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Fluorescently labeled neomycin as a probe of phosphatidylinositol-4,5-bisphosphate in membranes

Anna Arbuzova [a], Katherine Martushova [a], Gyöngyi Hangyás-Mihályné [a], Andrew J. Morris [b], Shoichiro Ozaki [c], Glenn D. Prestwich [c], Stuart McLaughlin [a,*]

[a] *Department of Physiology and Biophysics, State University of New York at Stony Brook, Stony Brook, NY, USA*
[b] *Department of Pharmacological Sciences, State University of New York at Stony Brook, Stony Brook, NY, USA*
[c] *Department of Medical Chemistry, University of Utah, Salt Lake City, UT, USA*

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Abstract

Phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$), a minor component of the plasma membrane, is important in signal transduction, exocytosis, and ion channel activation. Thus fluorescent probes suitable for monitoring the $PI(4,5)P_2$ distribution in living cells are valuable tools for cell biologists. We report here three experiments that show neomycin labeled with either fluorescein or coumarin can be used to detect $PI(4,5)P_2$ in model phospholipid membranes. First, addition of physiological concentrations of $PI(4,5)P_2$ (2%) to lipid vesicles formed from mixtures of phosphatidylcholine (PC) and phosphatidylserine (PS) enhances the binding of labeled neomycin significantly (40-fold for 5:1 PC/PS vesicles). Second, physiological concentrations of inositol-1,4,5-trisphosphate (10 μM $I(1,4,5)P_3$) cause little translocation of neomycin from PC/PS/$PI(4,5)P_2$ membranes to the aqueous phase, whereas the same concentrations of $I(1,4,5)P_3$ cause significant translocation of the green fluorescent protein/phospholipase C-δ pleckstrin homology (GFP-PH) constructs from membranes (Hirose et al., Science, 284 (1999) 1527). Third, fluorescence microscopy observations confirm that one can distinguish between PC/PS vesicles containing either 0 or 2% $PI(4,5)P_2$ by exposing a mixture of the vesicles to labeled neomycin. Thus fluorescently labeled neomycin could complement GFP-PH constructs to investigate the location of $PI(4,5)P_2$ in cell membranes.

Keywords: Phosphatidylinositol-4,5-bisphosphate; Neomycin; Fluorescence; Microscopy; Binding

Abbreviations: $PI(4,5)P_2$, phosphatidylinositol-4,5-bisphosphate; PC, phosphatidylcholine; PS, phosphatidylserine; $I(1,4,5)P_3$, inositol-1,4,5-trisphosphate; GFP-PH, green fluorescent protein/phospholipase C-δ pleckstrin homology; PLC, phospholipase C; PH, pleckstrin homology; PLD, phospholipase D; FITC, fluorescein-5-isothiocyanate; AMCA-S, 7-amino-3-((((succinimidyl)oxy)carbonyl)methyl)-4-methylcoumarin-6-sulfonic acid; AMCA-X, 6-((7-amino-4-methylcoumarin- 3-acetyl)amino)hexanoic acid, succinimidyl ester; DPH-PC, 2-(3-(diphenylhexatrienyl) propanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine; ES-MS, electron spray mass spectrometry; HPLC, high pressure liquid chromatography; LUVs, large unilamellar vesicles; $PI(3,4)P_2$, phosphatidylinositol-3,4-bisphosphate

* Corresponding author. Fax: +1-631-444-3432; E-mail: smcl@epo.som.sunysb.edu

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1. Introduction

Although phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) comprises only about 1% of the phospholipids in a mammalian cell plasma membrane [1,2], it plays at least five important roles in cellular processes. First, hydrolysis of $PI(4,5)P_2$ by phosphoinositide-specific phospholipase C (PLC) [3] produces the second messengers inositol-1,4,5-trisphosphate ($I(1,4,5)P_3$) and diacylglycerol, which release calcium from intracellular stores and activate protein kinase C [4–6]. Furthermore, $PI(4,5)P_2$ binds to the pleckstrin homology (PH) domain of PLC-δ with high (μM) affinity [7,8]. Second, PI-3 kinase phosphorylates $PI(4,5)P_2$ to produce phosphatidylinositol-3,4,5-trisphosphate ($PI(3,4,5)P_3$), a lipid that has been implicated in regulating cell metabolism, proliferation, and survival [9]. Third, $PI(4,5)P_2$ may play a role in cytoskeleton rearrangements [10,11]. Fourth, there is strong evidence that $PI(4,5)P_2$ is required for exocytosis [12–14]. Fifth, $PI(4,5)P_2$ regulates the activity of ion channels, e.g. K_{ATP} channels [15–18], and enzymes, e.g. phospholipase D (PLD) [19].

How can $PI(4,5)P_2$ play so many different roles? One widely discussed possibility is that localization of $PI(4,5)P_2$ to different cellular membranes and lateral domains within a membrane allows segregation of the different signaling pathways that depend on $PI(4,5)P_2$ [14,20]. Biochemical analysis, e.g. membrane fractionation and lipid extraction [2,21–24], indicate $PI(4,5)P_2$ is localized mainly in the plasma membrane, nucleus, and synaptic vesicles [12,25,26]. It is important to have probes specific for $PI(4,5)P_2$ that can be observed in living cells using a non-invasive method, such as fluorescence microscopy.

Constructs of the PLC-δ PH domain with green fluorescent protein (GFP) have been used to study $PI(4,5)P_2$ distribution in living cells [27,28]. Previous studies showed this PH domain binds specifically, with μM affinity to $PI(4,5)P_2$ [7,8]. The molecular basis for the specificity is understood. The PLC-δ PH domain is a rigid cage-like structure that does not undergo a significant change in conformation when it binds to the headgroup of $PI(4,5)P_2$ [29]. Fluorescence microscopy studies of the GFP-PH constructs show that a major pool of $PI(4,5)P_2$ in quiescent cells is in the plasma membrane [27,28], in agreement with the results from biochemical analyses and immunofluorescence studies with $PI(4,5)P_2$-specific antibodies [30,31]. Activation of PLC (e.g. by increasing the Ca^{2+} concentration in the cytoplasm [28] or stimulating the receptor for platelet activating factor [27]) produces rapid redistribution of the GFP-PH constructs to the cytosol. This redistribution of GFP-PH construct is interpreted as reflecting a decrease in the $PI(4,5)P_2$ concentration in the plasma membrane. A recent study [32] suggests, however, that translocation of the GFP-PH constructs to the cytosol might reflect an increase in cytosolic $I(1,4,5)P_3$ rather than a decrease in $PI(4,5)P_2$ in the plasma membrane. Previous studies [7,8] demonstrated that the PLC-δ PH domain binds 10-fold more strongly to $I(1,4,5)P_3$ than to $PI(4,5)P_2$, so a small increase in cytosolic $I(1,4,5)P_3$ could cause a significant redistribution of the GFP-PH probe. Hence, new probes that are specific for $PI(4,5)P_2$ but do not have a higher affinity for $I(1,4,5)P_3$ are needed to complement the GFP-PLC-δ PH constructs in studies of the inositol phospholipid role in cellular functions.

Here we explore whether fluorescently labeled neomycins may be used as probes for $PI(4,5)P_2$ in biological membranes. We have chosen neomycin because it is a small molecule that binds strongly to $PI(4,5)P_2$ [33–35]. Neomycin is an aminoglycoside antibiotic with six primary amine groups and an average charge of +4.5 in a solution containing 100 mM KCl, pH 7 [36][1]. Fig. 1 shows the chemical structures of fluorescently labeled neomycins used in this study. The affinity of neomycin for $PI(4,5)P_2$ in a $PC/PI(4,5)P_2$ membrane is about 10^5 M^{-1} [35]. We made three types of measurements. First, we determined if addition of a fluorophore to neomycin alters its interaction with $PI(4,5)P_2$. Second, we tested whether addition of $I(1,4,5)P_3$ to the mixture of fluorescently labeled neomycin and vesicles displaces the probe from the membrane. Third, we tested if the labeled neomycin can be used to distinguish between phospholipid membranes with or without

[1] In our hands small basic peptides that have been claimed to bind specifically to $PI(4,5)P_2$ (for a review see [14]), such as a peptide from PLC-δ PH domain, show little affinity for $PI(4,5)P_2$ in direct equilibrium binding measurements [54]. Neomycin is the only small organic molecule that, to our knowledge, binds with μM affinity to $PI(4,5)P_2$.

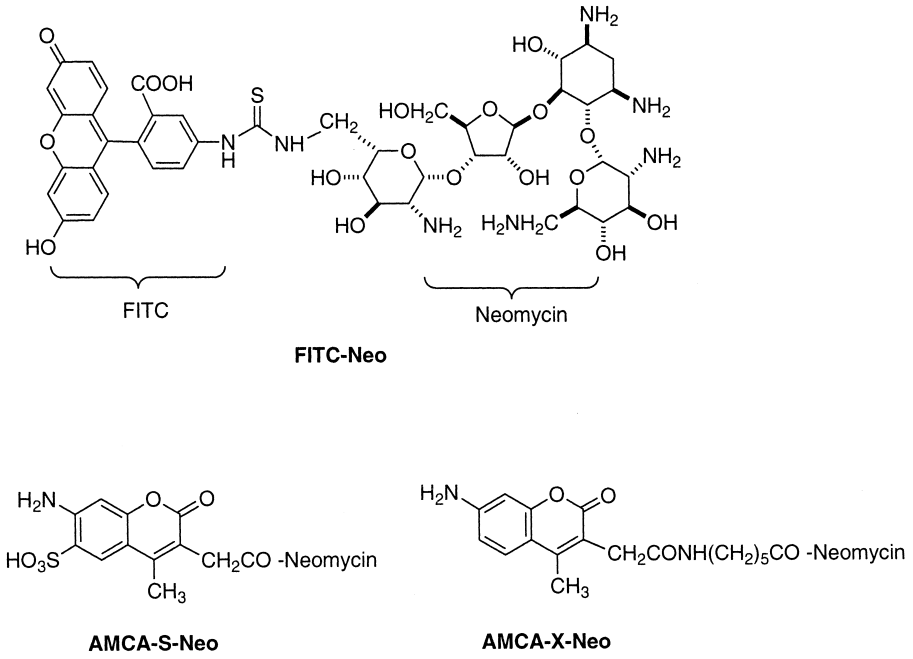

Fig. 1. Chemical structures of the fluorescent neomycin compounds used in this study. FITC is fluorescein-5-isothiocyanate, AMCA-S is 7-amino-3-((((succinimidyl)oxy)carbonyl)methyl)-4-methylcoumarin-6-sulfonic acid, and AMCA-X is 6-((7-amino-4-methylcoumarin-3-acetyl)amino)hexanoic acid. For each of these compounds, the neomycin can be modified at either of the two aminomethylene ($-CH_2NH_2$) groups, and the material used is an inseparable mixture of the two isomers.

physiological concentrations of $PI(4,5)P_2$, 1–5% total lipid. We conclude that fluorescently labeled neomycin is a potentially useful probe that could complement GFP-PH constructs in the study of $PI(4,5)P_2$ distribution in cellular membranes.

2. Materials and methods

2.1. Materials

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (PC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylserine (PS), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol (PG), and lissamine rhodamine B-dipalmitoyl-phosphatidylethanolamine (Rh-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). Radiolabeled 1,2-di[1-^{14}C]oleoyl-L-3-phosphatidylcholine ([^{14}C]PC) was purchased from Amersham (Arlington Heights, IL), fluorescein-5-isothiocyanate (FITC), 7-amino-3-((((succinimidyl)oxy)-carbonyl)methyl)-4-methylcoumarin-6-sulfonic acid (AMCA-S), 6-((7-amino-4-methylcoumarin-3-acetyl)-amino)hexanoic acid, succinimidyl ester (AMCA-X), and 2-(3-(diphenylhexatrienyl) propanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (DPH-PC) were from Molecular Probes (Eugene, OR). $PI(4,5)P_2$ (ammonium salt) was isolated from bovine brain extract (Sigma, St. Louis, MO) as described previously [37]. Phosphatidylinositol-3,4-bisphosphate, dipalmitoyl, $PI(3,4)P_2$ (ammonium salt) was purchased from Matreya (Pleasant Gap, PA). Neomycin trisulfate was from Sigma. $I(1,4,5)P_3$ was from Calbiochem-Novabiochem (San Diego, CA) and Sigma. Our standard buffer solution consists of 100 mM KCl, 1 mM MOPS, pH 7.

2.2. Fluorescently labeled neomycin

Neomycin was labeled with FITC, AMCA-S, or AMCA-X as described below. For the labeling with FITC, neomycin trisulfate (45.4 mg, 0.05 mmol) and fluorescein isothiocyanate (FITC; 3.75 mg, 0.0083 mmol) were dissolved in 1 ml of 1 N TEAB (triethylammonium bicarbonate, pH 7.5); the solution was stirred for 1 h and left unstirred for 36 h at room temperature. The solution was concentrated in vacuum, and the residue was dissolved in 1 ml of water.

Aliquots of the solution (50 μl each) were purified by HPLC on a C_{18} silica gel reversed-phase column (0.45×25 cm), with a linear gradient elution of buffer A (0.06% aq. trifluoroacetic acid) to buffer B (95% acetonitrile, 5% water, 0.052% trifluoroacetic acid), a flow rate of 1 ml/min, and absorbance monitoring at 494 nm. A single fraction midway through the gradient was collected from each run, and those fractions were combined, concentrated, and lyophilized to give a yellow powder (5.5 mg, yield 64%). Electron spray mass spectrometry (ES-MS) gives *m/z* 1003.63 for the product. Calculated molecular weight is 1004.2. For labeling with AMCA-S, a coumarin derivative, neomycin trisulfate (27 mg, 0.0297 mmol) and AMCA-S-SE (7-amino-3-(((succinimidyl)oxycarbonyl)methyl)-4-methyl-coumarin-6-sulfonic acid, 2.0 mg, 0.050 mmol) were dissolved in 0.2 ml of 0.1 M $NaHCO_3$ (pH 7.55) and the solution was stirred for 24 h at room temperature. The solution was concentrated in vacuum, the residue was dissolved in 1 ml of water, and aliquots were purified by the same HPLC system as above except that detection was at 353 nm. Fractions containing the product were pooled together and lyophilized as described above to give a pale yellow powder (2.5 mg, yield 55%). ES-MS gives *m/z* 910.02. Calculated molecular weight is 910.58. For labeling with AMCA-X, another coumarin derivative, neomycin trisulfate (51.1 mg, 0.056 mmol) and AMCA-X-SE 6-(7-amino-4-methylcoumarin-3-acetyl)amino)hexanoic acid, succinimidyl ester (2.5 mg, 0.0056 mmol) were dissolved in 0.2 ml of 1 N TEAB and 0.1 ml of dimethylformamide (DMF). The solution was stirred for 5 h and left unstirred for 72 h at room temperature. The solution was processed as described for AMCA-S-neomycin to give a yellow powder (2.8 mg, yield 51%). ES-MS gives *m/z* 943.00. Calculated molecular weight is 944.04. The chemical structures of the compounds we used are shown in Fig. 1. Fluorescently labeled neomycin was dissolved in water (Millipore filtered and double quartz distilled) and stored frozen in small aliquots. We determined the concentration of the probe by measuring the absorbance of the fluorophores (for fluorescein ε= 73 000 M^{-1} cm^{-1}, pH 9; for coumarin ε= 19 000 M^{-1} cm^{-1}, methanol).

The fluorescence intensity of FITC- and coumarin-labeled neomycin, either in solution or bound to a membrane, increases linearly over the range of concentrations we investigated (10–250 nM neomycin). Control experiments show fluorescently labeled neomycin (30–200 nM) remains in solution when the samples are centrifuged for 1 h. at 100 000×*g*. This result is consistent with our assumption that the labeled neomycin exists as a monomer in solution. There is, however, some loss of the probe on the walls of the centrifuge tube; we minimized the loss by pre-washing the tubes with unlabeled neomycin sulfate. For FITC-neomycin it was sufficient to pre-wash tubes with a solution containing the same concentration of neomycin that was used in the binding experiment. When the tubes were pre-washed, the concentration of labeled neomycin in buffer solution was the same before and after centrifugation, indicating this treatment prevents significant adsorption of the labeled aminoglycoside to the tube. Coumarin-labeled aminoglycosides adhere more strongly to the centrifuge tubes; hence we pre-washed the tubes with a solution containing a 10-fold higher concentration of unlabeled neomycin for experiments with this probes.

2.3. Vesicle preparation

We measured initial concentration of lipids in chloroform using a Cahn electrobalance. In some experiments, trace amounts of [^{14}C]PC were added to the lipid mixture to determine the final lipid concentration. The concentrations of I(1,4,5)P_3 and lipid in the solution were also confirmed by phosphate analysis [38]. Lipid mixtures were dried on a rotary evaporator and resuspended in a sucrose buffer (176 mM sucrose, 1 mM MOPS, pH 7) to produce multilamellar vesicles. Large unilamellar vesicles (LUVs) were made by extruding multilamellar vesicles 10 times through a stack of two polycarbonate filters (100 nm pore diameter) after 5 freeze–thaw cycles [39]. Vesicles with and without PI(4,5)P_2 were prepared on the same day from the same stock of lipid solutions in chloroform. PI(4,5)P_2 in a membrane has an effective net charge of −3 in a 100 mM KCl, pH 7 solution [36]. Thus we replaced 3 PS by 1 PI(4,5)P_2 in the PC/PS/PI(4,5)P_2 vesicles to keep the charge density the same as in the PC/PS vesicles.

All preparations, binding measurements, and microscopy observations were made at room temperature, 22–23°C.

2.4. Equilibrium binding measurements

Equilibrium binding of labeled neomycins was determined using a sucrose-loaded vesicles assay [40]. Briefly, labeled neomycin was mixed with the sucrose-loaded vesicles in 100 mM KCl and the mixture was centrifuged (1 h, 100 000 × *g*) to separate the vesicles, which form a pellet, from the unbound neomycin. The supernatant was removed carefully and its FITC fluorescence was measured (the structure of the labeled neomycin is shown in Fig. 1). In the experiments with FITC-neomycin, the FITC fluorescence of the supernatant reflects how much neomycin remained unbound. We obtained the same binding affinity (see Eqs. 1 and 3) using two different FITC-neomycin concentrations (100 and 200 nM), which controls for a number of possible artifacts (e.g. that FITC-neomycin does not change the vesicle surface charge density and that it binds as a monomer). We did not use pellet samples to calculate the binding because FITC-neomycin bound to a membrane gives a 5-fold lower signal than in solution. This is obviously a disadvantage of using this particular probe with biological membranes.

Coumarin fluorescence was the same from a labeled neomycin either in buffer or bound to a membrane for both AMCA-S- and AMCA-X-neomycin (the structures of the labels are shown in Fig. 1). Hence fluorescence measurements of both supernatant and pellet were used to calculate percent coumarin-neomycin bound as discussed in [40]. We obtained the same binding affinity (see Eqs. 1 and 3) using 35, 70, or 140 nM of AMCA-X-neomycin.

We describe neomycin binding to membranes with $PI(4,5)P_2$ in two ways. First, we determine a molar partition coefficient, K; this calculation does not require any assumptions about the mechanism(s) involved in the association of the probe with the membrane. Second, we assume that labeled neomycin forms a 1:1 complex with $PI(4,5)P_2$ and calculate the apparent specific association constant, K_{N-P}, for this complex; this assumption is consistent with the available data for unlabeled neomycin [35,36,41].

The molar partition coefficient, K, is the ratio of the molar concentration of the membrane-bound neomycin, $[N_b]$, to the concentration of lipid accessible to neomycin, [L], and the concentration of neomycin free in the aqueous solution, $[N_f]$:

$$K = \frac{[N_b]}{[L][N_f]}. \quad (1)$$

The accessible lipid concentration, the concentration of the lipid comprising the outer leaflet of the vesicles, equals about one half of the total lipid concentration for LUVs. In the limit of low neomycin concentration (when binding of neomycin does not change the affinity of the vesicle for the probe), K equals the reciprocal of the accessible lipid concentration that binds 50% of neomycin (i.e. $[N_b] = [N_f]$). Eq. 2 describes dependence of the percent neomycin bound on the accessible lipid concentration [L]:

$$\%\ \text{neomycin bound} = 100 \times K[L]/(1 + K[L]). \quad (2)$$

It is obtained from the mass conservation for neomycin and the definition of the molar partition coefficient (Eq. 1) with no assumptions about binding mechanism (for details see [42–44]). Note Eq. 2 is equivalent to an equation obtained assuming binding of one neomycin per one phospholipid. The molar partition coefficient was obtained by fitting Eq. 2 to the data with one unknown parameter, K.

If we assume that binding is due to formation of the specific 1:1 neomycin:$PI(4,5)P_2$ complex, the apparent association constant, K_{N-P}, for this complex is the ratio of the concentration of the neomycin-$PI(4,5)P_2$ complex, $[N–PIP_2]$, to the concentration of free accessible $PI(4,5)P_2$, $[PIP_2]$, and the concentration of neomycin free in aqueous solution, $[N_f]$:

$$K_{N-P} = \frac{[N - PIP_2]}{[PIP_2][N_f]}. \quad (3)$$

2.5. Measurements of the effect of $I(1,4,5)P_3$ addition on FITC-neomycin binding

We pre-mixed 80, 100, or 160 nM FITC-neomycin with sucrose-loaded 5:1 PC/PS vesicles with 1–2% $PI(4,5)P_2$ (several different lipid concentrations were used) and allowed the solution to equilibrate for 15 min. We then added different amounts of $I(1,4,5)P_3$ to the neomycin-vesicle solution. The samples were centrifuged (1 h, 100 000 × *g*), the superna-

tant was removed, and FITC fluorescence was measured. Percent FITC-neomycin bound was calculated as described for standard equilibrium binding experiments.

The association constant of neomycin with I(1,4,5)P_3 is

$$K_{N_f-IP3} = \frac{[N_f - IP_3]}{[IP_3][N_f]}, \quad (4)$$

where [N–IP_3] is the concentration of neomycin bound to I(1,4,5)P_3 and [IP_3] is the concentration of I(1,4,5)P_3 free in solution. Under our conditions, [N] is much lower than $[PIP_2]_{total}$ and $[IP_3]_{total}$. Thus the free concentrations of PI(4,5)P_2 and I(1,4,5)P_3 are approximately equal to the corresponding total concentrations: $[PIP_2] \approx [PIP_2]_{total}$ and $[IP_3] \approx [IP_3]_{total}$. Then combining Eqs. 3 and 4, and the mass conservation law for PI(4,5)P_2, I(1,4,5)P_3, and neomycin, we obtain Eq. 5 which describes the dependence of the fraction of neomycin bound to PI(4,5)P_2, [N–PIP_2]/[N], on the concentration of I(1,4,5)P_3 added to the aqueous phase:

$$\frac{[N - PIP_2]}{[N]} = \frac{K_{N-P}[PIP_2]}{1 + K_{N-P}[PIP_2] + K_{N-IP3}[IP_3]}. \quad (5)$$

2.6. Preparation of giant vesicles for microscopy

Giant unilamellar vesicles for microscopy studies were prepared using a gentle hydration method [45]. For each experiment, we prepared two sets of vesicles with the same surface charge density: one set contained PI(4,5)P_2; the other set contained a fluorescent marker, but no PI(4,5)P_2. For example, we prepared 66:28:2 PC/PS/PI(4,5)P_2 and 2:1 PC/PS vesicles, where the latter contained 0.3 mol% DPH-PC, allowing us to distinguish these vesicles from the unlabeled vesicles containing PI(4,5)P_2 in a mixed sample. An appropriate lipid mixture in chloroform was dried in a v-shaped flask on a rotary evaporator under vacuum for 30 min to form a thin film. The dried film was prehydrated for 20 min at 45°C with water-saturated argon, and 4–6 ml of the buffer solution was gently added to the flask. The sealed flask was incubated at 37°C for 12–24 h, and 100–200 μl of the upper part of the solution was harvested and used for microscopy studies. Vesicles with or without PI(4,5)P_2 were mixed together at approximately the same concentrations and the ratio was checked by viewing a slide. After adding labeled neomycin to the vesicle mixture, we transferred the sample to a glass slide and added agarose to inhibit vesicle movement. A typical sample contained about 1 mM of lipid, 0.2–1 μM of neomycin and 0.5% w/v agarose. (We cannot measure the exact concentration of lipid on a slide because the solution of giant vesicles is very heterogeneous.)

We conducted the same experiments using also 5:1 PC/PS giant vesicles with and without 2 mol% PI(4,5)P_2. We labeled the vesicles without PI(4,5)P_2 with either DPH-PC or Rh-DPPE. We obtained the same results using two different lipid markers. We also tested AMCA-X-neomycin.

2.7. Imaging

Images were obtained using a fixed stage microscope (Axioskop, Zeiss), Plan-NEOFLUOR 63×-oil objective (Zeiss), and Micro-Max Princeton CCD camera (Princeton). The fluorescence of FITC was measured using a short band path filter set 41001 from Chroma: exciter 460 ± 20 nm, beam splitter 505 nm, and emitter 535 ± 25 nm. The fluorescence of coumarin or DPH was measured using a short band path filter set 31000 from Chroma: exciter 360 ± 20 nm, beam splitter 400 nm, and emitter 460 ± 25 nm. Filters for rhodamine were from Omega Optical: exciter 510 ± 13.5 nm, emitter > 560 nm.

3. Results

We studied the binding of neomycin to phospholipid vesicles with and without PI(4,5)P_2. Because neomycin has an average charge of +4.5 in 100 mM KCl, pH 7 [36], it can bind to negatively charged membranes both via a non-specific electrostatic accumulation in the diffuse double layer (Gibbs surface excess, for a review see [46]) and by formation of a specific complex with the acidic lipid PI(4,5)P_2. To distinguish between these two possibilities, we measured the binding of fluorescently labeled neomycin to vesicles with the same surface charge density, but different concentrations of PI(4,5)P_2.

The cytoplasmic leaflet of the plasma membrane of

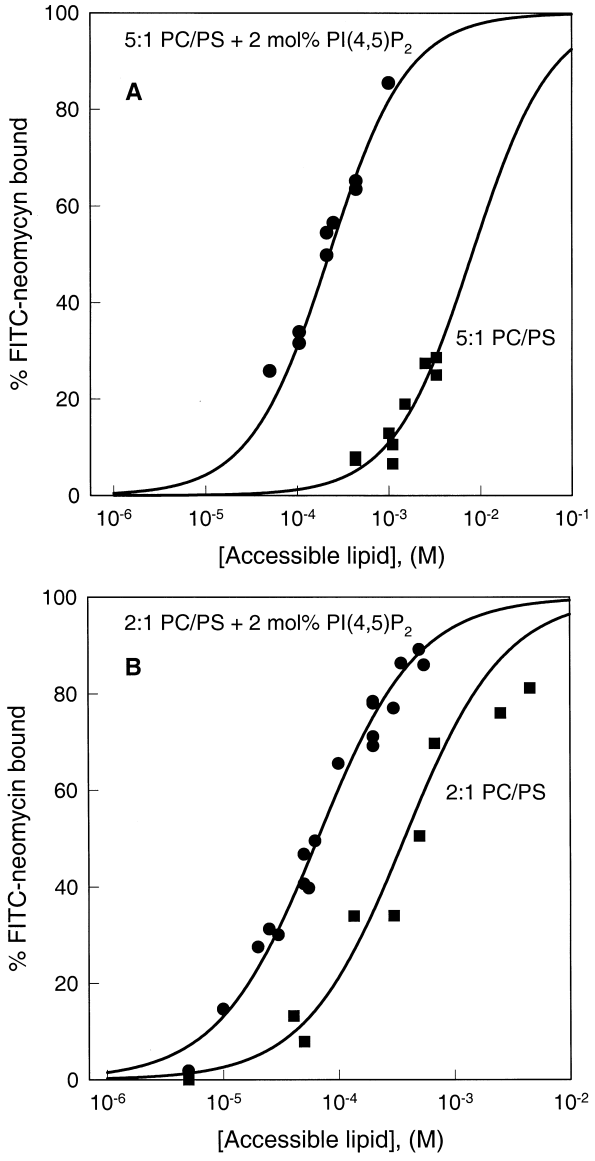

Fig. 2. $PI(4,5)P_2$ increases FITC-neomycin binding to 5:1 PC/PS (A) and 2:1 PC/PS (B) vesicles. In both figures the percent FITC-neomycin (100 nM) bound to vesicles is plotted against the concentration of the accessible lipid. (A) The vesicles were made from a 5:1 PC/PS (■) or a PC/PS/$PI(4,5)P_2$ (●) lipid mixture with the same surface charge density and 2 mol% $PI(4,5)P_2$. Each point is an average of duplicate samples. The solid curves are the best fits of Eq. 2 to the data with the molar partition coefficients of $K=1.2\times10^2$ M^{-1} and 4.5×10^3 M^{-1}. Addition of 2% $PI(4,5)P_2$ increases the binding of FITC-neomycin to the 5:1 PC/PS vesicles 40-fold. (B) The vesicles were made from a 2:1 PC/PS (■) or a PC/PS/$PI(4,5)P_2$ (●) lipid mixture with the same surface charge density and 2 mol% $PI(4,5)P_2$. Each point is an average of duplicate samples. The solid curves are the best fits of Eq. 2to the data with the molar partition coefficients of $K=2.7\times10^3$ M^{-1} and 1.5×10^4 M^{-1}. Addition of 2 mol% $PI(4,5)P_2$ increases the binding of FITC-neomycin to the 2:1 PC/PS vesicles 6-fold. The experiments in this figure and Fig. 3 were done using sucrose-loaded vesicles and our standard buffer, 100 mM KCl, 1 mM MOPS, pH 7.

a typical mammalian cell contains 15–30 mol% PS and 1–2 mol% $PI(4,5)P_2$ [1,21,22,25,47]. Hence we first tested FITC-neomycin binding to phospholipid vesicles with 17 mol% (5:1 PC/PS) and 33 mol% (2:1 PC/PS) PS. Then we substituted 6 mol% PS in the lipid mixture with 2 mol% $PI(4,5)P_2$ to keep the surface charge density constant and measured the binding of labeled neomycin to these $PI(4,5)P_2$ containing vesicles.

3.1. *$PI(4,5)P_2$ increases the binding of FITC-neomycin to phospholipid vesicles*

Fig. 2A shows binding isotherms for FITC-neomycin and 5:1 PC/PS (squares) or PC/PS/$PI(4,5)P_2$ (circles) vesicles with the same surface charge density. We first describe the data in terms of the molar partition coefficient, K, and then interpret the results assuming formation of a 1:1 neomycin:PI(4,5)P2 complex. The molar partition coefficients are $K=1.2\times10^2$ M^{-1} for 5:1 PC/PS vesicles without $PI(4,5)P_2$ and $K=4.5\times10^3$ M^{-1} for the vesicles with 2 mol% $PI(4,5)P_2$. Thus addition of $PI(4,5)P_2$ to the lipid mixture increases the binding 40-fold.

Fig. 2B shows binding isotherms for FITC-neomycin and 2:1 PC/PS (squares) or PC/PS/$PI(4,5)P_2$ (circles) vesicles with the same surface charge density. The molar partition coefficient is 6-fold higher for the vesicles containing 2 mol% $PI(4,5)P_2$ ($K=2.7\times10^3$ M^{-1} for 2:1 PC/PS[2] and 1.5×10^4 M^{-1} for PC/PS/$PI(4,5)P_2$ vesicles).

The results shown in Fig. 2A,B indicate that FITC-neomycin binds specifically to $PI(4,5)P_2$. If

[2] Binding of FITC-neomycin to PC/PS vesicles depends steeply on the mole fraction of PS in the membrane, as expected for this poly-basic compound. Binding of FITC-neomycin increases 25-fold when the mol% PS is increased 2-fold from 17 to 33 mol% (Fig. 2).

we calculate the apparent specific association constant, K_{N-P}, for a 1:1 neomycin:PI(4,5)P_2 complex from Eq. 3, we obtain $K_{N-P} = 2\times10^5$ M^{-1} and 5×10^5 M^{-1} for vesicles with a surface charge density equivalent to 17 and 33 mol% PS, respectively. We also measured FITC-neomycin binding to 98:2 PC/PI(4,5)P_2 vesicles and obtained a molar partition coefficient $K = 1.5\times10^3$ M^{-1} and an apparent specific association constant $K_{N-P} = 10^5$ M^{-1} (data not shown). The value of K_{N-P} is the same as that reported for unlabeled neomycin binding to PC/PI(4,5)P_2 vesicles [35]; thus, attachment of this fluorophore to neomycin does not affect significantly neomycin affinity for PI(4,5)P_2.

As an aside, we note that K_{N-P} is only an *apparent* binding constant because it depends on the surface charge density of the vesicles (mol% acidic lipid): for FITC-neomycin K_{N-P} equals 10^5 M^{-1} for PC/PI(4,5)P_2, 2×10^5 M^{-1} for 5:1 PC/PS+2 mol% PI(4,5)P_2, and 5×10^5 M^{-1} for 2:1 PC/PS+2 mol% PI(4,5)P_2 vesicles. The K_{N-P} depends on the surface charge density of the vesicles (surface potential) because the concentration of poly-basic neomycin close to the negatively charged membrane is enhanced by a non-specific long-range electrostatic attraction (Boltzmann factor) (see [35,48] for a detailed discussion).

3.2. The molar partition coefficient increases linearly with mol% PI(4,5)P_2 in the membrane

To study how the binding of neomycin depends on the mol% PI(4,5)P_2 in a membrane, we measured binding of the FITC-labeled neomycin to 2:1 PC/PS vesicles containing 0, 1, 2, or 4 mol% PI(4,5)P_2. The results are summarized in Table 1. The first column gives the composition of the vesicles used. The second column gives the values of K obtained by fitting Eq. 2 to the data from equilibrium binding experiments (data from Fig. 2B for 0 and 2 mol% PI(4,5)P_2, data not shown for 1 and 4 mol% PI(4,5)P_2). There is a linear relation between the amount of PI(4,5)P_2 in the vesicles and the molar partition coefficient: increasing the mol% PI(4,5)P_2 in membrane 2-fold increases K approximately 2-fold. The third column gives the apparent specific association constant, K_{N-P}, for neomycin and PI(4,5)P_2 calculated from the data using Eq. 3; as expected for a 1:1 complex, K_{N-P} is independent of the mol% PI(4,5)P_2 in the membrane.

All our equilibrium binding results are consistent with the formation of a 1:1 complex between PI(4,5)P_2 and FITC-neomycin, as was suggested by previous studies using unlabeled neomycin [35,36,41].

3.3. PI(4,5)P_2 increases the binding of coumarin-labeled neomycin to 2:1 PC/PS vesicles

We also tested neomycin labeled with the coumarin derivatives AMCA-S and AMCA-X and obtained qualitatively similar results to those reported above for FITC-neomycin. Fig. 3 shows AMCA-X binding isotherms for 2:1 PC/PS vesicles and PC/PS/PI(4,5)P_2 vesicles with the same surface charge density, with $K = 2\times10^4$ M^{-1} and 1×10^5 M^{-1}, respectively. Addition of 2 mol% PI(4,5)P_2 increases binding of AMCA-X-labeled neomycin to 2:1 PC/PS vesicles 5-fold.

AMCA-S-neomycin binds to membranes 5-fold less strongly (data not shown) and AMCA-X-neomycin almost 10-fold more strongly than FITC-neomycin (compare Figs. 2B and 3). This increase in the

Table 1
The molar partition coefficients and the apparent association constants for the FITC-neomycin binding to 2:1 PC/PS vesicles with 0–4 mol% PI(4,5)P_2

Lipid mixture (mol% PI(4,5)P_2 in the mixture)	Molar partition coefficient, K (M^{-1})	Apparent association constant, K_{N-P} (M^{-1})
66:34 PC/PS, 0 mol%	2.7×10^3	NA[a]
66:31:1 PC/PS/PI(4,5)P_2, 1 mol%	9×10^3	5×10^5
66:28:2 PC/PS/PI(4,5)P_2, 2 mol%	1.5×10^4	5×10^5
66:22:4 PC/PS/PI(4,5)P_2, 4 mol%	3.4×10^4	7×10^5

[a]NA, not applicable.

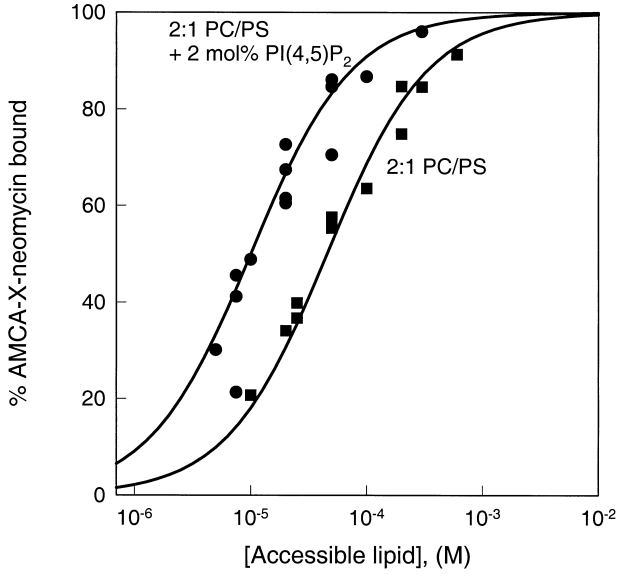

Fig. 3. $PI(4,5)P_2$ increases AMCA-X-neomycin binding to 2:1 PC/PS vesicles. AMCA-X-neomycin binding isotherms for 2:1 PC/PS (■) and 66:28:2 PC/PS/$PI(4,5)P_2$ (●) vesicles. The solid curves are the best fits obtained using Eq. 2 with the molar partition coefficients $K=2\times10^4$ M^{-1} and 1×10^5 M^{-1}, respectively. Addition of 2 mol% $PI(4,5)P_2$ increases the binding of AMCA-X-neomycin to the 2:1 PC/PS vesicles 5-fold.

binding of AMCA-X-neomycin to membranes compared to binding of unlabeled neomycin could be due to a hydrophobic interaction of the fluorophore with the lipid head group region. The AMCA-X fluorophore has a hydrophobic linker (see Fig. 1) that could allow both electrostatic interaction of neomycin and hydrophobic interaction of coumarin with the membrane, increasing the binding.

3.4. FITC-neomycin does not distinguish between $PI(3,4)P_2$ and $PI(4,5)P_2$

We measured the binding of FITC-neomycin to 2:1 PC/PS vesicles containing 1 mol% phosphatidylinositol-3,4-bisphosphate ($PI(3,4)P_2$) or phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) (data not shown). The FITC-neomycin binds equally well to vesicles with either 1 mol% $PI(3,4)P_2$ or with 1 mol% $PI(4,5)P_2$. $PI(4,5)P_2$ is the major bisphosphate inositol lipid in mammalian cells and the distribution of the probe should reveal mainly its location in a living cell.

3.5. $I(1,4,5)P_3$ causes only slight translocation of labeled neomycin from PC/PS/$PI(4,5)P_2$ bilayers

Upon activation of PLC in a cell, it cleaves $PI(4,5)P_2$ to produce $I(1,4,5)P_3$ that might compete for a $PI(4,5)P_2$-specific probe. Because $I(1,4,5)P_3$ is cytosolic, binding of $I(1,4,5)P_3$ to the probe can cause translocation of the probe into the cytosol even when the level of $PI(4,5)P_2$ in the membrane does not change significantly: this probably occurs with the GFP-PLC-δ PH constructs [32]. We tested whether addition of $I(1,4,5)P_3$ to the mixture of FITC-neomycin with 5:1 PC/PS vesicles containing 1–2 mol% $PI(4,5)P_2$ causes translocation of FITC-neomycin from the membrane into the aqueous solution. In our experiments, the concentration of accessible $PI(4,5)P_2$ (2–10 μM) is approximately equal to the concentration of $PI(4,5)P_2$ present in a cell plasma membrane. Eq. 5 describes how the fraction of neomycin bound to $PI(4,5)P_2$ depends on the concentration of $I(1,4,5)P_3$ added to the aqueous phase. We can describe our experimental data on depen-

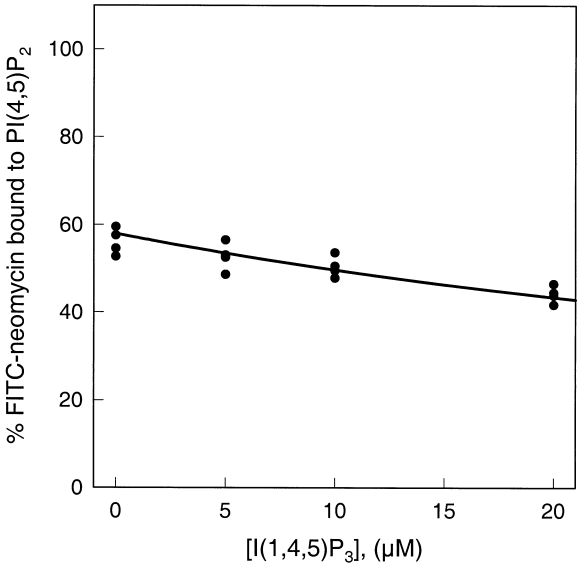

Fig. 4. $I(1,4,5)P_3$ produces little translocation of neomycin from the 5:1 PC/PS vesicles with 2 mol% $PI(4,5)P_2$. 100 nM FITC-neomycin was pre-mixed with sucrose loaded 5:1 PC/PS vesicles with 2 mol% $PI(4,5)P_2$ (300 μM accessible lipid, 6 μM accessible $PI(4,5)P_2$), then 5, 10, or 20 μM $I(1,4,5)P_3$ was added and the samples were centrifuged to determine the percent FITC-neomycin that remained bound to $PI(4,5)P_2$ (●). Results from two independent experiments are shown. The solid line is a theoretical curve obtained from Eq. 5 with $K_{IP3}=4\times10^4$ M^{-1}.

dence of [N-PI(4,5)P_2] on the amount of I(1,4,5)P_3 added to the aqueous phase with a value for K_{N-IP3} similar to the association constant of neomycin to PI(4,5)P_2. Fig. 4 shows a typical result. In the absence of I(1,4,5)P_3, 55% FITC-neomycin bound to 5:1 PC/PS vesicles with 2 mol% PI(4,5)P_2 (left hand data in Fig. 4). Addition of 5, 10, or 20 μM I(1,4,5)P_3 changed the percent FITC-neomycin bound to the vesicles only slightly. The theoretical curve obtained from Eq. 5 with $K_{N-IP3} = 4 \times 10^4$ M^{-1} describes the experimental data adequately. This value for K_{N-IP3} is comparable to, but slightly less than, the association constant for neomycin and PI(4,5)P_2 in a PC/PI(4,5)P_2 membrane, $K_{N-P} = 1 \times 10^5$ M^{-1}. For 2:1 PC/PS membranes with 2 mol% PI(4,5)P_2, or membranes with more PI(4,5)P_2, we observed even less translocation of FITC-neomycin than is shown in Fig. 4 (data not shown), as expected from Eq. 5 for conditions where K_{N-P} is higher. From these data we conclude that 1–10 μM I(1,4,5)P_3 should not cause significant translocation of labeled neomycin from the plasma membrane into the cytoplasm.

3.6. *Specific binding of FITC-neomycin to giant vesicles containing PI(4,5)P_2 can be detected using fluorescence microscopy*

We studied the distribution of fluorescently labeled neomycin on vesicles with and without PI(4,5)P_2 in a mixture of the two types of vesicles using fluorescence digital microscopy. Three kinds of images were taken from the same location on the slide. Phase contrast images revealed all the vesicles in focus at this location. DPH fluorescence images re-

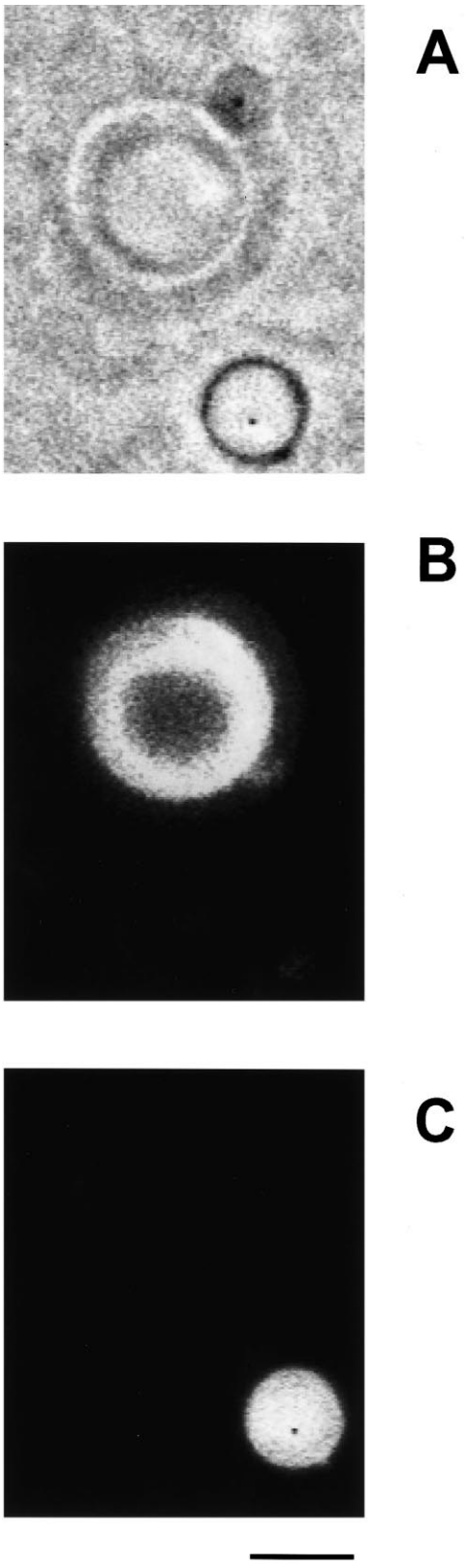

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Fig. 5. Preferential binding of FITC-neomycin to vesicles containing PI(4,5)P_2 in a mixture of vesicles can be detected using fluorescence microscopy. Two samples of 2:1 PC/PS vesicles, one with and the other without 2 mol% PI(4,5)P_2, were prepared separately, then mixed prior to addition of FITC-neomycin (0.5 μM). The vesicles without PI(4,5)P_2 contained 0.3 mol% DPH-PC as a marker. Three images were taken from the same location on the slide. (A) Phase contrast image shows two large vesicles. (B) DPH-PC fluorescence indicates that the upper vesicle contains no PI(4,5)P_2, but the lower vesicle contains PI(4,5)P_2. (C) The lower vesicle binds FITC-neomycin. Scale bar: 10 μm.

vealed vesicles prepared without $PI(4,5)P_2$. FITC fluorescence images revealed vesicles that bound FITC-neomycin. A typical example of the images obtained is shown in Fig. 5A–C. The phase contrast image in Fig. 5A shows two large vesicles: a ~20-μm vesicle in the center that is slightly out of focus and a ~10 μm vesicle in the lower right corner. (A dot adjacent to the upper right corner of the large vesicle is an artifact.) Fig. 5B shows the image obtained through the filter set for DPH dye: the central vesicle has DPH fluorescence and is from the preparation containing no $PI(4,5)P_2$; the lower vesicle has no DPH fluorescence, and thus contains $PI(4,5)P_2$. Fig. 5C shows the image obtained through the filter set for FITC dye: the lower vesicle reveals strong FITC fluorescence. This vesicle contains $PI(4,5)P_2$ and has FITC-neomycin bound to it, whereas there is almost no binding of FITC-neomycin to the upper vesicle that contains no $PI(4,5)P_2$. The results shown here are consistent with the direct binding measurements presented in Fig. 2B.

We also detected FITC-neomycin bound only to 5:1 PC/PS with 2 mol% $PI(4,5)P_2$ vesicles in a mixture with 5:1 PC/PS vesicles with and without $PI(4,5)P_2$. Preferential binding of AMCA-X-neomycin to giant unilamellar vesicles containing $PI(4,5)P_2$ was observed in fluorescence microscopy experiments similar to those shown in Fig. 5 using Rh-PE as a lipid marker for vesicles without $PI(4,5)P_2$ (data not shown). The results on $PI(4,5)P_2$ distribution obtained with coumarin-labeled neomycin are consistent with the results from the direct binding measurements shown in Fig. 3. We conclude that fluorescently labeled neomycin can be used to distinguish between membranes with and without $PI(4,5)P_2$ using fluorescence microscopy.

4. Discussion

We report three major results. First, incorporating physiological concentrations of $PI(4,5)P_2$ (e.g. 2%) into a lipid bilayer increases the binding of fluorescently labeled neomycin (Figs. 2 and 3). FITC-labeled neomycin binds to $PI(4,5)P_2$-containing membranes to about the same degree as the unlabeled neomycin [33,35], and our results are consistent with formation of a 1:1 neomycin:$PI(4,5)P_2$ complex with an apparent association constant of 10^5-10^6 M^{-1} (Table 1). Second, $I(1,4,5)P_3$ causes little translocation of FITC-neomycin from membrane to the aqueous phase under conditions designed to correspond to those in a living cell (Fig. 4). Third, we can detect the preferential binding of FITC or coumarin-labeled neomycin to vesicles containing 2% $PI(4,5)P_2$ in a mixture of vesicles with and without $PI(4,5)P_2$ using fluorescence microscopy on giant phospholipid vesicles (Fig. 5). These three results suggest that fluorescently labeled neomycin could be useful as a probe for monitoring $PI(4,5)P_2$ distribution within cells, complementing the use of GFP-PH constructs recently developed by several groups [27,28][3]

There are two advantages of using neomycin. First, it is a small molecule and may probe $PI(4,5)P_2$ on the cell membranes where larger molecules like the GFP-PH constructs could be sterically inhibited from binding to $PI(4,5)P_2$. Second, physiological concentrations of $I(1,4,5)P_3$ do not displace neomycin from the membrane (Fig. 4). In contrast, $I(1,4,5)P_3$ binds avidly to the PLC-δ PH domain and could produce translocation of the GFP-PH constructs from the membrane to the cytosol upon PLC activation; this could lead to erroneous conclusions regarding the $PI(4,5)P_2$ concentration in a membrane [32]. Thus fluorescently labeled neomycin, which is not displaced by $I(1,4,5)P_3$ from $PI(4,5)P_2$-containing membranes, could be used together with the GFP-PH constructs. A few technical considerations about fluorescently labeled neomycin that might be useful to cell biologists are discussed in Appendix A.

Acknowledgements

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[3] PH domains from different proteins bind selectively to different phosphoinositides [8,55–57]. Therefore, one can use them as selective indicators for different phosphoinositides in the membrane. For example, Bruton's tyrosine kinase PH binds specifically to $PI(3,4,5)P_3$ [55] and GFP fusion protein with the PH domain was used recently to study distribution of $PI(3,4,5)P_3$ in living cells [58].

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Appendix A

We expect that injection of <1 μM neomycin (a sufficient neomycin concentration to detect $PI(4,5)P_2$ using a fluorescence microscope) will not interfere significantly with the inositol pathways in a cell. At this concentration, neomycin should bind less than 5% of the $PI(4,5)P_2$. No cytotoxicity of neomycin was observed in human endothelial cells if added to the cells at low concentrations [49]. However, higher concentrations of neomycin are used routinely to inhibit PLC (for example, in [28,50]) and 100 μM to 5 mM of neomycin added to permeabilized cells perturbs turnover of the inositol phospholipids [33,51,52]. Therefore one should use low (<1 μM) concentrations of the labeled neomycin.

The available evidence suggests that cell membranes are not permeable to neomycin. Fluorescently labeled neomycin could be either injected into cells or added to permeabilized cells. It can also be incorporated into cells using electroporation, a technique used routinely to transfect cells with DNA or RNA. We used the electroporation technique to introduce FITC-neomycin into mammalian kidney cells. Cells treated with electroporation with 0.5, 1, or 2 μM of FITC-neomycin in the media were highly fluorescent and had some staining inside the cell. Diffuse fluorescence from the cytoplasm possibly indicates that neomycin binds to other negatively charged molecules in a cell: e.g. it is known that neomycin binds to 16S ribosomal RNA in prokaryotic cells [53] and could bind to some RNA in eukaryotic cells. Thus FITC-neomycin may be less useful for studies in living cells than other fluorescently labeled neomycins because its fluorescence decreases when it binds to membranes. Neomycin labeled with rhodamine B, X-rhodamine isothiocyanate, or other labels has been used to investigate the uptake of neomycin and $PI(4,5)P_2$ into cells (S. Ozaki, D.B. DeWald, J.C. Shope, J. Chen and G.D. Prestwich, personal communication). Labeling of neomycin with a polarity sensitive fluorophore that gives a higher fluorescence signal when the probe is bound to a membrane (e.g. acrylodan) might be advantageous. Fluorescently labeled neomycin derivatives may be obtained from one of the authors (G.D.P.) for collaborative projects. FITC, Texas Red, BODIPY, tetramethylrhodamine, rhodamine B, rhodamine-X, and other adducts have been prepared. Many of these are commercially available from Echelon Research Laboratories, (http://www.echelon-inc.com).

References

[1] R.H. Michell, Inositol phospholipids and cell surface receptor function, Biochim. Biophys. Acta 415 (1975) 81–147.
[2] E.J. Murphy, L. Joseph, R. Stephens, L.A. Horrocks, Phospholipid composition of cultured human endothelial cells, Lipids 27 (1992) 150–153.
[3] S.G. Rhee, Y.S. Bae, Regulation of phosphoinositide-specific phospholipase C isozymes, J. Biol. Chem. 272 (1997) 15045–15048.
[4] M.J. Berridge, Inositol trisphosphate and calcium signalling, Nature 361 (1993) 315–325.
[5] D.E. Clapham, Calcium signaling, Cell 80 (1995) 259–268.
[6] Y. Nishizuka, Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C, Science 258 (1992) 607–614.
[7] M.E. Cifuentes, T. Delaney, M.J. Rebecchi, D-myo-inositol 1,4,5-trisphosphate inhibits binding of phospholipase C-δ 1 to bilayer membranes, J. Biol. Chem. 269 (1994) 1945–1948.
[8] M.A. Lemmon, K.M. Ferguson, R. O'Brien, P.B. Sigler, J. Schlessinger, Specific and high-affinity binding of inositol phosphates to an isolated pleckstrin homology domain, Proc. Natl. Acad. Sci. USA 92 (1995) 10472–10476.
[9] L.E. Rameh, L.C. Cantley, The role of phosphoinositide 3-kinase lipid products in cell function, J. Biol. Chem. 274 (1999) 8347–8350.
[10] A. Hall, Rho GTPases and the actin cytoskeleton, Science 279 (1998) 509–514.
[11] P.A. Janmey, The cytoskeleton and cell signaling: component localization and mechanical coupling, Physiol. Rev. 78 (1998) 763–781.
[12] P. De Camilli, S.D. Emr, P.S. McPherson, P. Novick, Phosphoinositides as regulators in membrane traffic, Science 271 (1996) 1533–1539.
[13] O. Cremona, P. De Camilli, Synaptic vesicle endocytosis, Curr. Opin. Neurobiol. 7 (1997) 323–330.
[14] T.F.J. Martin, Phosphoinositide lipids as signaling molecules: common themes for signal transduction, cytoskeletal regulation, and membrane trafficking, Annu. Rev. Cell Dev. Biol. 14 (1998) 231–264.
[15] D.W. Hilgemann, R. Ball, Regulation of cardiac Na^+,Ca^{2+} exchange and K_{ATP} potassium channels by PIP_2, Science 273 (1996) 956–959.
[16] C.L. Huang, S. Feng, D.W. Hilgemann, Direct activation of

inward rectifier potassium channels by PIP_2 and its stabilization by $G_{\beta\gamma}$, Nature 391 (1998) 803–806.
[17] S.-L. Shyng, C.G. Nichols, Membrane phospholipid control of nucleotide sensitivity of $K_{(ATP)}$ channels, Science 282 (1998) 1138–1141.
[18] T. Baukrowitz, U. Schulte, D. Oliver, S. Herlitze, T. Krauter, S.J. Tucker, J.P. Ruppersberg, B. Fakler, PIP_2 and PIP as determinants for ATP inhibition of $K_{(ATP)}$ channels, Science 282 (1998) 1141–1144.
[19] V.A. Sciorra, M.A. Frohman, A.J. Morris, Regulation of phospholipase D signaling by phosphoinositides, in: S. Cockcroft (Ed.), The Frontiers of Molecular Biology, Biology of Phosphoinositides, Oxford University Press, Oxford, in press.
[20] T.F. Martin, Phosphoinositides as spatial regulators of membrane traffic, Curr. Opin. Neurobiol. 7 (1997) 331–338.
[21] D. Allan, R. Watts, R.H. Michell, Production of 1,2-diacylglycerol and phosphatidate in human erythrocytes treated with calcium ions and ionophore A23187, Biochem. J. 156 (1976) 225–232.
[22] M.A. Seyfred, W.W. Wells, Subcellular incorporation of ^{32}P into phosphoinositides and other phospholipids in isolated hepatocytes, J. Biol. Chem. 259 (1984) 7659–7665.
[23] K.T. Mitchell, J.E. Ferrell Jr., W.H. Huestis, Separation of phosphoinositides and other phospholipids by two-dimensional thin-layer chromatography, Analyt. Biochem. 158 (1986) 447–453.
[24] L.J. Pike, L. Casey, Localization and turnover of phosphatidylinositol 4,5-bisphosphate in caveolin-enriched membrane domains, J. Biol. Chem. 271 (1996) 26453–26456.
[25] A. Zachowski, Phospholipids in animal eukaryotic membranes: transverse asymmetry and movement, Biochem. J. 294 (1993) 1–14.
[26] C.S. D'Santos, J.H. Clarke, N. Divecha, Phospholipid signalling in the nucleus. Een DAG uit het leven van de inositide signalering in de nucleus, Biochim. Biophys. Acta 1436 (1998) 201–232.
[27] T.P. Stauffer, S. Ahn, T. Meyer, Receptor-induced transient reduction in plasma membrane $PtdIns(4,5)P_2$ concentration monitored in living cells, Curr. Biol. 8 (1998) 343–346.
[28] P. Varnai, T. Balla, Visualization of phosphoinositides that bind pleckstrin homology domains: calcium- and agonist-induced dynamic changes and relationship to myo-[^{3}H]inositol-labeled phosphoinositide pools, J. Cell Biol. 143 (1998) 501–510.
[29] K.M. Ferguson, M.A. Lemmon, J. Schlessinger, P.B. Sigler, Structure of the high affinity complex of inositol trisphosphate with a phospholipase C pleckstrin homology domain, Cell 83 (1996) 1037–1046.
[30] K. Fukami, K. Matsuoka, O. Nakanishi, A. Yamakawa, S. Kawai, T. Takenawa, Antibody to phosphatidylinositol 4,5-bisphosphate inhibits oncogene-induced mitogenesis, Proc. Natl. Acad. Sci. USA 85 (1988) 9057–9061.
[31] K. Fukami, T. Endo, M. Imamura, T. Takenawa, α-Actinin and vinculin are PIP_2-binding proteins involved in signaling by tyrosine kinase, J. Biol. Chem. 269 (1994) 1518–1522.
[32] K. Hirose, S. Kadowaki, M. Tanabe, H. Takeshiba, M. Iino, Spatiotemporal dynamics of inositol 1,4,5-trisphosphate that underlies complex Ca^{2+} mobilization patterns, Science 284 (1999) 1527–1531.
[33] J. Schacht, Inhibition by neomycin of polyphosphoinositide turnover in subcellular fractions of guinea-pig cerebral cortex in vitro, J. Neurochem. 27 (1976) 1119–1124.
[34] J. Schacht, Purification of polyphosphoinositides by chromatography on immobilized neomycin, J. Lipid Res. 19 (1978) 1063–1067.
[35] E. Gabev, J. Kasianowicz, T. Abbott, S. McLaughlin, Binding of neomycin to phosphatidylinositol 4,5-bisphosphate (PIP_2), Biochim. Biophys. Acta 979 (1989) 105–112.
[36] S. McLaughlin, M. Whitaker, Cations that alter surface potentials of lipid bilayers increase the calcium requirement for exocytosis in sea urchin eggs, J. Physiol. 396 (1988) 189–204.
[37] A.J. Morris, S.A. Rudge, C.E. Mahlum, J.M. Jenco, Regulation of phosphoinositide-3-kinase by G protein beta gamma subunits in a rat osteosarcoma cell line, Mol. Pharmacol. 48 (1995) 532–539.
[38] K. Itaya, M. Ui, A new micromethod for the colorimetric determination of inorganic phosphate, Clin. Chim. Acta 14 (1966) 361–366.
[39] M.J. Hope, M.B. Bally, G. Webb, P.R. Cullis, Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential, Biochim. Biophys. Acta 812 (1985) 55–65.
[40] C.A. Buser, S. McLaughlin, Ultracentrifugation technique for measuring the binding of peptides and proteins to sucrose-loaded phospholipid vesicles, Methods Mol. Biol. 84 (1998) 267–281.
[41] D.G. Reid, K. Gajjar, A proton and carbon 13 nuclear magnetic resonance study of neomycin B and its interactions with phosphatidylinositol 4,5-bisphosphate, J. Biol. Chem. 262 (1987) 7967–7972.
[42] L.K. Tamm, Physical studies of peptide–bilayer interactions, in: S.H. White (Ed.), Membrane Protein Structure, Experimental Approaches, Oxford University Press, 1994, pp. 281–313.
[43] R.M. Peitzsch, S. McLaughlin, Binding of acylated peptides and fatty acids to phospholipid vesicles pertinence to myristoylated proteins, Biochemistry 32 (1993) 10436–10443.
[44] N. Ben-Tal, B. Honig, C. Miller, S. McLaughlin, Electrostatic binding of proteins to membranes: theoretical predictions and experimental results with charybdotoxin and phospholipid vesicles, Biophys. J. 73 (1997) 1717–1727.
[45] K. Akashi, H. Miyata, H. Itoh, K. Kinosita Jr., Preparation of giant liposomes in physiological conditions and their characterization under an optical microscope, Biophys. J. 71 (1996) 3242–3250.
[46] D. Murray, N. Ben-Tal, B. Honig, S. McLaughlin, Electrostatic interaction of myristoylated proteins with membranes simple physics, complicated biology, Structure 5 (1997) 985–989.

[47] P. Gascard, D. Tran, M. Sauvage, J.C. Sulpice, K. Fukami, T.C.M. Takenawa, F. Giraud, Asymmetric distribution of phosphoinositides and phosphatidic acid in the human erythrocyte membrane, Biochim. Biophys. Acta 1069 (1991) 27–36.

[48] S. McLaughlin, The electrostatic properties of membranes, Annu. Rev. Biophys. Biophys. Chem. 18 (1989) 113–136.

[49] G.F. Hu, Neomycin inhibits angiogenin-induced angiogenesis, Proc. Natl. Acad. Sci. USA 95 (1998) 9791–9795.

[50] L.M. Neri, P. Borgatti, S. Capitani, A.M. Martelli, Nuclear diacylglycerol produced by phosphoinositide-specific phospholipase C is responsible for nuclear translocation of protein kinase C-α, J. Biol. Chem. 273 (1998) 29738–29744.

[51] S. Cockcroft, T.W. Howell, B.D. Gomperts, Two G-proteins act in series to control stimulus–secretion coupling in mast cells: use of neomycin to distinguish between G-proteins controlling polyphosphoinositide phosphodiesterase and exocytosis, J. Cell Biol. 105 (1987) 2745–2750.

[52] M. Whitaker, M. Aitchison, Calcium-dependent polyphosphoinositide hydrolysis is associated with exocytosis in vitro, FEBS Lett. 182 (1985) 119–124.

[53] D. Moazed, H.F. Noller, Interaction of antibiotics with functional sites in 16S ribosomal RNA, Nature 327 (1987) 389–394.

[54] M. Rebecchi, A. Peterson, S. McLaughlin, Phosphoinositide-specific phospholipase C-δ 1 binds with high affinity to phospholipid vesicles containing phosphatidylinositol 4,5-bisphosphate, Biochemistry 31 (1992) 12742–12747.

[55] K. Salim, M.J. Bottomley, E. Querfurth, M.J. Zvelebil, I. Gout, R. Scaife, R.L. Margolis, R. Gigg, C.I. Smith, P.C. Driscoll, M.D. Waterfield, G. Panayotou, Distinct specificity in the recognition of phosphoinositides by the pleckstrin homology domains of dynamin and Bruton's tyrosine kinase, EMBO J. 15 (1996) 6241–6250.

[56] L.E. Rameh, A. Arvidsson, K.L. Carraway, A.D. Couvillon, G. Rathbun, A. Crompton, B. VanRenterghem, M.P. Czech, K.S. Ravichandran, S.J. Burakoff, D.S. Wang, C.S. Chen, L.C. Cantley, A comparative analysis of the phosphoinositide binding specificity of pleckstrin homology domains, J. Biol. Chem. 272 (1997) 22059–22066.

[57] J.M. Kavran, D.E. Klein, A. Lee, M. Falasca, S.J. Isakoff, E.Y. Skolnik, M.A. Lemmon, Specificity and promiscuity in phosphoinositide binding by pleckstrin homology domains, J. Biol. Chem. 273 (1998) 30497–30508.

[58] P. Varnai, K.I. Rother, T. Balla, Phosphatidylinositol 3-kinase-dependent membrane association of the Bruton's tyrosine kinase pleckstrin homology domain visualized in single living cells, J. Biol. Chem. 274 (1999) 10983–10989.