Effect of Monolayer Surface Pressure on the Activities of Phosphoinositide-Specific Phospholipase C-β_1, -γ_1, and -δ_1[†]

Valentina Boguslavsky,[‡] Mario Rebecchi,[‡] Andrew J. Morris,[‡] Deok-Young Jhon,[§] Sue Goo Rhee,[§] and Stuart McLaughlin*,[‡]

Health Sciences Center, State University of New York, Stony Brook, New York 11794-8661, and Laboratory of Biochemistry, NHLBI/National Institutes of Health, Bethesda, Maryland 20892

Received October 8, 1993; Revised Manuscript Received December 30, 1993[⊛]

ABSTRACT: Three isoforms of phospholipase C, either PLC-β_1, PLC-γ_1, or PLC-δ_1, were added to the aqueous subphase beneath phospholipid monolayers formed at an air–solution interface, and the initial rate of hydrolysis of phosphatidylinositol 4,5-bisphosphate was measured after addition of 10 μM free Ca^{2+}. The monolayers were formed from mixtures of phosphatidylcholine (65% PC), phosphatidylserine (33% PS), and phosphatidylinositol 4,5-bisphosphate (2% PIP_2). Increasing the surface pressure of the monolayer, π, from 15 to 25 mN/m decreases the rate of hydrolysis 16-, 13-, and 5-fold for PLC-β_1, PLC-γ_1, and PLC-δ_1, respectively. The simplest interpretation of these results is that a portion of each of the enzymes of area A_p must insert into the monolayer, doing work πA_p, prior to hydrolysis of PIP_2; binding studies with simple model compounds of known cross-sectional area are consistent with this interpretation. Removing the monovalent acidic lipid PS from the monolayer decreases the initial rates of hydrolysis of PIP_2 about 3-fold for each PLC isoform, which suggests that negative electrostatic surface potentials increase the PLC activity.

The intracellular phosphoinositide-specific phospholipase C (PLC)[1] enzymes catalyze hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), producing the two second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (Berridge, 1993). At least eight different PLC isoenzymes have been identified in mammalian cells, and these have been grouped into three major classes: β, γ, and δ (Dennis et al., 1991; Rhee & Choi, 1992; Cockcroft & Thomas, 1992). Members of the G_q class of guanine nucleotide-binding proteins (G proteins) activate members of the PLC-β class (Sternweis & Smrcka, 1992). Receptor tyrosine kinases, such as the epidermal growth factor receptor, phosphorylate and activate members of the PLC-γ class (Carpenter, 1992). The physiologically important factors that regulate the PLC-δ class have not been identified, although Ca^{2+} ions, the $\beta\gamma$ subunits of G proteins (Park et al., 1993), and sphingomyelin (Pawelczyk & Lowenstein, 1992) are possible factors.

The molecular mechanisms by which G proteins activate PLC-β and receptor tyrosine kinases activate PLC-γ are not understood. Thus it is important to characterize the physical factors that affect the enzymatic activity of this family of proteins, which, like other phospholipases, operate at the membrane–solution interface. A large body of work demonstrates that the activities of many extracellular lipases, such as phospholipase A_2, depend critically on the physical state of their lipid substrates (Verger & de Haas, 1976; Dennis, 1983; Waite, 1987; Jain & Berg, 1989). We demonstrated previously that the activity of PLC-δ_1 increases 100-fold as the surface pressure of a phospholipid monolayer containing PIP_2 decreases from 40 to 20 mN/m (Rebecchi et al., 1992b). (The surface pressure $\pi = \gamma_o - \gamma$ where γ_o is the surface tension at the air–water interface, and γ is the surface tension of the monolayer.) To account for this observation, we proposed that a portion of PLC-δ with an area $A_p = 1$ nm^2 inserts into the monolayer and does work πA_p prior to activation of the enzyme. In this study we compare how the enzymatic activities of representative members of the three classes of PLC depend on the surface pressure of a phospholipid monolayer containing PIP_2. We also measure the monolayer adsorption of two model compounds of known cross-sectional area A to test our proposal that the adsorption should be proportional to the Boltzmann factor, $\exp(-\pi A/kT)$.

MATERIALS AND METHODS

Lipids and Chemicals. We used the triammonium salt of PIP_2 (Calbiochem, La Jolla, CA) when forming monolayers or bilayers (Toner et al., 1988; Gabev et al., 1989). Radioactive [^{3}H]PIP_2 was obtained from NEN Products (Boston, MA); 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC), 1,2-dicapryloyl-*sn*-glycero-3-phosphocholine (diC_8PC), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (PS) were obtained from Avanti Polar Lipids (Birmingham, AL); myristic acid was obtained from Sigma (St. Louis, MO). The concentration of lipids in chloroform was determined both by evaporating and weighing a measured volume on a Cahn electrobalance and by phosphate analysis (Lowry & Tinsley, 1974).

We used 18 MΩ water (Super-Q, Millipore Corp., Bedford, MA) that was subsequently bidistilled in an all-quartz still to prepare the aqueous solutions, which were buffered to pH 7.0

[†] This work was supported by NSF Grant MCB 91-17526 to S.M., NIH Grant GM-43422 to M.R., and American Heart Association Grant-in-Aid 911524 to A.J.M.

* To whom correspondence should be addressed. Telephone, 516-444-3615; Fax, 516-444-3432.

[‡] State University of New York, Stony Brook.

[§] National Institutes of Health.

[⊛] Abstract published in *Advance ACS Abstracts,* February 15, 1994.

[1] Abbreviations: A, cross-sectional area of myristic acid or the short chain phospholipid diC_8PC; A_p, effective area of phospholipase that inserts into monolayer; DTT, dithiothreitol; G proteins, guanine nucleotide-binding proteins; IP_3, inositol 1,4,5-trisphosphate; k, Boltzmann constant; PC, phosphatidylcholine; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phosphoinositide-specific phospholipase C; PS, phosphatidylserine; S, area of monolayer; T, temperature; π, surface pressure of a monolayer; γ_o, surface tension of the air–water interface; γ, surface tension of a monolayer.

0006-2960/94/0433-3032$04.50/0

with 25 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (HEPES) from Calbiochem (La Jolla, CA).

Purification of Enyzmes. PLC-γ_1 and β_1 were purified from bovine brain as described in Ryu et al. (1987); PLC-δ_1 was purified from bovine brain cytosol as described in Rebecchi et al. (1992a). 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 (Cifuentes et al., 1993).

Some experiments were performed using recombinantly expressed bovine PLC-β_1 (rPLC-β_1). Using PCR amplification with specific primer–adapters, a *Not*1 restriction site was introduced into the PLC-β_1 cDNA 40 bp upstream of the initiating methionine codon, allowing directional insertion into *Not*1/*Eco*R1 sites of the PVL1392 transfer vector (Invitrogen, San Diego, CA). *Spodoptera frugiperda* cells (Sf9 cells) were transfected with a mixture of the PLC-β_1/PVL1390 vector and linearized wild-type *Autographica california* polyhedrosis virus DNA to generate recombinant baculoviruses that were purified and amplified (Lucklow & Sumners, 1988). Recombinant PLC-β_1 proteins were purified from 1-L suspension cultures of Sf9 cells (2×10^6 cells/mL). The cells were harvested by centrifugation, washed once in phosphate-buffered saline, and subjected to nitrogen cavitation. rPLC-β_1 was purified from supernatants obtained by ultracentrifugation of the nitrogen-cavitated cell suspension by sequential chromatography on Q-Sepharose and heparin-Sepharose. Approximately 200 μg of purified protein was obtained from a 1-L culture of Sf9 cells. rPLC-β_1 is catalytically active and G-protein regulated, displaying activities comparable to those reported for PLC-β_1 purified from bovine brain (A. J. Morris, details to be published elsewhere).

Monolayers. In a typical experiment (e.g., Figures 1, 2, and 3) we formed a monolayer from a mixture of PC (65%), PS (33%), and [^{3}H]PIP_2 + PIP_2 (2%) dissolved in chloroform. A measured volume of the chloroform solution was deposited on the surface of the subphase, which was contained in a circular teflon trough. We used a small (5-cm diameter) trough to minimize the amount of enzyme required for these experiments. Other experimental details are described in Rebecchi et al. (1992b).

Once the chloroform had evaporated (10 min), we used a square piece of filter paper (Whatman No. 2) and a balance as described by Fromherz (1975) to measure the surface pressure and ensure that it had the correct value; Figure 1 of Rebecchi et al. (1992b) illustrates how the surface pressure of these monolayers varies with the average area/molecule. We then added PLC—typically 4 μg/15 mL of PLC-β_1 = 1.9 nM, 4μg/15 mL of PLC-γ_1 = 1.8 nM, or 80 ng/15 mL of PLC-δ_1 = 62 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. We then added Ca^{2+} to the aqueous subphase to bring the free concentration to 10 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 typically collected 1, 2, 5, 10, 20, and 30 min after addition of calcium and analyzed for radioactive IP_3. The surface pressure of the monolayer was measured at the end of the experiment to make sure that it had not changed significantly.

Control experiments demonstrated that for PLC-δ the enzymatic hydrolysis in the absence of Ca^{2+} was negligible (<3%) at all surface pressures compared to the hydrolysis produced by enzyme in the presence of 10 μM Ca^{2+}. Ten micromolar Ca^{2+} produced about a 10-fold increase in the activity of both PLC-β_1 and PLC-γ_1 at low surface pressures (π = 15, 20 mN/m). As illustrated in Figure 1, however, the Ca^{2+}-stimulated activity of PLC-γ_1 (and PLC-β_1) is very low for $\pi > 25$ mN/m, and the Ca^{2+}-independent activity is relatively more important at high surface pressures. For example, at π = 30 mN/m the Ca^{2+}-independent activity of PLC-γ_1 is 50% of the Ca^{2+}-dependent activity. Thus the data reported at these higher pressures (Figure 2) represent overestimates of the Ca^{2+}-stimulated activity. We show the data only to illustrate that the activity remains low as π is increased.

PLC Assay. In several experiments we measured the concentration of active PLC in the aqueous subphase 5 and 30 min after enzyme addition. We withdrew 20-μL samples from the subphase and measured the rates of PIP_2 hydrolysis in a detergent/phospholipid mixed micelle system. The reaction mixture contained 0.2 mM PIP_2 + [^{3}H]PIP_2, 0.5% sodium cholate, 0.9 mM Ca^{2+}, 0.3 mM EGTA, 0.3 mM DTT, 100 mM KCl, and 25 mM HEPES, pH 7.0, in a total volume of 60 μL. The reactions were terminated by addition of ice-cold 10% trichloroacetic acid in water (250 μL). Twenty-five microliters of 10% Triton X-100 in water was added to form a precipitate and the samples were centrifuged at 12000*g* for 0.5 min. The supernatant containing the [^{3}H]IP_3 product was mixed with 1 mL of $CHCl_3$/MeOH (2:1). The sample formed two phases: the upper (aqueous) phase was transferred to scintillation vials and the radioactivity determined in a liquid scintillation spectrometer. In each case, the specific activity or free concentration of active PLC in the aqueous subphase below the monolayer, as measured by this mixed micelle assay, fell only slightly (less than 30%) by 30 min, presumably because of inactivation of the enzyme or adsorption onto the teflon trough. Neither the monolayer composition nor the surface pressure affected this reduction in activity.

Monolayer Adsorption Measurements. We used an apparatus designed by Fromherz (1975), purchased from Mayer Feintechnik (Göttingen, Germany), to measure directly the adsorption of solutes of known cross-sectional area to phospholipid monolayers This apparatus monitors the surface pressure of the monolayer by means of a Wilhelmy plate and has a feedback circuit that increases the area of the monolayer as the solute adsorbs to the surface, maintaining a constant surface pressure. The monolayers were formed from palmitoyloleoyl-PC over a solution containing 0.1 M KCl and 0.005 M $KHPO_4$, pH 7.0, T = 25 °C. We added small volumes of myristic acid or diC_8PC in this solution to the subphase to produce concentrations of 1.5 μM myristic acid or 0.5 μM diC_8PC and measured the change in area, ΔS. [The diC_8PC has no net charge. The pK of myristic acid is 5, but the neutral HA form adsorbs about 300-fold more strongly than the charged A^- form (Peitzsch & McLaughlin, 1993), so 75% of the molecules that adsorb to the monolayer are the HA form. This minimizes the production of a surface charge, which impedes further adsorption of A^-. The surface potential effect is negligible under our experimental conditions.]

The mole fraction, X, of myristic acid (or diC_8PC) in the phospholipid monolayer is ($n \ll n_L$): $X = n/n_L = (\Delta S/S)(A_L/A)$ where n is the number of myristic acid molecules adsorbed to the monolayer, n_L is the number of phospholipids in the monolayer, $\Delta S/S$ is the fractional increase in area of the monolayer, A_L is the area of a phospholipid, and A is the area of a myristic acid molecule in the monolayer at that surface pressure. For small X ($\Delta S/S < 0.1$), X is

proportional to the mole fraction (or concentration) of solute such as myristate (data not shown) or dibucaine (Seelig, 1987) in the aqueous subphase, as expected theoretically. We write the proportionality constant (the adsorption or partition coefficient) as the product of K and $\exp(-\pi A/kT)$, where K is a function that may depend on π. Thus, $\Delta S/S = (A/A_L)K$[myristate] $\exp(-\pi A/kT)$. We then assume that (A/A_L), K, and A are independent of π. The first assumption is reasonable; although the area of each PC decreases from about 0.8 to 0.65 nm^2 as π increases from 20 to 35 mN/m (Evans et al., 1987; Rebecchi et al., 1992b), the area occupied by an adsorbed myristate probably also decreases. The constant K depends on the binding energy of myristate. This arises mainly from the hydrophobic effect (Peitzsch & McLaughlin, 1993), which should be independent of π. However, K may also depend on the accessible area in the monolayer, which for one lipid is the difference between the area occupied by PC molecule and the minimal area a PC molecule could occupy; therefore K may decrease with π (Pethica, 1955; McGreggor & Barnes, 1974). Finally, A almost certainly decreases with π, but this will blunt the decrease in binding predicted by the exponential term, and the effect will tend to cancel any decrease in K produced by the increase in π. Thus, to a first approximation it may be valid to assume that the binding is proportional to the Boltzmann factor $\exp(-\pi A/kT)$, where A is taken as the minimal cross-sectional area of the penetrating molecule. Ter-Minassian-Saraga (1991) has recently reviewed the more sophisticated theoretical approaches that have been used to describe the penetration of soluble surfactants into insoluble monolayers. Our approach in this report is to test the prediction that the penetration is proportional to $\exp(-\pi A/kT)$ by measuring the binding of two surfactants with known cross-sectional areas. The measured minimal cross-sectional area of a saturated chain PC lipid is 0.4 nm^2 (Marsh, 1990), and the minimal cross-sectional area of myristic acid must be about 0.2 nm^2. The simple Boltzmann relation predicts that the binding of diC_8PC should decrease by a factor of 4 and the binding of myristate by a factor of 2 as π increases from 20 to 35 mN/m.

RESULTS

Our main objective was to determine if the surface pressure of a monolayer affects the activity of either PLC-β_1 or PLC-γ_1. Figure 1 illustrates a typical experiment conducted with PLC-γ_1: an increase in surface pressure produces a decrease in the activity of the enzyme, which we define as the slope of a linear fit to the data. Increasing the surface pressure from 15 to 20 mN/m has a smaller effect (2-fold decrease) than increasing the pressure from 20 to 25 mN/m (10-fold decrease). The results of these and other experiments with PLC-γ_1 and of comparable experiments with PLC-β_1 are summarized in Figure 2.

Figure 2 illustrates how the specific activities of PLC-β_1 (left) and PLC-γ_1 (right) depend on the surface pressure of the monolayer. It is apparent that raising the surface pressure decreases enzymatic activity: increasing the pressure from 15 to 25 mN/m reduces the activity of PLC-β_1 and PLC-γ_1 16- and 13-fold, respectively. The curves in Figure 2 are drawn to guide the eye and have no theoretical significance. [As discussed under Materials and Methods, the data obtained at high surface pressures, $\pi \geq 30$ mN/m, are overestimates of the Ca^{2+}-stimulated activity. It is apparent from Figure 1 that <10% of the PIP_2 in the monolayer is hydrolyzed by PLC-γ_1 (and PLC-β_1) under these conditions.] Experiments

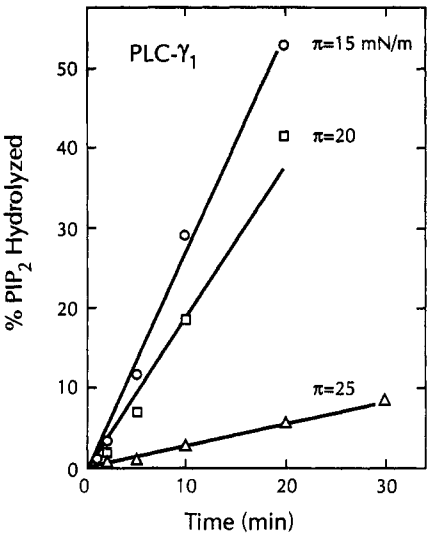

FIGURE 1: Percentage of radioactive PIP_2 in a PC/PS/PIP_2 monolayer hydrolyzed by PLC-γ_1 plotted as a function of time after enzyme activation by Ca^{2+}. The monolayers were formed at pressures of 15 mN/m (circles), 20 mN/m (squares), or 25 mN/m (triangles) over 15-mL subphases containing 100 mM KCl, 25 mM HEPES, 1 mM DTT, and 1 mM EGTA, pH 7.0. Five minutes after addition of PLC-γ_1 (4 μg) to the subphase, the reaction was initiated by addition of Ca^{2+} (free [Ca^{2+}] = 10 μM). Samples (200 μL) of the aqueous subphase were taken at the indicated times and analyzed for radioactive IP_3. The slopes of the lines (least-square best fits) are defined as the activity (% PIP_2 hydrolyzed/time).

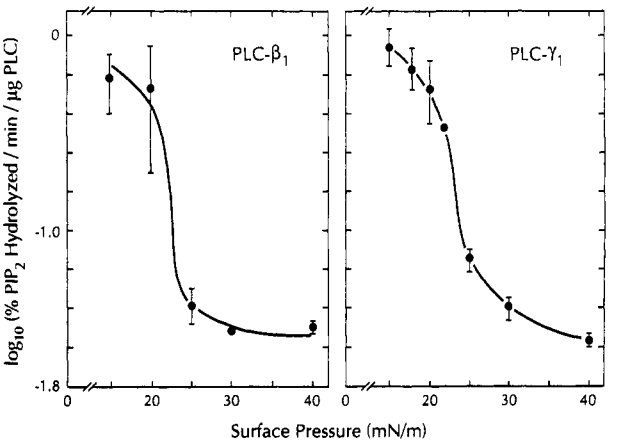

FIGURE 2: (Left) Specific activity of PLC-β_1 plotted as a function of the surface pressure of the PC/PS/PIP_2 monolayer. The specific activity of PLC-β_1 is deduced from experiments similar to those illustrated in Figure 1. The data points represent the average (±SD) of 3, 6, 2, 1, and 5 experiments for surface pressures of 15, 20, 25, 30 and 40 mN/m, respectively. (Right) Specific activity of PLC-γ_1 plotted as a function of the surface pressure of the PC/PS/PIP_2 monolayer. The specific activity is deduced from the slopes of the curves in Figure 1 and similar experiments. The data points represent the average (±SD) of 2, 3, 8, 1, 4, 3, and 3 experiments for pressures of 15, 18, 20, 22, 25, 30, and 40 mN/m, respectively.

with recombinantly expressed PLC-β_1 at $\pi = 15$ and 25 mN/m agreed, within experimental error, with the results illustrated in Figure 2 (data not shown). These results also agree qualitatively with the results obtained previously by Hirasawa et al. (1981), who studied a pig brain cytosol extract that presumably contained a mixture of phosphoinositide-specific PLCs. They observed a sharp "cutoff" in the extract's ability to hydrolyze phosphatidylinositol (PI) when monolayer surface pressure was increased.

Figure 3 summarizes the results we obtained with PLC-δ_1, which are very similar to those reported previously for this isoform (Rebecchi et al., 1992b). As we observed with the

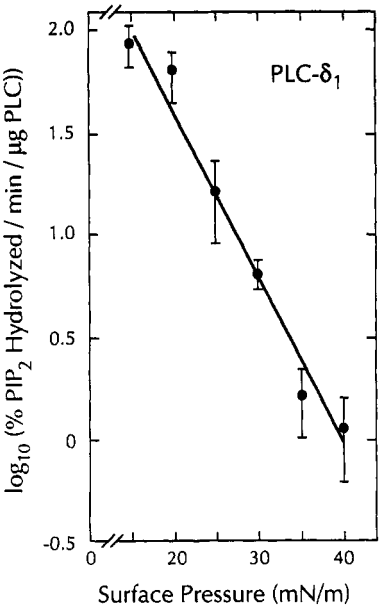

FIGURE 3: Specific activity of PLC-δ_1 plotted as a function of the surface pressure of the PC/PS/PIP_2 monolayer. The specific activity is deduced from experiments similar to those illustrated in Figure 1. The data points represent the average of three separate experiments (±SD). The line, which is the least-squares best fit to the data, indicates the activity is proportional to the exponent of the surface pressure, $\exp(-\pi A_p/kT)$, where A_p = 0.7 nm^2.

β_1 and γ_1 isoforms, an increase in surface pressure produces a large decrease in activity of PLC-δ_1: increasing the surface pressure from 15 to 25 mN/m reduces the activity 5-fold and increasing it to 40 mN/m reduces the activity about 100-fold. The results obtained with PLC-δ_1 differ from those obtained with the -β_1 and -γ_1 isoforms in two respects. First, the slope of the curve obtained with PLC-δ_1 (Figure 3) is less steep than the slope of the curves obtained with PLC-β_1 and -γ_1 (Figure 2), at least for $15 < \pi < 25$ mN/m, where we can obtain reliable data with PLC-β_1 and -γ_1. Second, PLC-δ_1 is about 100-fold more active in this monolayer system than either PLC-γ_1 or PLC-β_1 (note the different ordinate scales in Figures 2 and 3).

While the specific activity of PLC-δ_1 on monolayers is about 100-fold greater than the specific activity of either PLC-β_1 or -γ_1 (Figures 2 and 3), it is only about 10-fold greater in the particular detergent/phospholipid mixed micelle assay (cholate/PIP_2: see Materials and Methods) we used to ensure that the enzyme activity remained constant over time in the aqueous subphase below the monolayer. The specific enzyme activities of PLC-β_1, -γ_1, and -δ_1 determined from this micelle assay were 6 ± 3 (n = 12), 5 ± 2 (n = 10), and 70 ± 10 (n = 8) μmol of PIP_2 hydrolyzed/(min·mg of enzyme) (±S/D), respectively.[2]

Our interpretation of the results illustrated in Figures 2 and 3 is that a portion of the enzyme molecule with cross-sectional area A_p must insert into the monolayer and do work

[2] One difference between the monolayer and the mixed micelle assay systems is that most of the lipase molecules in the monolayer system are free in the aqueous subphase (because of the small quantity of lipid present), whereas most of the lipase molecules in the detergent micelle system are presumably bound to the micelles (because of the high concentration of micelles). A difference in the affinity of the lipases for interfaces (e.g., if PLC-δ_1 had a 10-fold greater affinity for the monolayer than PLC-β_1 or -γ_1) could explain the higher relative activity of the δ_1 isoform in the monolayer system. The binding of PLC-δ_1 to phospholipid vesicles has been measured (Rebecchi et al., 1992a; Pawelczyk & Lowenstein, 1993), but we know of no binding data for the other isoforms.

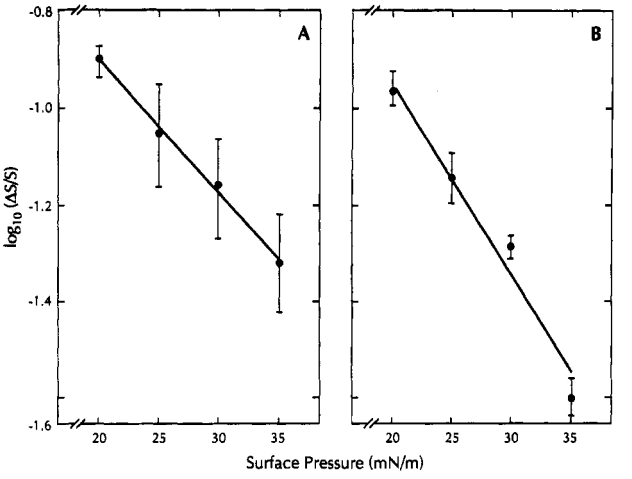

FIGURE 4: Binding of molecules of known cross-sectional area to palmitoyloleoyl-PC monolayers maintained at different surface pressures. The surface pressure was maintained constant with a feedback device that increases the area of the monolayer as the molecules bind to the surface. ΔS is the change in monolayer area, S, produced by the adsorption of either myristic acid (A) or a short chain phospholipid, diC_8PC (B). The straight lines are the least-square fit of an equation that assumes the binding is proportional to $\exp(-\pi A/kT)$, where π is the surface pressure, A is the cross-sectional area of the adsorbing molecule, k is Boltzmann's constant, and T is the temperature. The values of A deduced from the fit to the data are 0.25 nm^2 for myristic acid and 0.42 nm^2 for diC_8PC, which correspond well to the known values of about 0.2 and 0.4 nm^2 for the minimal cross-sectional areas of these molecules. The aqueous solutions contained 0.1 M KCl and 0.005 M $KHPO_4$, pH 7.0, T = 25 °C and either 1.5 μM myristic acid (A) or 0.5 μM diC_8PC (B). The bars illustrate the SD of the six (A) and four (B) separate experiments.

πA_p to activate the enzyme. Thus the activity should be proportional to the Boltzmann factor $\exp(-\pi A_p/kT)$. A corollary of this hypothesis is that the partitioning of any solute that penetrates deeply into a phospholipid monolayer should be proportional to this Boltzmann factor. We tested this corollary directly by measuring the adsorption of solutes of known area to monolayers formed from palmitoyloleoyl-PC. We formed the PC monolayer on an apparatus of the Fromherz design, which has a feedback device that monitors the surface pressure and maintains it at a constant preset value by increasing the area of the monolayer as the solute adsorbs. We studied the binding of myristic acid and a short chain phospholipid, diC_8PC (Figure 4). Although the data are consistent with the prediction that the binding is proportional to $\exp(-\pi A/kT)$ where A is the minimal cross-sectional area, it was not possible to obtain sufficiently accurate data to rule out other nonexponential relationships between binding and π; the least-square lines in Figure 5 are drawn with A = 0.25 and 0.42 nm^2, values that agree well with the limiting areas of 0.2 and 0.4 nm^2 for myristic acid and PC, respectively (Marsh, 1990).

Removing the monovalent acidic lipid PS from the monolayer decreases the activity of PLC-γ_1 about 3-fold (Figure 5). A similar effect is observed with PLC-β_1 (data not shown) and PLC-δ_1 (Rebecchi et al., 1992b). Removing PS from PC/PS/PIP_2 vesicles produces a similar (4-fold) decrease in the binding of PLC-δ_1 to phospholipid vesicles (Rebecchi et al., 1992a). One simple interpretation of these results is that 33% PS produces a negative electrostatic surface potential of about −30 mV (McLaughlin, 1989), which increases the free concentration of the enzyme in the diffuse double layer adjacent to the membranes or monolayers. Another possibility is that basic residues in the enzymes, such as those in the "P box" of PLC-β_1 (Wu et al., 1993), a region required for

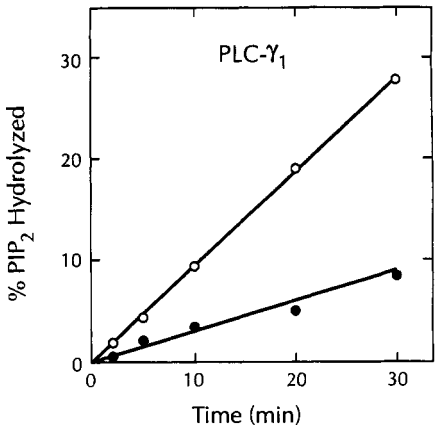

FIGURE 5: Effect of the monovalent acidic lipid PS on the activity of PLC-γ_1: the open circles illustrate data obtained with monolayers containing PC(65%), PS(33%), and PIP_2 (2%). The filled circles illustrate data obtained with monolayers containing PC (98%) and PIP_2 (2%). In both cases free $[Ca^{2+}] = 10\ \mu M$. The surface pressure was 20 mN/m, and the PLC-γ_1 concentration was 4 μg/15 mL. (One example of duplicate experiments.)

membrane association, bind to monovalent acidic lipids. The binding of basic residues to acidic lipids is well documented for simple peptides (Mosior & McLaughin, 1992a,b; Montich et al., 1993).

DISCUSSION

Figures 2 and 3 show that increasing the surface pressure of a monolayer containing PIP_2, the enzyme's substrate, reduces the activity of all three isoforms of PLC. The simplest interpretation of this result is that a portion of the enzyme with effective area A_p must insert into the monolayer and do work πA_p before the enzyme is active. A quantitative analysis of the data obtained with PLC-δ_1 suggests that $A_p \approx 1\ nm^2$ for this isoform (Figure 3). The steeper dependence of activity on π for PLC-γ_1 and -β_1 (Figure 2), at least for $15 \leq \pi \leq 25$ where we can obtain reliable data, suggests that a larger area of these enzymes inserts into the monolayer.

The data in Figure 4 provide some support for our interpretation that penetration of an area A_p of a molecule into a monolayer should be proportional to the simple Boltzmann factor $\exp(-\pi A_p/kT)$. Seelig (1987) made similar measurements of the binding of the local anesthetic dibucaine to monolayers, and her data are also consistent with this interpretation.[3] While any quantitative interpretation of the data in Figures 1–3 is suspect, they do provide evidence that a portion of all three lipases inserts into the monolayer.

Although no structural data are available for these PLC enzymes, direct evidence for this type of insertion mechanism has recently been obtained for annexin V: Ca^{2+} induces the exposure of a small tryptophan-containing region of this protein (Concha et al., 1993), which then inserts into membranes (Meers & Mealy, 1993). In addition, activation of a fungal lipase involves movement of a short helical segment or "lid" and exposure of a small tryptophan-containing hydrophobic region (Brzozowski et al., 1991), which presumably inserts into membranes.

Evans and Skalak (1980, pp 85–91) and Cevc and Marsh (1987, pp 42–46 and 350–357) discuss the relationship between the internal surface pressure of a bilayer, which cannot be determined directly in a closed membrane system, and the surface pressure of a monolayer. Several lines of evidence suggest the packing of phospholipids in a bilayer or biological membrane is comparable to that in a monolayer with a surface pressure $30 < \pi < 35$ mN/m (Demel et al., 1975; Blume, 1979; Seelig, 1987, 1992; Moreau et al., 1988).[4] Thus any factor that disrupts the packing of phospholipids in a bilayer and increases their effective area should contribute to the activation of these lipases. One obvious and testable speculation is that the G_q class of G proteins, which are known to stimulate different members of the PLC-β class (Smrcka et al., 1991; Taylor et al., 1991; Waldo et al., 1991; Wu et al., 1992a,b; Park et al., 1993; Smrcka & Sternweis, 1993), may disrupt the packing of the boundary lipids that surround them and thus faciliate the penetration of these PLC isoforms into the bilayer component of a biological membrane.

[3] For 1 mM dibucaine in the aqueous subphase, the binding to a PC monolayer decreased 5-fold as the surface pressure increased from 27 to 37 mN/m (Seelig, 1987). She estimates that the cross-sectional area of dibucaine is $A = 0.55\ nm^2$, so the Boltzmann relation, $\exp(-\pi A/kT)$, predicts that the binding should have decreased 4-fold, in good agreement with the result. However, the binding of peptides such as substance P antagonists (Seelig, 1992) and signal peptides (Tamm, 1986) does not decrease as much as predicted from the Boltzmann relation when π increases, possibly because these peptides do not penetrate deeply into the monolayer.

[4] For example, the partition coefficient of myristic acid onto a large unilamellar PC bilayer vesicle [see the pH 7 data in Figure 7 of Peitzsch and McLaughlin (1993)] is identical to the partition coefficient of myristic acid onto a PC monolayer (Figure 4A) formed at a surface pressure of 35 mN/m.

REFERENCES

Berridge, M. J. (1993) *Nature 361*, 315–325.
Blume, A. (1979) *Biochim. Biophys. Acta 557*, 32–44.
Brzozowski, A. M., Derewenda, U., Derewenda, Z. S., Dodson, G. G., Lawson, D. M., Turkenburg, J. P., Bjorkling, F., Huge-Jensen, B., Patkar, S. A., & Thim, L. (1991) *Nature 351*, 491–494.
Carpenter, G. (1992) *FASEB J. 6*, 3283–3289.
Cevc, G., & Marsh, D. (1987) *Phospholipid Bilayers*, Wiley-Interscience, New York.
Cifuentes, M., Honkanen, L., & Rebecchi, M. (1993) *J. Biol. Chem. 268*, 11586–11593.
Cockcroft, S., & Thomas, G. M. H. (1992) *Biochem. J. 288*, 1–14.
Concha, N. O., Head, J. F., Kaetzel, M. A., Dedman, J. R., & Seaton, B. A. (1993) *Science 261*, 1321–1324.
Demel, R. A., Geurts van Kessel, W. S .M., Zwaal, R. F .A., Roelofsen, B., & van Deenen, L. L. M. (1975) *Biochim. Biophys. Acta 406*, 97–107.
Dennis, E. A. (1983) *The Enzymes 16*, 307–353.
Dennis, E. A., Rhee, S. G., Billah, M. M., & Hannun, Y. A. (1991) *FASEB J. 5*, 2068–2077.
Evans, E. A., & Skalak, R. (1980) *Mechanics and Thermodynamics of Biomembranes*, CRC Press, Boca Raton, FL.
Evans, R. W., Williams, M. A., & Tinoco, J. (1987) *Biochem. J. 245*, 455–462.
Fromherz, P. (1975) *Rev. Sci. Instrum. 46*, 1380–1385.
Gabev, E., Kasianowicz, J., Abbott, T., & McLaughlin, S. (1989) *Biochim. Biophys. Acta 979*, 105–112.
Hirasawa, K., Irvine, R. F., & Dawson, R. M. C. (1981) *Biochem. J. 193*, 607–614.
Jain, M. K., & Berg, O. G. (1989) *Biochim. Biophys. Acta 1002*, 127–156.
Lowry, R. R., & Tinsley, I. J. (1974) *Lipids 9*, 491–492.
Lucklow, V. A., & Sumners, M. D. (1988) *Bio/Technology 6*, 47–55.
Marsh, D. (1990) *Handbook of Lipid Bilayers*, pp 163–170, CRC Press, Boca Raton, FL.
McGreggor, M. A., & Barnes, G. T. (1974) *J. Colloid Interface Sci. 49*, 362–367.
McLaughlin, S. (1989) *Annu. Rev. Biophys. Biophys. Chem. 18*, 113–136.
Meers, P., & Mealy, T. (1993) *Biochemistry 32*, 5411–5418.

Montich, G., Scarlata, S., McLaughlin, S., Lehrmann, R., & Seelig, J. (1993) *Biochim. Biophys. Acta 1146*, 17–24.
Moreau, H., Pieroni, G., Jolivet-Reynaud, C., Alouf, J. E., & Verger, R. (1988) *Biochemistry 27*, 2319–2323.
Mosior, M., & McLaughlin, S. (1992a) *Biochemistry 31*, 1767–1773.
Mosior, M., & McLaughlin, S. (1992b) *Biochim. Biophys. Acta 1105*, 185–187.
Park, D., Jhon, D.-Y., Lee, C.-W., Lee, K.-H., & Rhee, S. G. (1993) *J. Biol. Chem. 268*, 4573–4576.
Pawelczyk, T., & Lowenstein, J. M. (1992) *Arch. Biochem. Biophys. 297*, 328–333.
Pawelczyk, T., & Lowenstein, J. M. (1993) *Biochem. J. 291*, 693–696.
Peitzsch, R. M., & McLaughlin, S. (1993) *Biochemistry 32*, 10436–10443.
Pethica, B. A. (1955) *Trans. Faraday Soc. 51*, 1402–1411.
Rebecchi, M. J., Peterson, A., & McLaughlin, S. (1992a) *Biochemistry 31*, 12742–12747.
Rebecchi, M., Boguslavsky, V., Boguslavsky, L., & McLaughlin, S. (1992b) *Biochemistry 31*, 12748–12753.
Rhee, S. G., & Choi, K. D. (1992) *J. Biol. Chem. 267*, 12393–12396.
Ryu, S. H., Cho, K. S., Lee, K.-Y., Suh, P.-G., & Rhee, S. G. (1987) *J. Biol. Chem. 262*, 12511–12518.
Seelig, A. (1987) *Biochim. Biophys. Acta 899*, 196–204.
Seelig, A. (1992) *Biochemistry 31*, 2897–9204.
Smrcka, A. V., Hepler, J. R., Brown, K. O., & Sternweis, P. C. (1991) *Science 251*, 804–807.
Smrcka, A. V., & Sternweis, P. C. (1993) *J. Biol. Chem. 268*, 9667–9674.
Sternweis, P. C., & Smrcka, A. V. (1992) *Trends Biochem. Sci. 17*, 502–506.
Tamm, L. K. (1986) *Biochemistry 25*, 7470–7476.
Taylor, S. J., Chae, H. Z., Rhee, S. G., & Exton, J. H. (1991) *Nature 350*, 516–518.
Ter-Minassian-Saraga, L. (1991) in *Cationic Surfactants* (Rubingh, D., & Holland, P. M., Eds.) pp 249–321, Marcel Dekker, New York.
Toner, M., Vaio, G., McLaughlin, A., & McLaughlin, S. (1988) *Biochemistry 27*, 7435–7443.
Verger, R., & de Haas, G. H. (1976) *Annu. Rev. Biophys. Bioeng. 5*, 77–117.
Waite, M. (1987) *The Phospholipases* (*Handbook of Lipid Research*) Vol. 5, pp 191–241, Plenum Press, New York.
Waldo, G. L., Boyer, J. L., Morris, A. J., & Harden, T. K. (1991) *J. Biol. Chem. 266*, 14217–14225.
Wu, D., Lee, C.-H., Rhee, S. G., & Simon, M. I. (1992a) *J. Biol. Chem. 267*, 1811–1817.
Wu, D., Katz, A., Lee, C.-H., & Simon, M. I. (1992b) *J. Biol. Chem. 267*, 25798–25802.
Wu, D., Jiang, H., Katz, A., & Simon, M. I. (1993) *J. Biol. Chem. 268*, 3704–3709.