Phosphoinositide-Specific Phospholipase C-δ_1: Effect of Monolayer Surface Pressure and Electrostatic Surface Potentials on Activity†

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ABSTRACT: We added phospholipase C-δ_1 (PLC-δ) to the aqueous subphase beneath monolayers formed from mixtures of phosphatidylinositol 4,5-bisphosphate (2% PIP_2), phosphatidylserine (33% PS), and phosphatidylcholine (65% PC) and then measured the initial rate of hydrolysis of PIP_2 after addition of 10 μM free calcium. Increasing the surface pressure of the monolayer, π, from 20 to 40 mN/m decreased the rate of hydrolysis 200-fold. The rate of hydrolysis depends exponentially on the surface pressure: rate $\propto \exp(-\pi A_p/kT)$ where k is the Boltzmann constant, T is the temperature, and $A_p \simeq 1$ nm^2. Similar results were obtained with different (1 and 100 μM) free [Ca^{2+}] and with different mole fractions of PIP_2. The results are consistent with a model in which PLC-δ binds to PIP_2 with high affinity ($K_a = 10^6$ M^{-1}) in the absence of calcium ions [Rebecchi, M. J., Peterson, A., & McLaughlin, S. (1993) *Biochemistry* (preceding paper in this issue)], and a portion of PLC-δ of area A_p inserts into the monolayer doing work = πA_p prior to hydrolysis of PIP_2. Removing the monovalent acidic lipid PS from the monolayer decreases the activity of PLC-δ 4-fold, this effect of PS on activity is similar to the effect of monovalent acidic lipids on the binding of PLC-δ to PIP_2 in bilayer vesicles.

The phosphoinositide-specific phospholipase C (PLC)[1] family of enzymes is an important component of the calcium/phospholipid second messenger system; PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) into the two second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). The biochemistry and molecular biology of these phospholipases has been reviewed recently by Rhee et al. (1989), Kriz et al. (1990), Majerus et al. (1990), Bansal and Majerus (1990), Dennis et al. (1991), Rhee (1991), and Rhee and Choi (1992).

The β, γ, and δ isoforms of mammalian PLC have two large homologous domains but different patterns of activation (Rhee, 1991). The β isoform is activated by the α subunit of a pertussis toxin-insensitive G protein, G_q (Smrcka et al., 1991; Taylor et al., 1991). The γ isoform is activated by epidermal growth factor (EGF) receptors (Todderud et al., 1990) as well as other receptor tyrosine kinases (Rhee, 1991). The *src*-homology regions in PLC-γ are important in its binding to receptors (Anderson et al., 1990; Kriz et al., 1990; Matsuda et al., 1990) but are not essential for catalytic activity (Emori et al., 1989; Dennis et al., 1991). The activated EGF receptor phosphorylates PLC-γ, but it is not yet clear how this tyrosine phosphorylation activates PLC-γ to hydrolyze PIP_2 (Goldschmidt-Clermont et al., 1990).

The δ isoform is considerably smaller than either β or γ; it lacks *src*-homology regions and is therefore unlikely to be a substrate for a receptor tyrosine kinase. It is not known how PLC-δ is activated in vivo; two obvious possibilities are that activation of this isoform requires only an increase in intracellular calcium or that an unidentified G protein is involved in its stimulation.

It is also unclear what steps limit the rate of calcium-stimulated phosphoinositide hydrolysis catalyzed by the PLC isoforms in vitro. The phospholipids that PLCs and other phospholipases attack in the living cell exist in the form of a bilayer, and it is reasonable to suspect that a portion of the enzyme inserts itself at least partially into one of the constituent monolayers prior to hydrolysis of the lipid. One approach to investigating this phenomenon is to study monolayers formed at the air–water interface because the surface pressure, π, can be controlled and measured. (By definition, $\pi = \gamma_0 - \gamma$ where γ_0 is the surface tension of the air–water interface and γ is the surface tension of the monolayer.) If a portion of the protein penetrates a monolayer maintained at a constant pressure π, displacing an area A_p of lipid, then work, W, must

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[1] Abbreviations: A, area of a phospholipid in a monolayer; A_p, area occupied by PLC-δ when it penetrates a monolayer; DAG, diacylglycerol; 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, Boltzmann constant; LUVs, large unilamellar vesicles; PC, phosphatidylcholine; PG, phosphatidylglycerol; PS, phosphatidylserine; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLA_2, phospholipase A_2; PLC, phospholipase C; T, temperature; π, surface pressure of monolayer.

[2] Demel et al. (1975) compared the action of purified phospholipases on monolayers formed at different pressures with their action on the outer monolayer of human erythrocyte membranes. They concluded that the lipid packing in the erythrocyte outer monolayer was comparable to that in a monolayer with a lateral surface pressure $31 < \pi < 35$ mN/m. Moreau et al. (1988) obtained similar results with a different phospholipase. Blume (1979) examined transitions from the gel to liquid crystalline state and concluded that "the behavior of the bilayer system is very similar to that of the respective monolayer system at a lateral pressure of approximately 30 dyne/cm, because at this pressure the absolute area and the area change in both systems are the same." Seelig (1987) compared the binding of the amphipathic local anesthetic dibucaine to palmitoyl-oleoyl-PC monolayers formed at different surface pressures with the binding of dibucaine to palmitoyl-oleoyl-PC lipid vesicles. She obtained agreement between monolayer and bilayer binding data for $31 < \pi < 33$ mN/m. Finally, the area of an egg PC lipid in a vesicle has been estimated from X-ray diffraction studies to be about 0.65–0.70 nm^2 (Shipley, 1973; Rand, 1981; McIntosh et al., 1989), corresponding to a surface pressure of about 30–35 mN/m in a monolayer (e.g., Figure 1). However, arguments have been made for both higher (MacDonald & Simon, 1987) and lower surface pressures (Schindler, 1979).

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be done equal to the product

$$W = \pi A_p \quad (1)$$

We expected the activity of the enzyme would decrease as π was increased because the lipase must perform more work to push aside an area A_p of lipids. For example, if A_p = 1 nm^2 and π = 20 mN/m the work is equal to 2 × 10^{-20} J (2.9 kcal/mol) whereas if π = 40 mN/m the work increases to 4 × 10^{-20} J (5.8 kcal/mol), which is 10-fold larger than the thermal energy (kT = 4 × 10^{-21} J at 25 °C). Several lines of evidence[2] suggest that the packing of the lipids in a bilayer or biological membrane is comparable to that in a phospholipid monolayer with a surface pressure 30 < π < 35 mN/m.

There is a long history of using surface pressure measurements to investigate the penetration of lipases into monolayers. Hughes (1935) showed that the rate of hydrolysis of phosphatidylcholine (PC) by phospholipase A_2 (PLA_2) decreases as the surface pressure of the monolayer increases. Verger and de Haas (1976) showed that the number of pancreatic PLA_2 molecules adsorbed to a monolayer also decreases with an increase in surface pressure. The structures of several PLA_2 enzymes have recently been deduced (Scott et al., 1990, 1991), which will facilitate a molecular interpretation of results obtained with monolayers. Ransac et al. (1991) recently reviewed the extensive monolayer literature concerned with PLA_2 and other phospholipases. We are aware of only one report describing the effect of surface pressure on the activity of an intracellular phosphoinositide-specific PLC. Hirasawa et al. (1981) investigated the ability of a pig brain cytosol extract, which presumably contained a mixture of phosphoinositide-specific PLCs, to hydrolyze phosphatidylinositol (PI) in monolayers formed from mixtures of lipids when the aqueous subphase contained 1 mM calcium ions. They found that "as the monolayer pressure was increased a sharp cutoff of enzymic hydrolysis occurred at 33 mN/m".

In this study, we examine how the monolayer surface pressure affects the rate at which purified PLC-δ hydrolyzes the substrate PIP_2. We also investigate how the electrostatic potential at the surface of the monolayer affects the activity of PLC-δ. The pioneering work of Bangham and Dawson (1959) demonstrated that electrostatic surface potentials markedly affect the activity of phospholipases, and Waite (1987; see chapter 10) has summarized many of the subsequent reports. Our previous results (Rebecchi et al., 1993) demonstrated that monovalent acidic lipids enhance the binding of PLC-δ to PIP_2 in vesicles. We examine here if monovalent acidic lipids produce a comparable enhancement in the activity of PLC-δ.

MATERIALS AND METHODS

Lipids and Chemicals. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (PS), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (PG) were obtained from Avanti Polar Lipids (Birmingham, AL). The triammonium salt of phosphatidylinositol 4,5-bisphosphate (PIP_2) was obtained from Calbiochem (La Jolla, CA) or Boehringer Mannheim (Indianapolis, IN); it is crucial to use the ammonium salt of PIP_2, which is soluble in chloroform, for monolayer experiments (Toner et al., 1988; Gabev et al., 1989). Radioactive [^{3}H]-PIP_2 was obtained from NEN Products (Boston, MA). The concentration of lipids in chloroform was determined both by evaporating and by weighing a measured volume on a Cahn electrobalance and by phosphate analysis (Lowry & Tinsley, 1974).

Aqueous solutions were prepared with 18-MΩ water (Super-Q; Millipore Corp., Bedford, MA) that was subsequently bidistilled in an all-quartz still. They were buffered to pH 7.5 with 25 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) from Calbiochem (La Jolla, CA).

Purification of PLC-δ$_1$. Phosphoinositide-specific phospholipase C-δ$_1$ was purified from bovine brain cytosol as described in Rebecchi et al., (1993). The identity of PLC-δ$_1$ was confirmed by sequence-specific antibodies prepared against synthetic peptides based on the sequence of the bovine brain PLC-δ$_1$ isotype (M. Cifuentes, M. and M. Rebecchi, unpublished observation).

Monolayers. In a typical experiment (e.g., Figures 1 and 2), we formed a monolayer from a mixture of PC (65%), PS (33%), and PIP_2 (2%) dissolved in chloroform. A measured volume of the chloroform solution was carefully deposited on the surface of the subphase, which was contained in a circular Teflon trough. The pressure–area curve reported in Figure 1 was obtained in a trough with a 10-cm diameter by depositing different amounts of lipid onto the surface. The pressures obtained with a smaller, 5-cm-diameter trough were shifted slightly (5%) to the left of those illustrated in Figure 1, presumably because of a loss of lipid onto the perimeter of the trough. This loss was not deemed to be a problem for the experiments illustrated in Figure 2 because we measured the pressure in each experiment with enzyme; we used the smaller 5-cm-diameter trough to minimize the amount of enzyme required for these experiments.

The trough, milled out of virgin Teflon, resembles a 5-cm-diameter Petri dish with a 0.5-cm-deep well in the center that accommodates a Teflon-coated magnetic stir bar to facilitate mixing of the aqueous subphase. In separate experiments, we injected a dye into the subphase and observed that the mixing time was about 15 s. (The well is important for effective mixing of the subphase; when we attempted the experiments with simple Petri dishes, we observed a lag of several minutes between addition of calcium ions and hydrolysis of PIP_2.) The trough also has a small hole drilled at an angle through the wall to allow addition of enzyme and calcium ions and withdrawal of subphase samples without breaking the surface of the monolayer. The monolayer apparatus was contained in a large perspex box to control the atmosphere and prevent dust from settling on the monolayer surface. Control experiments in a nitrogen or normal atmospheric environment gave identical results, and most experiments were done with the box open to the atmosphere.

Once the chloroform had evaporated (10 min), we measured the surface pressure using a square piece of filter paper (Whatman no. 2) and a balance as described by Fromherz (1975). We then added PLC-δ, typically 80 ng/15 mL = 63 pM, to the aqueous subphase. Five minutes after adding enzyme, we removed a 0.2-mL sample from the 15-mL subphase to check that no significant hydrolysis of PIP_2 had occurred; control experiments demonstrated that in the absence of calcium ions no significant hydrolysis occurred in 20 min.[3] We then added calcium ions to the aqueous subphase to bring the free concentration to either 1, 10, or 80 μM. (Separate experiments were performed with a calcium-sensitive electrode to determine the total concentration required to increase the free [Ca^{2+}] to these levels.) Samples of the subphase (0.2 mL) were collected at 1, 2, 5, 10, and 20 min after addition of calcium and analyzed for radioactive IP_3. The surface

[3] PLC-δ is activated by low concentrations of calcium ions at low surface pressures. A free [Ca^{2+}] = 100 nM significantly increases PIP_2 hydrolysis for π = 20 mN/m (data not shown).

pressure of the monolayer was measured at the end of the experiment to make sure that it had not changed significantly.

In addition to the mixing time, the time resolution of our experiments is limited by diffusion of calcium from the subphase and IP_3 from the monolayer through the aqueous unstirred layer. Irrespective of how fast one stirs the bulk aqueous phase, a region of thickness $\delta \simeq 100$ μm adjacent to the monolayer remains unstirred. A brief description of the Nernstian unstirred layer and references to more detailed discussions can be found elsewhere (McLaughlin & Eisenberg, 1975; Jain & Berg, 1989). The time for a molecule of diffusion coefficient $D \simeq 5 \times 10^{-6}$ cm^2/s to diffuse through this layer is about $t = \delta^2/2D = 10$ s according to the Einstein (1905) relation. Thus, measurements obtained at 1 min could be underestimates of the actual fraction of PIP_2 that is hydrolyzed, and we did not attempt to obtain data at shorter times.

PLC Assay. In a few experiments, we measured the concentration of active PLC in the aqueous subphase 5 and 30 min after enzyme addition. We withdrew 200-μL samples from the subphase and measured the rates of PIP_2 hydrolysis in a detergent/phospholipid mixed micelle system. The free concentration of PLC fell only 30% (±10%, $n = 4$) after 30 min, presumably because of inactivation of the enzyme or adsorption onto the Teflon trough. The reduction in activity was independent of the monolayer composition or surface pressure.

Use of [^{32}P]PIP_2 in Monolayers. In a separate series of experiments, we followed the approach of Bangham and Dawson (1960) and monitored [^{32}P]PIP_2 hydrolysis by means of a Geiger–Muller tube suspended above the monolayer. We prepared the [^{32}P]PIP_2 by labeling human erythrocyte membranes with [γ-^{32}P]ATP, extracting the lipids, and isolating the [^{32}P]PIP_2 by chromatography on a weak anion-exchange HPLC column (*n*-propylamine-bonded phase) as described by Low (1990). [^{32}P]PIP_2 in monolayers was hydrolyzed by PLC-δ upon addition of calcium ions. The initial rate of hydrolysis decreased with an increase in π (data not shown), as we observed with [^{3}H]PIP_2 (Figure 2). To our surprise, however, the enzyme never cleaved more than 50% of the ^{32}P-labeled substrate under any condition. We examined the [^{32}P]PIP_2 by deacylating the lipid and analyzing the inositol phosphate products on a SAX HPLC column as described by Auger et al. (1990). Only 50% of the lipid was authentic phosphatidylinositol 4,5-bisphosphate. The remainder of the lipid was a different isomer(s) of PIP_2, possibly the 3,4-bisphosphate isomer, which is not susceptible to hydrolysis by PLC-δ (Serunian et al., 1989). We abandoned this approach and used instead the [^{3}H]PIP_2 available commercially from NEN (Boston, MA). Analysis of this material (deacylation and SAX HPLC) indicated it was the pure 4,5 isomer. Furthermore, in control experiments we demonstrated that all the [^{3}H]PIP_2 could be cleaved by PLC-δ in detergent/phospholipid micelles prepared as described previously (Rebecchi & Rosen, 1987).

RESULTS

Figure 1 illustrates how the surface pressure, π, varies with the average area/molecule, A, for our PC/PS/PIP_2 monolayers. These π–A results are very similar to those that we (data not shown) and others [e.g., Shah and Shulman (1967), Phillips et al. (1975), Evans et al. (1987), and Moreau et al. (1988)] have obtained with either palmitoyl-oleoyl-PC or egg PC. The relationship between the surface pressure and the average area per phospholipid can be described by an equation of state similar to that for a Van der Waals gas (Aveyard & Haydon, 1973; Evans & Skalak, 1980; Cevc & Marsh, 1987; Mingins et al., 1992). X-ray diffraction studies (Shipley, 1973; Rand, 1981; McIntosh et al., 1989) indicate that the area occupied by an egg PC lipid in a bilayer vesicle is 0.65–0.7 nm^2, which corresponds to a surface pressure in Figure 1 of 30–35 mN/m. Experiments using other techniques[2] also suggest that a monolayer surface pressure of $30 < \pi < 35$ mN/m corresponds to the equilibrium state of the bilayer.

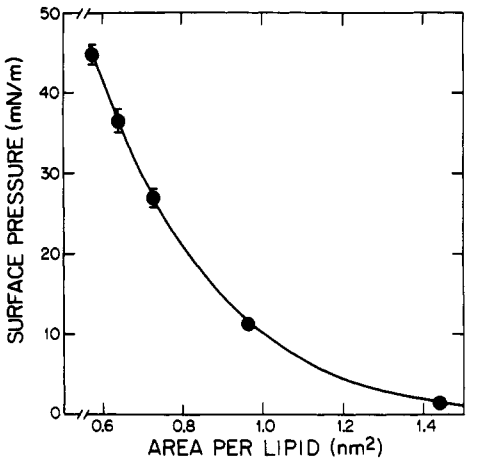

FIGURE 1: Surface pressure of a monolayer, π, vs the average area per lipid head group, A. The monolayer consisted of a mixture of PC (65%), PS (33%), and PIP_2 (2%). The aqueous subphase contained 100 mM KCl, 25 mM HEPES, 100 μM EGTA, and 1 mM DTT, pH 7.5. The standard deviations of the five independent measurements are indicated when larger than the symbol size. The curve has no theoretical significance.

We then examined the effect of monolayer surface pressure on the activity of the enzyme. We formed a PC/PS/PIP_2 monolayer, measured the surface pressure to ensure that it had the desired value, added PLC-δ to the subphase, and 5 min later added calcium ions to the subphase to increase the free [Ca^{2+}] to 10 μM. Samples of subphase were collected at the times indicated on the graph (measured as time after the addition of calcium), and radioactive IP_3 was measured.[4] Figure 2 illustrates how monolayer surface pressures of 20–35 mN/m affect PLC-δ activity. The salient feature of Figure 2 is that an increase in π reduces the activity of the enzyme, defined as the slopes of the lines, about 100-fold as the pressure increases from 20 to 35 mN/m. These activities, divided by the nanograms of PLC-δ in the 15-mL aqueous phase, are plotted as open symbols in Figure 3.

It is apparent from Figure 2 that the low activity at $\pi = 35$ mN/m precludes accurate measurements at higher surface pressures under these conditions. To investigate the activity

[4] In Figure 2, we illustrate only the initial, linear portion of the reaction progress curves. An analysis of the complete curves indicated the hydrolysis of PIP_2 by PLC-δ is not a simple first-order process. In the $\pi = 20$ mN/m curves illustrated in Figure 2, for example, the curve appeared to level off when 70% of the PIP_2 was hydrolyzed; apparent saturation, or a much slower phase of hydrolysis, was also observed at higher values of π. Similar results were also obtained with LUVs. The apparent saturation is not due to impurities in the sample of labeled PIP_2; in control experiments with micelles, PLC-δ hydrolyzed 100% of this PIP_2. It is not due to a decrease in the mole fraction of PIP_2 in the membrane as the reaction proceeds; when experiments similar to those illustrated in Figure 2 were done with 0.25, 0.5, and 1% rather than 2% PIP_2 in the monolayer, the initial slopes (% PIP_2 hydrolyzed/min) were unchanged. These experiments argue that the apparent saturation cannot be due to the accumulation of DAG in the monolayer or IP_3 in the subphase. IP_3 can inhibit hydrolysis of PIP_2 in LUVs by PLC-δ, but only when present at micromolar concentration, whereas the concentration in the subphase produced by PIP_2 hydrolysis in the monolayer reaches only the nanomolar concentration range.

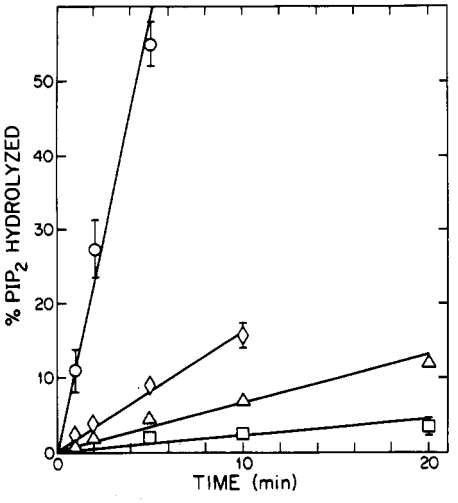

FIGURE 2: Percentage of radioactive PIP_2 in a monolayer hydrolyzed by PLC-δ plotted as a function of time after addition of calcium to the subphase; monolayer and subphase composition are as in Figure 1. The monolayer was formed at a pressure of 20 (circles), 25 (diamonds), 30 (triangles), or 35 (squares) mN/m. PLC-δ (63 pM) was added to the subphase 5 min prior to calcium (free $[Ca^{2+}] = 10$ μM), which was used to start the reaction. Aliquots of the aqueous subphase were taken at the indicated times and analyzed for radioactive IP_3. The slopes of the lines (least-squares best fits) are defined as the activity (% PIP_2 hydrolyzed/time). The vertical bars through the symbols indicate the standard deviations of the 4 (π = 20 mN/m), 3 (25), 2 (30), and 2 (35) independent experiments when these are larger than the symbol size.

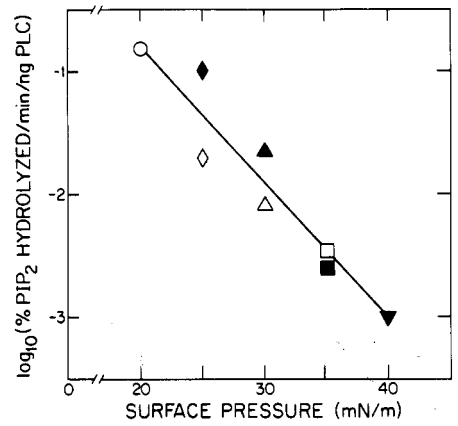

FIGURE 3: Specific activity of PLC-δ plotted as a function of the surface pressure of the monolayer. The ordinate is the % of PIP_2 in the monolayer hydrolyzed per minute per nanogram of PLC-δ in the 15-mL subphase. Open symbols represent data from Figure 2 where [PLC-δ] = 63 pM. Filled symbols represent data obtained in separate experiments with [PLC-δ] = 126 pM. The straight line is the least-squares best fit of log (specific activity) vs π and is also the theoretical fit to the model presented in the Discussion.

at surface pressures >35 mN/m, we repeated the measurements illustrated in Figure 2 with a 2-fold higher concentration of PLC-δ. Under these experimental conditions, the rate of hydrolysis of PIP_2 at π = 20 mN/m is too rapid to measure accurately, but we were able to measure the rate of hydrolysis at higher pressures; the activity of PLC-δ continued to decrease as the pressure increased from 35 to 40 mN/m.

Independent experiments (data not shown) demonstrate that at each pressure the activity depends linearly on the enzyme concentration in the subphase. This is reasonable because our estimate of the association constant of PLC-δ with PIP_2 in a PC/PS/PIP_2 vesicle, about 10^6 M^{-1} (Rebecchi et al., 1993), suggests that only about 1 in 10^4 of the PIP_2 molecules in the monolayer is bound to PLC-δ. We normalized the activities or initial rates we obtained with the 2-fold higher concentration of the enzyme by dividing the rate by the nanograms of PLC-δ in the 15-mL subphase; these measurements are the filled symbols in Figure 3. There is reasonable agreement between the two sets of measurements. Figure 3 illustrates that the activity of the enzyme decreases exponentially with the surface pressure; increasing the pressure from 20 to 40 mN/m reduces the activity 200-fold.

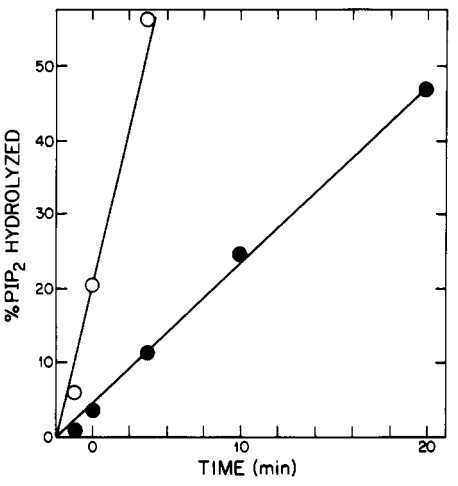

FIGURE 4: Effect of monovalent acidic lipids on the activity of PLC-δ. The open circles were obtained with a monolayer that contained PC (65%), PS (33%), and PIP_2 (2%). The filled circles were obtained with a monolayer that contained only PC and PIP_2 (2%). In both cases, π = 20 mN/m and free $[Ca^{2+}] = 10$ μM.

We observed a comparable dependence of PLC-δ activity on the surface pressure of the monolayer when we repeated the experiments with higher or lower free concentrations of calcium ions, $[Ca^{2+}]$. When $[Ca^{2+}] = 80$ μM, the enzyme activity at each surface pressure is about 5-fold higher than illustrated in Figure 3, but we again observed about a 100-fold reduction in activity as the surface pressure increased from 20 to 40 mN/m (data not shown). When $[Ca^{2+}] = 1$ μM, the enzyme activity at π = 20 and 30 mN/m (the only surface pressures we examined) was 5–10-fold lower than illustrated in Figure 3; although the data obtained at lower $[Ca^{2+}]$ were less reliable, we did observe that increasing π significantly reduced activity.

The dependence of PLC-δ activity on π is not a function of the mol % PIP_2 in the monolayer. We repeated the measurements illustrated in Figure 2 with 0.25, 0.5, 1, and 2% PIP_2 at π = 20 (and 30) mN/m. The initial slopes of % PIP_2 hydrolyzed vs time curves are identical for all mole fractions of PIP_2 in the monolayer. In other words, the rate of PIP_2 hydrolysis is linearly proportional to the mol % PIP_2 in the monolayer and, for a given mol % PIP_2, is about 10-fold higher at π = 20 than at 30 mN/m.

Finally, we investigated how acidic monovalent lipids such as PS and PG affect the activity of the enzyme. Our previous work demonstrated that PLC-δ binds with high affinity and specificity to PIP_2 in PC vesicles and binds about 3-fold more strongly when the PC vesicles also contain 33% PS (Rebecchi et al., 1993). We speculated that the rate of hydrolysis of PIP_2 catalyzed by PLC-δ could be proportional to the number of PLC molecules bound to the surface. Figure 4 illustrates that deleting PS from the monolayer does indeed reduce the enzyme activity about 5-fold when π = 20 mN/m; we observed a similar (3-fold) effect at pressures of 25 and 30 mN/m. Replacing PS in a monolayer (π = 25 mN/m) with the monovalent acidic lipid PG did not affect the activity. Thus, the ability of PLC-δ to hydrolyze PIP_2 in a monolayer increases about 4-fold if the monolayer contains 33% monovalent acidic

lipid (Figure 4). Acidic lipids produce a comparable increase in the PLC-δ binding to PIP_2 in phospholipid vesicles (Rebecchi et al., 1993). The simplest explanation for these results is that 33% PS (or PG) produces an electrostatic potential of −30 mV at the surface of the membrane (McLaughlin, 1989), which could increase the free concentration of enzyme in the aqueous diffuse double layer adjacent to the membrane. The thickness of the double layer, or Debye length, is about 1 nm in 0.1 M salt. In this case the effective valence, i.e., the net charge in the vicinity of the site on the enzyme that binds PIP_2 or, more exactly, the valence that must be used in the Boltzmann equation to account for the accumulation of the enzyme in the aqueous diffuse double layer, would need to be +1 to account for the effect of PS (or PG) on the binding and activity of the enzyme. Other explanations for the results illustrated in Figure 4 (e.g., PLC binds directly to either PS or PG as well as to PIP_2) cannot be ruled out.

DISCUSSION

Figure 3 illustrates our most important result; the activity of PLC-δ depends exponentially on the surface pressure of the monolayer. Specifically, the rate, R, at which the enzyme hydrolyzes PIP_2 may be expressed as

$$R = B \exp(-\pi A_p/kT) \quad (2)$$

where A_p and B are constants, k is the Boltzmann constant, and T is the temperature. The line in Figure 3 illustrates the least-squares best fit of eq 2 to the data with $A_p = 1.0$ nm^2.

We interpret this equation in terms of a simple model. In the absence of calcium ions, PLC-δ binds to PIP_2 in a PC/PS/PIP_2 membrane with an apparent association constant of 10^6 M^{-1} but is essentially inactive (Rebecchi et al., 1993); we assume it binds to PIP_2 in a monolayer in a similar manner. We postulate two membrane-bound states of PLC-δ: the inactive or I state and the active or J state. Calcium ions must bind to PLC-δ molecules in the I state to convert then into the J state.[5,6] We assume that the rate of hydrolysis of PIP_2 is proportional to the concentration of PLC-δ in the J state ($R = C$[J] where C is a constant). Finally, we assume there is a Boltzmann distribution between the number of bound enzymes in the I and J states:

$$R = C[\mathrm{J}] = C[\mathrm{I}] \exp(-U/kT) \quad (3)$$

where U is the free energy difference between the two states. In the absence of calcium ions, U must be sufficiently large that most of the enzyme is inactive, [J] ≪ [I]. For simplicity, we further assume that most of the enzyme remains in the inactive or I state; [I] is thus a constant approximately equal to the total concentration of PLC-δ bound to the membrane, $[\text{PLC-}\delta]_b$:

$$[\mathrm{I}] \simeq [\text{PLC-}\delta]_b \quad (4)$$

(This assumption should be valid for the conditions illustrated in Figures 3 and 4, at least for $\pi > 20$ mN/m.) We now split the free energy U into two components:

$$U = \pi A_p + W \quad (5)$$

πA_p is simply the work that must be done to insert a portion of the enzyme with area A_p at least partially into the monolayer, displacing this area of lipid. We assume that the other component, W (which includes all the other free energy terms involved in activating the enzyme), is independent of the surface pressure. If we insert eqs 4 and 5 into eq 3, we obtain a theoretical equation identical in form to the empirical eq 2, where $B = C[\text{PLC-}\delta]_b \exp(-W/kT)$. (In this model W decreases when calcium ions bind to PLC-δ).

Our results suggest that the area of protein that inserts into the membrane, A_p, comprises less than 1% of the total surface area of the enzyme but do not indicate the location of this region. This area is less than the area occupied by two phospholipids, and it is not unreasonable that such a small portion of the enzyme inserts into the monolayer. Our model is highly oversimplified and assumes that the protein completely displaces the lipids in one monolayer; if the protein only partially displaces the lipids as seems more likely, our model will underestimate A_p. For example, molecules that form amphipathic helices such as melittin only partially penetrate phospholipid surfaces (Tamm, 1991). The binding of amphipathic mitochondrial signal (Tamm, 1986) and substance P antagonist (Seelig, 1992) peptides as well as intact Sec A proteins (Breukink et al., 1992) to phospholipid monolayers does decrease as the surface pressure increases.

In contrast to our results with PLC-δ, Hirasawa et al. (1981) observed a stronger dependence of activity on π (cutoff pressure) when they examined the relationship between surface pressure and PLC activity with their cytoplasmic extract. It is possible that their preparation consisted mainly of PLC isozymes that must penetrate into the monolayer to a greater extent than the δ isoform; our analysis indicates that a larger A_p will produce a stronger dependence of activity on π. Other PLC isozymes must be examined in monolayer experiments to resolve this question.

Is the monolayer a reasonable model system for investigating the mechanism of action of PLC-δ? One way of addressing the question is to compare the catalytic activity of the enzyme on a monolayer with its catalytic activity on a bilayer vesicle, which is a better model system for a biological membrane. We first estimate the turnover number, n, of a PLC-δ bound to the PC/PS/PIP_2 monolayer and exposed to free [Ca^{2+}] = 10 μM from the expression

$$n = V/\{\text{PLC}\} \quad (6)$$

where V is the velocity of the reaction or the moles of PIP_2 hydrolyzed cm^{-2} s^{-1} and {PLC} is the surface concentration of bound PLC-δ in mol cm^{-2}. The numerator may be calculated from the data in Figure 2. For π = 30–35 mN/m, a surface pressure range that correspons to the average area per lipid in bilayer vesicles and biological membranes[2], the rate of hydrolysis, R (fraction of PIP_2 hydrolyzed per second), is (3–10) × 10^{-5} s^{-1}; if $\{PIP_2\}$ is the surface concentration of PIP_2 in mol cm^{-2}, then the numerator in eq 6 is $R\{PIP_2\}$. The denominator, the surface concentration of the bound enzyme, can be calculated from $\{\text{PLC}\} = K[\text{PLC}]\{PIP_2\}$ where K = 10^6 M^{-1} is the equilibrium association constant determined for the binding of PLC to PIP_2 in PC/PS/PIP_2 vesicles in the absence of calcium ions and [PLC] is the concentration of enzyme in the aqueous phase (Rebecchi et al., 1993). Thus $n = R/K[\text{PLC}] = ((3\text{–}10) \times 10^{-5}\ \text{s}^{-1})/(10^6\ \text{M}^{-1})(60 \times 10^{-12}\ \text{M}) = 0.5 - 2\ \text{s}^{-1}$. (This should be regarded as an approximate calculation for several reasons.[5]) This turnover number agrees with the number calculated for PLC-δ hydrolyzing PIP_2 in PC/PS/PIP_2 vesicles ($n \simeq 1$ s^{-1}; M. Rebecchi, unpublished results). Thus, at least as judged by this criterion, the

[5] We assume that addition of calcium ions does not affect the binding of PLC-δ to monolayers or bilayers.

[6] Activation of PLC-δ by calcium ions cannot be due solely to penetration of the enzyme into a phospholipid surface. Our work with phosphoinositides having short acyl chains demonstrates that PLC-δ also requires calcium ions to hydrolyze these monomeric (soluble) lipids.

monolayer appears to be reasonable[7] model system for investigating the mechanism of action of PLC-δ.

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[7] The dependence of PLC-δ activity on $[Ca^{2+}]$, however, may vary with the system used to assay activity. With micelles and soluble short chain phosphoinositides, our PLC-δ exhibits maximal activity with about 1 μM $[Ca^{2+}]$, a result similar to those obtained previously by other investigators using micelles and vesicles (Wilson et al., 1984; Homma et al., 1988; Fukui et al., 1988; McDonald & Mamrack, 1989; Haber et al., 1991; Pawelczyk & Lowenstein, 1992). With monolayers, however, the PLC-δ activity increases an order of magnitude as the $[Ca^{2+}]$ increases from 1 to 100 μM.