Furthermore, neomycin had a similar effect on the surface potential of the two PC/PIP_2 monolayers (Fig. 3), a result consistent with our hypothesis that neomycin forms 1:1 complexes with PIP_2.

Discussion

Our results confirm the previous observations of Schacht and his co-workers that neomycin binds with high affinity to PIP_2. The electrophoretic mobility results illustrated in Fig. 1 suggest that neomycin forms an electroneutral complex with PIP_2; high concentrations of neomycin cause the mobility to approach zero asymptotically and do not reverse the charge on the vesicles. We can describe the data obtained with vesicles formed with different PC/PIP_2 ratios (Fig. 1) with the

* We acknowledge that it would be more correct to write a series of Boltzmann relations, one for each charged form (e.g., valence = 6, 5, 4 etc.) of neomycin. Joel Cohen (personal communication) analyzed this problem and showed that the use of an average valence and a single Boltzmann relation to calculate the total concentration of polyvalent cation at the membrane solution interface is surprisingly accurate for potentials less than $kT/e = 25$ mV.

* We assumed the dipole potential of the PC and the PC/PIP_2 monolayers were identical to deduce that the charges on PIP_2 produced a diffuse double layer potential of −50 mV. We checked this assumption in the following manner. We know that high concentrations of neomycin reduce the zeta potential to zero (Fig. 1). Thus the *change* in the surface potential above a monolayer produced by high concentrations of neomycin should equal the initial value of the diffuse double layer potential produced by the charges on PIP_2. We obtain the same number using both approaches about −50 mV for a PC/PIP_2 monolayer with 17 mol% PIP_2 (see Fig. 3), which supports our assumption that this concentration of PIP_2 does not significantly affect the dipole potential of a PC membrane.

Gouy-Chapman-Stern theory using the same value for the 1:1 neomycin:PIP_2 intrinsic association constant, 10^5 M^{-1}. Thus our results are consistent with our assumption that neomycin forms a 1:1 complex with PIP_2. A recent NMR study is also consistent with this assumption [74]. In contrast, zeta potential measurements suggest neomycin binds in a co-operative manner to monovalent anionic lipids such as phosphatidylinositol, PI, [39]. Similar results were obtained with phosphatidylserine, PS, and phosphatidylglycerol, PG (data not shown). The binding of neomycin to PC/PI, PC/PS, and PC/PG vesicles depends on the surface concentration of monovalent negative lipid in the vesicle and the results imply that one neomycin forms a positively charged complex with more than one negatively charged lipid [39].

We used two different techniques to study the binding of neomycin to PIP_2 because different artifacts arise in making electrophoretic mobility and monolayer surface potential measurements. For example, the effect of neomycin on the surface potential of the PC/PIP_2 monolayer (Fig. 3) could be due to neomycin changing the dipole rather than the double layer potential. However, the electrophoretic mobility does not respond to changes in the dipole potential and similar results were obtained with mobility measurements (Fig. 1).

The slopes of the data points in Figs. 1, 2 and 3 agree with the values of the slopes predicted by the Gouy-Chapman-Stern theory. This agreement implies that no significant discreteness-of-charge or Esin-Markov effect occurs [48]. Of course, our model is highly oversimplified. A more sophisticated model would account for the binding of hydrogen and potassium ions to PIP_2 [58] and the competition between neomycin and these ions for binding sites on PIP_2. Furthermore, the charges on the monoester groups of PIP_2 might not be at the membrane/solution interface and the finite size of neomycin should be considered in a more realistic model [75]. In the absence of detailed molecular information about these parameters, the simple Gouy-Chapman-Stern model would appear to provide the best available description of the adsorption of neomycin to PIP_2 in bilayer membranes.

What is the relevance of our results to cell biologists and biochemists interested in the generation of second messengers from PIP_2 in biological membranes? PS and other anionic phospholipids produce a negative electrostatic potential at the cytoplasmic surface of most plasma membranes. For example, the human erythrocyte has about 30% PS on its inner monolayer. PS should produce a surface potential between -30 and -60 mV (see Eqn. 2), depending on the free concentration of magnesium [54] and spermine [76] in the cytoplasm. A recent study suggests the surface potential of the cytoplasmic leaflet of the human erythrocyte is -40 mV under physiological conditions [53]. We anticipate that the cytoplasmic leaflet of most biological membranes will have a surface potential more negative than -15 mV. These surface potentials will attract neomycin ions, which have a valence > 4 at pH 7, to concentrations that are at least one order of magnitude higher than the bulk aqueous concentration (see Eqn. 4). PIP_2 is present only at trace concentrations (about 1% in human erythrocytes) in the plasma membranes of most cells and the binding of neomycin ($< 10^{-4}$ M) to PIP_2 will not significantly change the charge density and surface potential of these membranes. In other words, the charge density and surface potential of the erythrocyte membrane are determined mainly by the surface concentration of the monovalent anionic lipid phosphatidylserine ({PS}). Low concentrations of neomycin ($< 10^{-4}$ M) will not affect {PS} significantly. Thus, if all the PIP_2 in a biological membrane is free, one should be able to describe the adsorption of low concentrations of neomycin to PIP_2 with a simple Langmuir adsorption isotherm, Eqn. 3. Of course, the surface potential of these membranes will affect the adsorption, as indicated by the combination of Eqns. 3 and 4. If the surface potential is -15 mV, 10^{-6} and 10^{-5} M neomycin should bind 50% and 90%, respectively, of the PIP_2 in the plasma membrane and might be expected to interfere with the turnover of PIP_2 and the generation of second messengers. In platelets, 10^{-5} M neomycin does interfere with the production of second messengers [35]. In mast cells and sea urchin eggs, however, 100-fold higher concentrations of neomycin are required [36,37].

It is not clear why high concentrations of neomycin are required to block the turnover of PIP_2 in many plasma membranes. One possible explanation is that most of the PIP_2 in these biological membranes is bound to positively charged regions of intrinsic proteins that are close to the membrane/solution interface. There are several independent, albeit indirect, lines of support for this explanation. First, a simple electrostatic calculation indicates that a strong association should occur between PIP_2 and the positively charged regions of proteins (McLaughlin, unpublished). More importantly, Anderson and Marchesi [77] observed experimentally that PIP_2 binds to glycophorin, which has several positive residues near the cytoplasmic surface of the membrane. Furthermore, most of the PIP_2 in erythrocytes [78] and WRK-1 mammary tumor cells [79] is somehow compartmentalized in a metabolically inactive pool and Muller et al. [78] suggested it may be bound to proteins. Finally, we (McDaniel, Hubbell and McLaughlin, unpublished) have attached covalently a fluorescent probe onto PIP_2 and demonstrated that membrane-bound, cationic, molecules associate with PIP_2. Thus, the available evidence is consistent with a hypothsis that much of the PIP_2 in plasma membranes is associated electrostatically with positive regions of intrinsic proteins. The biological significance of any electrostatically induced

two-dimensional lateral phase separation that might occur with PIP_2 is unknown.

Acknowledgments

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References

1 Berridge, M.J. (1984) Biochem. J. 220, 345–360.
2 Berridge, M.J. and Irvine, R.F. (1984) Nature 312, 315–321.
3 Majerus, P.W., Neufeld, E.J. and Wilson, D.B. (1984) Cell 37, 701–703.
4 Nishizuka, Y. (1984) Nature 308, 693–698.
5 Hokin, L.E. (1985) Annu. Rev. Biochem. 54, 205–235.
6 Nishizuka, Y. (1986) Science 233, 305–312.
7 Sekar, M.C. and Hokin, L.E. (1986) J. Membr. Biol. 89, 193–210.
8 Berridge, M.J. (1987) Annu. Rev. Biochem. 56, 159–193.
9 Combettes, L., Berthon, B. and Claret, M. (1987) Biochimie 69, 281–286.
10 Drummond, A.H. and MacIntyre, D.E. (1987) Platelets in Biology and Pathology, Vol. 3, Chapter 13, Elsevier Science Publishers, New York.
11 Exton, J.H. (1987) Kidney Int. 32, S68–S76.
12 Fisher, S.K. and Agranoff, B.W. (1987) J. Neurochem. 48, 999–1017.
13 Hokin, L.E. (1987) Trends Pharmacol. Sci. 8, 53–56.
14 Putney, Jr., J.W. (1987) Trends Pharmacol. Sci. 8, 481–486.
15 Schacht, J. (1976) J. Neurochem. 27, 1119–1124.
16 Schacht, J. (1978) J. Lipid Res. 19, 1063–1067.
17 Schacht, J. (1979) Arch. Otorhinolaryngol. 224, 129–134.
18 Wang, B.W., Weiner, N.D., Ganesan, M.G. and Schacht, J. (1984) Biochem. Pharmacol. 33, 3787–3791.
19 Lodhi, S., Weiner, N.D. and Schacht, J. (1976) Biochim. Biophys. Acta 426, 781–785.
20 Lodhi, S., Weiner, N.D. and Schacht, J. (1979) Biochim. Biophys. Acta 557, 1–8.
21 Lodhi, S., Weiner, N.D., Mechigian, I. and Schacht, J. (1980) Biochem. Pharmacol. 29, 597–601.
22 Wang, B.M., Weiner, N.D., Takada, A. and Schacht, J. (1984) Biochem. Pharmacol. 33, 3257–3262.
23 Au, S., Weiner, N.D. and Schacht, J. (1987) Biochim. Biophys. Acta 902, 80–86.
24 Orsulakova, A., Stockhorst, E. and Schacht, J. (1976) J. Neurochem. 26, 285–290.
25 Lang, V., Prythitka, G. and Buckley, J.T. (1977) Can. J. Biochem. 55, 1007–1012.
26 Schibeci, A. and Schacht, J. (1977) Biochem. Pharmacol. 26, 1769–1774.
27 Stockhurst, E. and Schacht, J. (1977) Acta Otolaryngol. 83, 401–409.
28 Griffin, H.D., Sykes, M. and Hawthorne, J.N. (1980) J. Neurochem. 34, 750–752.
29 Downes, C.P. and Michell, R.H. (1981) Biochem. J. 198, 133–140.
30 Lipsky, J.J. and Lietman, P.S. (1982) J. Pharmacol. Exp. Ther. 220, 287–292.
31 Schwertz, D.W., Kreisberg, J.I. and Venkatachalam, M.A. (1984) J. Pharmacol. Exp. Ther. 231, 48–55.
32 Carney, D.H., Scott, D.L., Gordon, E.A. and LaBelle, E.F. (1985) Cell 42, 479–488.
33 Siess, W. and Lapetina, E.G. (1986) FEBS Lett. 207, 53–57.
34 Tysnes, O.B., Verhoeven, A.J.M. and Holmsen, H. (1987) Biochem. Biophys. Res. Commun. 144, 454–462.
35 Rock, C.O. and Jackowski, S. (1987) J. Biol. Chem. 262, 5492–5498.
36 Cockcroft, S., Howell, T.W. and Gomperts, B. (1987) J. Cell Biol. 105, 2745–2750.
37 Whitaker, M. and Aitchison, M. (1985) FEBS Lett. 182, 119–124.
38 Cockcroft, S. and Gomperts, B.D. (1985) Nature 314, 534–536.
39 McLaughlin, S. and Whitaker, M. (1988) J. Physiol. 396, 189–204.
40 Lassing, I. and Lindberg, U. (1988) Exp. Cell Res. 174, 1–15.
41 Prentki, M., Deeney, J., Matschinsky, F.M. and Joseph, S.K. (1986) FEBS Lett. 197, 285–288.
42 Polascik, T., Godfrey, P.P. and Watson, S.P. (1987) Biochem. J. 243, 815–819.
43 Nakashima, S., Tohmatsu, T., Shirato, L., Takenaka, A. and Nozawa, Y. (1987) Biochem. Biophys. Res. Commun. 146, 820–826.
44 Herrmann, E. and Jakobs, K.H. (1988) FEBS Lett. 229, 49–53.
45 Ferrell, J.E. and Huestis, W.H. (1984) J. Cell Biol. 98, 1992–1998.
46 Op den Kamp, J. (1979) Annu. Rev. Biochem. 48, 47–71.
47 Middelkoop, E., Lubin, B., Bevers, E., Op den Kamp, J., Comfurius, P., Chiu, D., Zwaal, R., Van Deenen, L. and Roelofsen, B. (1988) Biochim. Biophys. Acta 937, 281–288.
48 Winiski, A., McLaughlin, A., McDaniel, R., Eisenberg, M. and McLaughlin, S. (1986) Biochemistry 25, 8206–8214.
49 Marra, J. (1986) Biophys. J. 815–825.
50 Winiski, A., Eisenberg, M., Langner, M. and McLaughlin, S. (1988) Biochemistry 27, 386–392.
51 Langner, M., Winiski, A., Eisenberg, M., McLaughlin, A. and McLaughlin, S. (1988) in New Trends in Ganglioside Research (Ledeen, R., Hogan, E., Tettamanti, G., Yates, A. and Yu, R., eds.), pp. 121–131, Liviana Press, Padova, Italy.
52 Lin, G., Macey, R. and Mehlhorn, R. (1983) Biochim. Biophys. Acta 732, 683–690.
53 Iglic, A., Brumen, M. and Svetina, S. (1987) Period. Biol. 89, 363–366.
54 McLaughlin, S., Mulrine, N., Gresalfi, T., Vaio, G. and McLaughlin, A. (1981) J. Gen. Physiol. 77, 445–473.
55 Aveyard, R. and Haydon, D.A. (1973) An Introduction to the Principles of Surface Chemistry, Cambridge University Press, Cambridge.
56 McLaughlin, S. (1977) in Current Topics in Membranes and Transport, Vol. 9, pp. 71–144, Academic Press, New York.
57 Eisenberg, M., Gresalfi, T., Riccio, T. and McLaughlin, S. (1979) Biochemistry 18, 5213–5223.
58 Toner, M., Vaio, G., McLaughlin, A. and McLaughlin, S. (1988) Biochemistry 27, 7435–7443.
59 Wiersema, P.H., Loeb, A.L. and Overbeek, J. (1966) J. Colloid Interface Sci. 22, 78–99.
60 O'Brien, R.W. and White, L.R. (1978) J. Chem. Soc. Farad. Trans. II, 74, 1607–1626.
61 Hunter, R.J. (1981) Zeta Potential in Colloid Science, Academic Press, New York.
62 Pasquale, L., Winiski, A., Oliva, C., Vaio, G. and McLaughlin, S. (1986) J. Gen. Physiol. 88, 697–718.
63 Donath, E. and Pastushenko, V. (1979) J. Electroanal. Chem. 104, 543–554.
64 Levine, S., Levine, M., Sharp, K. and Brooks, D. (1983) Biophys. J. 42, 127–135.
65 McDaniel, R.V., Sharp, K., Brooks, D., McLaughlin, A., Winiski, A., Cafiso, D. and McLaughlin, S. (1986) Biophys. J. 741–752.
66 Lowry, R.R. and Tinsley, I.J. (1974) Lipids 9, 491–492.
67 Dawson, R.M.C. (1969) Methods Enzymol. 14, 633–648.
68 Schacht, J., Weiner, N.D. and Lodhi, S. (1978) Cyclitols and Phosphoinositides (Wells, W.W. and Esinberg, F., eds.), pp. 153–165, Academic Press, New York.
69 Van Paridon, P.A., De Kruijff, B., Ouwerkerk, R. and Wirtz, K.W.A. (1986) Biochim. Biophys. Acta 877, 216–219.
70 McIntosh, T.J. and Simon, S.A. (1986) Biochemistry 25, 4058–4066.

71 Bangham, A.D. and Mason, W. (1979) Br. J. Pharmacol. 66, 259–265.
72 Reyes, J., Greco, F., Motais, R. and Latorre, R. (1983) J. Membr. Biol. 72, 93–103.
73 Haydon, D.A. and Elliot, J.R. (1986) Biochim. Biophys Acta 867, 337–340.
74 Reid, D.G. and Gajjar, K. (1987) J. Biol. Chem. 262, 7967–7972.
75 Carnie, S. and McLaughlin, S. (1983) Biophys. J. 44, 325–332.
76 Chung, L., Kaloyanides, G., McDaniel, R., McLaughlin, A. and McLaughlin, S. (1985) Biochemistry 24, 442–452.
77 Anderson, R.A. and Marchesi, V.T. (1985) Nature 318, 295–298.
78 Muller, E., Hegewald, H., Jaroszewicz, K. Cumme, G.A., Hoppe, H. and Frunder, H. (1986) Biochem. J. 235, 775–783.
79 Koreh, K. and Monaco, M.E. (1986) J. Biol. Chem. 261, 88–91.