Phosphoinositide-Specific Phospholipase C-δ_1 Binds with High Affinity to Phospholipid Vesicles Containing Phosphatidylinositol 4,5-Bisphosphate†

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ABSTRACT: We studied the binding of phosphoinositide-specific phospholipase C-δ_1 (PLC-δ) to vesicles containing the negatively charged phospholipids phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidylserine (PS). PLC-δ did not bind significantly to large unilamellar vesicles formed from the zwitterionic lipid phosphatidylcholine (PC) but bound strongly to vesicles formed from mixtures of PC and PIP_2. The apparent association constant for the putative 1:1 complex formed between PLC-δ and PIP_2 was $K_a \simeq 10^5$ M^{-1}. The binding strength increased further ($K_a \simeq 10^6$ M^{-1}) when the vesicles also contained 30% PS. High-affinity binding of PLC-δ to PIP_2 did not require Ca^{2+}. PLC-δ bound only weakly to vesicles formed from mixtures of PC and either PS or phosphatidylinositol (PI); binding increased as the mole fraction of acidic lipid in the vesicles increased. We also studied the membrane binding of a small basic peptide that corresponds to a conserved region of PLC. Like PLC-δ, the peptide bound weakly to vesicles containing monovalent negatively charged lipids; unlike PLC-δ, it did not bind strongly to vesicles containing PIP_2. Our data suggest that a significant fraction of the PLC-δ in a cell could be bound to PIP_2 on the cytoplasmic surface of the plasma membrane.

The different isoforms of intracellular phosphoinositide-specific phospholipase C (PLC)[1] are all key enzymes in the Ca^{2+}/phospholipid signal transducing pathway (Rhee et al., 1989; Kriz et al., 1990; Majerus et al., 1990; Bansal & Majerus, 1990; Dennis et al., 1991; Rhee, 1991; Rhee & Choi, 1992). These enzymes cleave phosphatidylinositol 4,5-bisphosphate (PIP_2) into the two second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG); IP_3 releases Ca^{2+} stored in subcellular organelles and DAG activates protein kinase C. Although the principal substrate for these enzymes, PIP_2, is a component of the plasma membrane, all isoforms of PLC are found in both the cytoplasmic and membrane fractions of cells (Lee et al., 1987; Katan & Parker, 1987; Rebecchi & Rosen, 1987). The amino acid sequences of these proteins have no significant stretches of hydrophobic amino acids analogous to the transmembrane segments of intrinsic membrane proteins (Suh et al., 1988; Stahl et al., 1988; Katan et al., 1988; Emori et al., 1989). Some isoforms may bind to membrane proteins—e.g., PLC-γ to EGF receptors (Anderson et al., 1990; Todderud et al., 1990; Kriz et al., 1990) and PLC-β to G_q (Smrcka et al., 1991; Taylor et al., 1991; Harden et al., 1991)—but PLC-δ has not been found associated with membrane proteins. Thus, the physical basis for the partitioning of PLC-δ onto the membrane surface is unclear.

We considered the possibility that PLC-δ may bind significantly to anionic phospholipids such as phosphatidylserine (PS) and PIP_2. PS comprises 30% (Op den Kamp, 1979; Bishop & Bell, 1988; Deveaux, 1991, 1992) and PIP_2 3% (Christensen, 1986; Mitchell et al., 1986) of the phospholipids on the inner monolayer of human erythrocyte plasma membranes. PS, which has one net negative charge, is the predominant anionic phospholipid on the inner monolayer of most mammalian plasma membranes. PIP_2, which has net charge of about −3 under physiological conditions (Toner et al., 1988), is the principal substrate for PLC-δ. One or both of these phospholipids could bind the enzyme to the membrane. For example, there is evidence that a different (62-kDa) phosphatidylinositol-specific phospholipase C binds strongly to PIP_2 (Xu & Nelsesteun, 1992). If PLC-δ binds to PIP_2, it would be concentrated at the surface that contains both its substrates and possible regulatory elements such as a G protein.

We measured the binding of PLC-δ, a soluble isozyme isolated from bovine brain cytosol, to phospholipid vesicles formed from mixtures of phosphatidylcholine (PC), PIP_2, and PS. We also compared the behavior of the native enzyme to that of several basic peptides and neomycin, a small, basic aminoglycoside antibiotic that binds to PIP_2 with high affinity and specificity.

MATERIALS AND METHODS

Enzyme Preparation. PLC-δ was isolated to homogeneity from bovine brain cytoplasm using the method of Rebecchi and Rosen (1987) with the following modifications. (1) Following ammonium sulfate precipitation, DEAE-Sepharose was substituted for the three fractionation procedures that precede step 6, the Fast-S Sepharose step of Rebecchi and Rosen (1987). The PLC-δ activity eluted at about 70 mM KCl from the DEAE-Sepharose column. (2) Heparin–agarose was substituted for the hydroxylapatite and PI–Sepharose affinity chromatography steps: the protein was applied in 100 mM NaCl, 1 mM EGTA, 0.5 mM dithiothreitol (DTT), 20 mM HEPES, pH 7.2, and eluted with a linear gradient of NaCl (100–700 mM). PLC-δ was recovered between 500 and 600 mM NaCl. A Millipore Minitan ultrafiltration manifold with 50-kDa membranes was used to dialyze the

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[1] Abbreviations: DOPC, dioleoylphosphatidylcholine; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; IP_3, inositol 1,4,5-trisphosphate; K_a, apparent association constant for complex between PIP_2 and PLC-δ (M^{-1}); K, partition constant onto surface (μm); LUVs, large unilamellar vesicles; MOPS, 4-morpholinepropanesulfonic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phosphoinositide-specific phospholipase C; PS, phosphatidylserine.

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active fractions between each step. In some of the gel filtration experiments, we used a preparation that contained only 50% PLC-δ by weight as judged by Coomassie-stained SDS–PAGE; in most experiments the purity was >90%. The identity of the PLC-δ isozyme was confirmed by its reaction with a monoclonal antibody specific for the δ isoform (kindly performed by S. G. Rhee) and by reaction with antiserum raised against peptides from the published sequence of bovine PLC-δ (Suh et al., 1988; M. Cifuentes and M. Rebecchi, unpublished results).

Peptide Preparation. A positively charged peptide corresponding to residues 430–442 of bovine PLC-δ_1 (PLC(430–442), amino acid sequence acetyl-QLRGKILLKGKKL-amide; Suh et al., 1988) was synthesized by Multiple Peptide Systems (San Diego, CA; 80% pure) and further purified to 95% by reverse-phase HPLC. This sequence is part of a highly conserved region of PLC-β, -δ, and -γ (Suh et al., 1988; Stahl et al., 1988; Katan et al., 1988; Emori et al., 1989). Pentamers of lysine (Lys_5) and arginine (Arg_5) were synthesized by Research Plus (Bayone, NJ). Lys_5 was >95% pure and Arg_5 was >80% pure as determined by reverse-phase HPLC.

Vesicle Preparation. Multilamellar vesicles for electrophoretic mobility measurements were formed by the method of Bangham et al. (1974). Extruded large (diameter = 0.1 μm) unilamellar vesicles (LUVs) were formed from multilamellar vesicles using an Extruder (Lipex Biomembranes, Vancouver, BC, Canada) as described elsewhere (Hope et al., 1985; Kim et al., 1991). The buffer solutions used in forming vesicles were 100 mM KCl, 25 mM HEPES, pH 7.2 or 180 mM sucrose, 20 mM KCl, 5 mM HEPES, pH 7.2 (sucrose-loaded vesicles) for the PLC-δ experiments and 100 mM KCl, 1 mM MOPS, pH 7.0 for peptide and neomycin experiments. The sucrose-loaded LUVs were diluted into the 100 mM KCl, 25 mM HEPES solution, which was isotonic with the entrapped sucrose solution. The phospholipids used included 1-palmitoyl-2-oleoylphosphatidylcholine (PC), 1-palmitoyl-2-oleoylphosphatidylglycerol (PG), 1-palmitoyl-2-oleoylphosphatidylserine and bovine brain phosphatidylserine (PS), and bovine liver phosphatidylinositol (PI), all from Avanti (Alabaster, AL). The ammonium salt of bovine brain phosphatidylinositol 4,5-bisphosphate (PIP_2) was purchased from Calbiochem (San Diego, CA) and Boehringer Mannheim (Indianapolis, IN); [^{3}H]PIP_2 was from New England Nuclear (Boston, MA) and Amersham (Arlington Heights, IL); [^{14}C]DOPC was from Amersham.

Binding of PLC-δ to LUVs Using Equilibrium Gel Filtration. Equilibrium binding of PLC-δ to LUVs was done at room temperature using a 1.8-mL Sephadex G-200 column (Pharmacia, Piscataway, NJ) equilibrated with 0.4 μg/mL PLC-δ, 100 mM KCl, 1 mM EGTA, 0.5 mM DTT, 0.1% gelatin, 25 mM HEPES, pH 7.1. Gelatin was used to minimize nonspecific adsorption of PLC-δ. Our experimental procedure is based on Huang and Charlton's (1972) adaptation of the Hummel and Dreyer (1962) technique. LUVs containing trace quantities of [^{14}C]DOPC were applied to the column, and the amount of PLC-δ binding to LUVs was determined from the increase in enzyme activity in fractions containing vesicles. (We used a small column to conserve PLC-δ; compared to that of Huang and Charlton (1972), our small column gave less ideal separation between the elution peak and the depletion trough.) An apparent association constant, K_a (M^{-1}), for PLC-δ binding to PIP_2 was calculated by assuming they form a 1:1 complex:

$$K_a = [PLC]_b/[PLC]_f[PIP_2] \quad (1)$$

where $[PLC]_f$ is the free PLC-δ concentration and $[PLC]_b$ is the bound PLC-δ concentration as determined from the increase in PLC-δ activity in the vesicle peak fraction.

A partition constant for PLC-δ binding to a vesicle surface was calculated from

$$K = \{PLC\}/[PLC]_f \quad (2)$$

where {PLC} is the moles of bound PLC per unit surface area of a vesicle (assuming the area per lipid is 0.7 nm^2). The constant K, which has units of length, was used to compare binding of PLC-δ to different vesicle surfaces because it contains no assumptions about binding stoichiometry. K may be interpreted as the distance one must move from a planar membrane to find the same number of PLC-δ molecules in the aqueous phase as are bound to a unit area of the membrane.

When we define K in this manner, we are adopting Gibbs' convention that the surface phase of the membrane to which PLC adsorbs is an infinitely thin plane. Alternatively, if we adopt the thermodynamically equivalent Guggenheim convention that the surface phase has thickness d, we can describe the partitioning of PLC into this phase with a dimensionless partition coefficient k, where

$$k = K/d \quad (3)$$

As d must be of the order of 1 nm, a value of K = 1 μm corresponds to a $k = 10^3$.

Binding of PLC-δ to Sucrose-Loaded LUVs. We developed another method to determine the equilibrium binding of PLC-δ to LUVs; we loaded the LUVs with sucrose to increase their density and then separated free and bound PLC-δ by centrifugation. Sucrose-loaded LUVs and PLC-δ were mixed in 0.1 mL of solution containing 100 mM KCl, 0.1 % (w/v) gelatin, and 25 mM HEPES, pH 7.2, at room temperature. Free PLC-δ was separated from bound by sedimentation at 100000g for 30 min. $[PLC]_f$ was determined by measuring the enzymatic activity of PLC-δ in the supernatant. $[PLC]_b$ was calculated by subtracting $[PLC]_f$ from the total [PLC], which was measured as the enzyme activity of PLC-δ prior to addition of vesicles. K_a and K were calculated from eq 1 and 2. In some experiments, we measured directly the PLC-δ bound to the sucrose-loaded LUVs by solubilizing them in SDS, subjecting the sample to electrophoresis on polyacrylamide gels (Rebecchi & Rosen, 1987) and transferring to nitrocellulose (Towbin et al., 1979), and then determining the amount of PLC in the sample with sequence-specific antibodies. The results of this analysis (not shown) coincided with the results of the enzymatic assay reported in Table II.

Peptide Binding to LUVs. Mixtures of the peptide PLC-(430–442) and LUVs were filtered through 10-kDa molecular mass cutoff filters (Amicon, Beverly, MA) as described previously (Kim et al., 1991). A total of 90–95% of the free peptide passed through the filters, whereas LUVs and peptides bound to LUVs were retained. Free peptide in the filtrate was quantitated by a fluorescamine assay (Weigele et al., 1972).

ζ Potential Measurements. The ζ potentials of multilamellar vesicles composed of 10:1 PC/PIP_2 were calculated from their electrophoretic mobilities measured in a Rank Brothers Mark I instrument (Bottisham, Cambridge, U.K.) as described previously (Cafiso et al., 1989; McLaughlin et al., 1981). The ζ potential, which is equal to the electrostatic

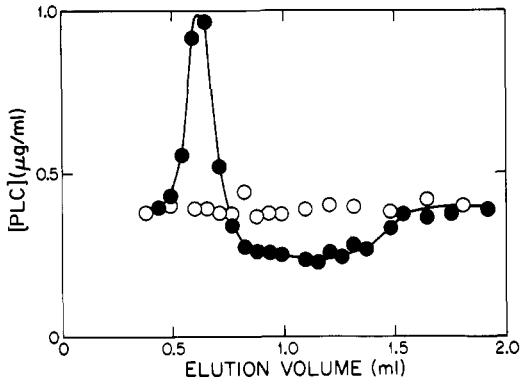

FIGURE 1: Equilibrium binding of PLC-δ to large unilamellar phospholipid vesicles (LUVs) as measured by elution from a size-exclusion column. The column was equilibrated with 0.4 μg/mL PLC-δ, 100 mM KCl, 1 mM EGTA, 0.5 mM DTT, 0.1% gelatin, 25 mM HEPES, pH 7.2. Either 10 μg (weight dry lipid) of LUVs formed from 2:1 PC/PS + 2% PIP_2 (filled circles) or 250 μg of LUVs formed from 2:1 PC/PS (open circles) was added to the column. PLC-δ concentration was determined from enzyme activity. The vesicles eluted with the peak of PLC-δ activity. The equilibrium association constant for the complex formed between PIP_2 and PLC-δ (eq 1) calculated from this experiment is $K_a \simeq 10^6$ M^{-1}.

potential at the hydrodynamic plane of shear of the vesicles (~0.2 nm from the surface), becomes less negative as positively charged molecules bind to the vesicle surface.

PLC Assay. PLC-δ enzymatic activity was assayed at 30 °C in a reaction mixture containing 260 μM PIP_2, 26 000 cpm [^{3}H]PIP_2, 100 mM KCl, 1.4 mM $CaCl_2$, 1% (w/v) octyl glucoside, 0.03% (w/v) gelatin, 0.5 mM DTT, 25 mM HEPES, pH 7.2 (total volume = 30 μL). A 40-min incubation with 0.12 ng of PLC-δ typically yielded 1000 cpm of [^{3}H]IP_3. Under these conditions, the reaction rate was constant with time and could be linearly related to enzyme concentration.

RESULTS

PLC-δ Binding to Phospholipids. We used the method of Hummel and Dreyer (1962) to measure the equilibrium binding of PLC-δ to phospholipid LUVs in the absence of calcium ions.[2] As LUVs passed through a size-exclusion column equilibrated with PLC-δ, they bound the protein until an equilibrium was reached between the free and bound PLC-δ in the vesicle fractions. LUVs that contained PIP_2 bound large amounts of PLC-δ, whereas LUVs without PIP_2 did not bind the enzyme detectably. Calcium ions were not required for binding. The filled circles in Figure 1 show the elution profile of enzyme activity with 2:1 PC/PS vesicles that also contained 2% PIP_2; the open circles show the elution profile with a 25-fold greater concentration of 2:1 PC/PS vesicles. These experiments indicate that the apparent association constant K_a (eq 1) for the putative 1:1 complex formed between PLC-δ and PIP_2 is 1.8×10^6 M^{-1} (Table I). K_a was unaffected when the concentration of PIP_2 in the vesicles was 4-fold lower (Table I). Equivalently, the partition constant K (eq 2) is proportional to the surface concentration of PIP_2 in the vesicles: K was 4-fold lower for PC/PS vesicles with 0.5% PIP_2 than for PC/PS vesicles with 2% PIP_2 (Table I). These data are consistent with the hypothesis that PLC-δ and PIP_2 form a 1:1 complex. (We obtained similar K_a values when column were run with about half the concentration of LUVs suggesting that binding equilibrium had been reached.)

Table I: Apparent Association Constant K_a (eq 1) of PLC-δ with PIP_2 in LUVs and Partition Constant K (eq 2) of PLC-δ onto LUVs Determined from Hummel and Dreyer Type Measurements Illustrated in Figure 1

vesicle composition	K_a (M^{-1})[a]	K (μm)[a]
2:1 PC/PS		<0.3
+0.5% PIP_2	1.4×10^6 ($n = 1$)	17
+2% PIP_2	$(1.8 \pm 0.3) \times 10^6$ ($n = 2$)	83
PC + 2% PIP_2	0.2×10^6 ($n = 1$)	10

[a] Values are reported as means ± standard deviations.

Table II: Apparent Association Constant K_a (eq 1) of PLC-δ with PIP_2 in LUVs and Partition Constant K (eq 2) of PLC-δ onto LUVs Determined from Centrifugation Experiments with Sucrose-loaded LUVs

vesicle composition	K_a (M^{-1})[a]	K (μm)[a]
2:1 PC/PS		0.4 ± 0.02 ($n = 6$)
+0.5% PIP_2	$(1.0 \pm 0.1) \times 10^6$ ($n = 5$)	11 ± 2 ($n = 5$)
+2% PIP_2	$(1.3 \pm 0.6) \times 10^6$ ($n = 21$)	62 ± 30 ($n = 21$)
PC + 2% PIP_2	$(0.4 \pm 0.2) \times 10^6$ ($n = 13$)	18 ± 10 ($n = 13$)

[a] Values are reported as means ± standard deviations.

In the absence of PIP_2, PLC-δ bound detectably to LUVs only if the vesicles contained ≥50 mol % PS. We calculated K values of about 1 μm and 10 μm for PLC-δ binding to 1:1 PC/PS and PS LUVs, respectively (data not shown). The presence of 30 μM Ca^{2+} in the elution buffer did not significantly alter the binding to PS LUVs. The presence of PS in LUVs containing PIP_2 considerably enhanced the binding of PLC-δ to the vesicles: K_a (and K) was about 10-fold higher for 2:1 PC/PS + 2% PIP_2 vesicles than for PC + 2% PIP_2 vesicles (Table I). Thus, even though PLC-δ bound only slightly to PS, the presence of PS at a concentration found in a typical biological membrane enhanced the binding of PLC-δ to LUVs containing PIP_2. In the Discussion section, we consider two possible explanations for this phenomenon: increased electrostatic accumulation of PLC-δ at the vesicle surface due to the more negative surface potential produced by PS in the vesicles and binding of PLC-δ to both PIP_2 and PS.

We also studied the binding of PLC-δ to LUVs with a different technique that required less enzyme: LUVs loaded with a sucrose solution were mixed with PLC-δ and then sedimented via centrifugation. We determined the concentration of PLC-δ free in solution, $[PLC]_f$, by assaying for PLC activity in the supernatant. This method yielded results very similar to those obtained by the gel filtration method (Table II). Specifically, PLC-δ bound to PC vesicles containing 2% PIP_2 with a K_a for PIP_2 of 0.4×10^6 M^{-1}. If the vesicles also contained PS, K_a increased to 1.3×10^6 M^{-1}. This value agrees within experimental error with the association constant deduced from the gel filtration experiments. PLC-δ bound only weakly to LUVs containing only PC or PC/PS mixtures (Tables I and II).

As with the gel filtration experiments, the amount of PLC-δ bound to LUVs increased as the mol % PIP_2 increased (Table II): the relation between K (μm) and mol % PIP_2 appeared linear over a 4-fold range, consistent with a 1:1 stoichiometry for the binding of PLC-δ to PIP_2. However, the standard deviations are sufficiently large that we cannot rule out other stoichiometries from these data.

We also examined the binding of PLC-δ to PI, which like PS is a monovalent acidic lipid, but unlike PS is a substrate

[2] Control experiments demonstrate that PLC-δ hydrolyzes a negligible fraction of PIP_2 in the absence of calcium ions under our experimental conditions. Specifically, parallel experiments were performed with PC/PIP_2 vesicles that contained [^{3}H]PIP_2: [^{3}H]IP_3 was not released from vesicles exposed to PLC-δ.

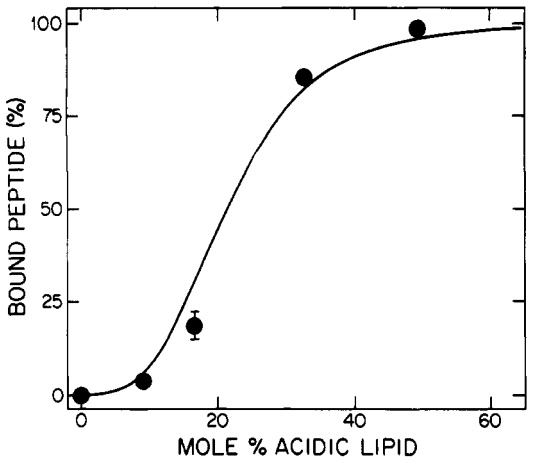

FIGURE 2: Binding of the peptide PLC(430–442) to LUVs formed from mixtures of PC and PG as determined from filtration experiments. The aqueous phase contained 10 mM total lipid, 30 μM total peptide, 100 mM KCl, 1 mM MOPS, pH 7. The theoretical curve was calculated from a Gouy–Chapman/mass action theory discussed briefly in the text and in more detail elsewhere (Mosior & McLaughlin, 1992a,b). Standard deviations are shown when larger than the size of symbols.

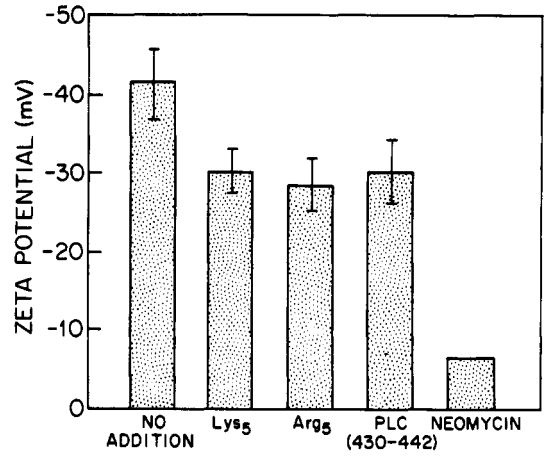

FIGURE 3: Binding of 10^{-5} M peptides or neomycin to 10:1 PC/PIP_2 multilamellar vesicles determined by ζ potential measurements. Vesicles were formed in a 100 mM KCl, 1 mM MOPS, pH 7 solution. Neomycin data are from Gabev et al. (1989).

for PLC. PLC-δ binds weakly to PI compared to PIP_2 (data not shown).

PLC(430–442) Binding to Phospholipids. Nothing is known about the location of the binding site(s) for PIP_2 on PLC-δ. We speculated that a region of the protein containing a high concentration of basic amino acids might be a good candidate for a binding site and selected a region where 5 of 12 residues are basic and none are acidic. We measured the equilibrium binding of a peptide corresponding to this region, PLC(430–442), to LUVs using a filtration method (Kim et al., 1991). As illustrated in Figure 2, PLC(430–442) bound to LUVs containing the anionic phospholipid PG, and the amount of peptide bound increased sigmoidally with increasing mole fraction PG. The binding profile is similar to that of other +5 valence basic peptides (Mosior & McLaughlin, 1991, 1992a,b). The theoretical curve in Figure 2 is identical to the curve used to describe the binding of acetyl-Lys_5-amide or acetyl-$(Arg\text{-}Ala_2)_4$-Arg-amide to membranes (Mosior & McLaughlin, 1992a). ζ potential measurements on PC/PS vesicles in the presence of PLC(430–442) agreed with these filtration experiments and demonstrated the peptide binds equally well to PS and PG (data not shown). Inclusion of 2 mol % PIP_2 in 2:1 PC/PG LUVs produced a comparatively small increase in binding of the PLC(430–442) peptide; with 3 μM peptide and 1 mM total lipid, 40% of the peptide bound to 2:1 PC/PG LUVs ($K \simeq 3$ μm from eq 2) and 60% bound to 2:1 PC/PG + 2% PIP_2 LUVs ($K \simeq 7$ μm). Thus, the peptide has a much lower specificity for PIP_2 than the intact protein, which bound 100-fold more strongly to LUVs containing 2 % PIP_2 than to LUVs that contained only monovalent acidic lipids (Tables I and II).

We compared the membrane binding of the PLC(430–442) peptide to that of two other +5 basic peptides, Lys_5 and Arg_5, by making electrophoretic mobility measurements. The electrophoretic mobility or ζ potential of a vesicle is, to a first approximation, linearly related to the number of cationic peptides bound per unit area (Cafiso et al., 1989). Figure 3 shows that the three peptides all reduced the ζ potential of 10:1 PC/PIP_2 vesicles comparably. In contrast neomycin, a +5 aminoglycoside, had a much larger effect on the ζ potential. The intrinsic association constant of neomycin with PIP_2 is 10^5 M^{-1} (Gabev et al., 1989), which is approximately equal to the apparent association constant of PLC-δ with PIP_2 in a PC vesicle (Tables I and II). The intrinsic association constants[3] of the three peptides with PIP_2 are estimated to be of order of 10^3 M^{-1} from the data in Figure 3.

DISCUSSION

Our most important result is that PLC-δ binds with high affinity and specificity to PIP_2 in phospholipid vesicles; calcium ions are not required for this binding although PLC-δ requires calcium ions to hydrolyze substrate (Rebecchi & Rosen, 1987; Ryu et al., 1987; McDonald & Mamrack, 1989). The data obtained using both the Hummel and Dreyer filtration technique (Table I) and a centrifugation assay (Table II) indicate that the association constant, K_a, of PLC-δ with PIP_2 in PC/PS vesicles is about 10^6 M^{-1}. The measurements made with both techniques also indicate the partition constant for the enzyme is about 100-fold higher for PC/PS vesicles containing 2% PIP_2 ($K \simeq 100$ μm) than for PC/PS vesicles ($K < 1$ μm).

While PLC-δ binds only weakly to PC/PS vesicles in the absence of PIP_2, addition of negatively charged PS to PC membranes containing PIP_2 significantly increases the affinity of PLC-δ for the membranes: K is about 3-fold higher for PC/PS/PIP_2 vesicles than for PC/PIP_2 vesicles (Table II). This increase in the affinity of the enzyme for the membranes is associated with a similar increase in the rate of PIP_2 hydrolysis in both vesicles and monolayers (Rebecchi et al., 1993).

There are two simple mechanisms that could account for PS enhancing the binding of the enzyme to PIP_2. First, the enzyme may have >1 phospholipid binding site and can bind both to PIP_2 and to 1 or more PS molecules. Second, addition of PS produces an electrostatic potential at the membrane interface, and this could increase the concentration of PLC-δ in the aqueous phase at the vesicle surface. For example, addition of 31% PS to vesicles containing 2% PIP_2 increases the surface potential from −15 mV to −50 mV (McLaughlin,

[3] The apparent association constants, K_a, of the peptides with PIP_2 were calculated from a mass action expression identical to eq 1. The intrinsic association constant, K_i, attempts to correct for the Boltzmann accumulation of the peptide in the aqueous diffuse double layer adjacent to the membrane. $K_i = K_a \exp(+z_{eff}F\psi/RT)$ where z_{eff} is the effective valence of the peptide, F is the Faraday constant, ψ is the electrostatic potential at the surface of the membrane, R is the gas constant, and T is the temperature.

1989); even if the effective valence of PLC-δ (i.e., the net charge of the region of the enzyme that binds to membranes) is as small as +1, the Boltzmann accumulation of PLC-δ could account for a 3-fold increase in K.

Neomycin, a much simpler molecule than PLC-δ, also binds with high specificity and affinity to PIP_2 in membranes (Figure 3). This positively charged aminoglycoside binds strongly to PIP_2 in PC bilayers by forming electroneutral 1:1 complexes (Gabev et al., 1989). Although neomycin binds only weakly to membranes containing monovalent acidic lipids (McLaughlin & Whitaker, 1988), the percentage of bound neomycin increased markedly (from 44% to 92% of the total 2 μM) when 17% monovalent negatively charged lipid was added to 1.5 mM PC vesicles containing 1% PIP_2. Thus, monovalent acidic lipids enhance the binding of both neomycin and PLC-δ to PIP_2 in membranes. In the case of neomycin, the electrostatic potential produced by the monovalent lipids enhances the binding by accumulating the basic drug in the aqueous diffuse double layer. Unlike neomycin, PLC-δ bears a net negative charge at pH 7 ($pI \simeq 5$; Rebecchi, unpublished results), but this does not preclude the possibility that the PIP_2 binding site bears a net positive charge and most negative charges are more than a Debye length from the membrane.

Since PLC-δ and neomycin bind comparably to membranes containing PIP_2, we looked for a region of the enzyme that has a net charge (≃+5) similar to that of neomycin. We identified a highly conserved, 12 amino acid sequence with five basic and no acidic residues. The corresponding synthetic peptide, PLC(430–442), binds to membranes containing monovalent acidic phospholipids. The sigmoidal shape of the binding curve (Figure 2) is characteristic of basic peptides binding to membranes containing acidic lipids; the reduction of dimensionality that occurs when the first basic residue in a peptide binds to an acidic lipid in a membrane and the electrostatic potential produced by acidic lipids produce apparent cooperativity, as discussed in detail elsewhere (Mosior & McLaughlin, 1992b). The binding of a small cluster of basic residues on PLC-δ to monovalent acidic lipids could account for the modest affinity of the enzyme for membranes containing PS; indeed, the peptide PLC(430–442) has a higher affinity for membranes containing PS than does the native protein. The peptide PLC(430–442) does not, however, exhibit the high specificity for PIP_2 observed with either PLC-δ or neomycin: there is only a small enhancement in the binding of PLC(430–442) in the presence of PIP_2. Furthermore, when we tested several other peptides with +5 net charge,[4] none exhibited the high affinity for PIP_2 we observe with neomycin (Figure 3) or PLC-δ (Figure 1).

Neither the identity of the region(s) of PLC-δ that binds to PIP_2 nor the relationship between the membrane binding site and the catalytic site of PLC-δ is known, but one experiment suggests they are distinct. Enzymatic removal of the amino-terminal region of PLC-δ destroys high-affinity binding to PIP_2 but not the calcium-dependent catalytic activity (M. Cifuentes and M. Rebecchi, unpublished results). If the sites are distinct, the membrane binding site may serve to moor the enzyme to the surface containing its substrates. Other lipases appear to have separate catalytic and membrane binding sites; e.g., a ring of basic and hydrophobic residues probably attach phospholipase A_2 to the membrane, forming a channel through which substrates move to the active site (Dijkstra et al., 1984; Jain & Berg, 1989; Scott et al., 1990, 1991).

In conclusion, we speculate that the high-affinity binding of PLC-δ to PIP_2 we observe in vitro may serve an important function in cells. The cytoplasmic monolayers of many mammalian cell membranes (e.g., human erythrocytes) contain roughly 3% PIP_2 and 30% PS (Mitchell et al., 1986). It is not known what fraction of the PIP_2 is free, since it binds to cytoplasmic proteins such as profilin (Lassing & Lindberg, 1985; Goldschmidt-Clermont et al., 1990, 1991), myosin I (Adams & Pollard, 1989), gelsolin (Janmey & Stossel, 1987; Janmey et al., 1992), cofilin (Yonezawa et al., 1990), and gCap39 (Yu et al., 1990) as well as to intrinsic membrane proteins such as glycophorin (Anderson & Marchesi, 1985), but there is indirect evidence that about 50% of the PIP_2 is free in erythrocytes (Hagelberg & Allan, 1990). Even if only 5% is free, this is equivalent to a $[PIP_2] = 10^{-6}$ M if the lipid is considered to be distributed uniformly throughout a spherical cell of radius 3 μm. This implies that about 50% of the PLC-δ should be present as PLC-PIP_2 complexes since $K_a = 10^6\ M^{-1}$. Binding of the enzyme to the membrane surface reduces the dimensionality (Adam & Delbrück, 1968; Sargent & Schwyzer, 1986; Mosior & McLaughlin, 1992b) and may facilitate its interaction with both substrates and putative regulatory elements.

[4] Janmey et al. (1992) provided indirect evidence that peptides with sequences that mimic a basic region in gelsolin may bind with high affinity to PIP_2. Yu et al. (1992) suggested that a similar region (434–441) from PLC-δ is important for the high-affinity phosphoinositide-specific binding, but our experiments with a slightly larger peptide, PLC(430–442), demonstrate that the peptide does not exhibit high-affinity binding to PIP_2 comparable to that exhibited by the native PLC-δ and neomycin (Figure 3).

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