Dependence of the Activity of Phospholipase Cβ on Surface Pressure and Surface Composition in Phospholipid Monolayers and Its Implications for Their Regulation[†]

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ABSTRACT: We have examined the influence of surface pressure and phospholipid composition on hydrolysis of phosphatidylinositol (4,5)-bisphosphate (PIP_2) by phospholipase Cβ1 (PLCβ1) and PLCβ2 in mixed composition phospholipid monolayers. Increasing the monolayer surface pressure from 15 to 36 mN/m reduced the rate at which PIP_2 was hydrolyzed by PLCβ1 and PLCβ2 by 4−6-fold, although PLCβ1 was more active than PLCβ2, even at high surface pressures. Reduced enzyme activity was accompanied by an increase in reaction induction times, suggesting that increasing surface pressure reduced the penetration rate of the enzymes into the monolayer. Quantitation of interfacial enzyme concentration using ^{35}S-labeled PLCβ1 confirmed that less enzyme was associated with the monolayer at higher pressures. The relationship between PLC activity and substrate concentration was examined at a single surface pressure of 30 mN/m. This relationship was not hyperbolic, and increases in the mole percentage (mol %) of PIP_2 in the monolayer resulted in an upwardly-curving increase in PLC activity. Thus, PLCβ1 activity increased 7-fold and PLCβ2 activity increased 4-fold when the mol % of PIP_2 in the monolayer increased from 17.9% to 29%, increasing further thereafter. Paradoxically, increasing the mol % of PIP_2 from 0 to 60% was accompanied by a 3-fold decrease in interfacial enzyme concentrations. Taken together, these data show that the catalytic activity of PLCβ involves some element of penetration of lipid interfaces, and suggest that the organization of the substrate facilitates PLC activity, giving credence to the substrate theory of interfacial activation of phospholipases. We present a hypothesis suggesting that PIP_2 moleclues coalesce into enriched lateral domains which favor PLCβ activity.

The second messengers inositol (1,4,5)-trisphosphate (IP_3)[1] and 1,2-diacylglycerol (DG) are generated in cells in response to stimulation by hormones and growth factors (Berridge, 1993). The generation of these signals is catalyzed by a family of PLC isozymes which hydrolyze the membrane phospholipid PIP_2. Three families of PLC have been identified, termed β, γ, and δ (Rhee & Choi, 1992; Lee & Rhee, 1995); PLCβ family members are activated by subunits of heterotrimeric GTP-binding proteins, and PLCγ is activated by tyrosine phosphorylation.

The molecular basis for activation of PLC isoforms is not clear, nor is it completely understood how PLCs interact with membrane substrates. The activity of all PLC isoforms is affected by the surface pressure of monolayer substrates (Hirasawa *et al.*, 1981; Rebecchi *et al.*, 1992; James *et al.*, 1994; Boguslavsky *et al.*, 1994), a feature common to many lipid-metabolizing enzymes that is consistent with the idea that the enzyme must do work to gain access to its substrate. Furthermore, it has been demonstrated that the overall catalytic efficiency of PLC isoforms is enhanced by multiple stable contacts with interfacial phosphoinositide molecules (Wahl *et al.*, 1992; Cifuentes *et al.*, 1993; James *et al.*, 1995, 1996). All isoforms of PLC are thought to have a pleckstrin homology (PH) domain within their tertiary structures (Parker *et al.*, 1994) which are protein modules apparently involved in binding both lipid (Harlan *et al.*, 1994) and proteins (Pitcher *et al.;* 1995; Yao *et al.*, 1994). The PH domain of PLCδ binds phosphoinositides (Garcia *et al.*, 1995) and may have a role in the membrane localization of this PLC isoform (Paterson, H. F., *et al.*, 1995), but there is little evidence of a role for the PH domains which are thought to be present in PLCβ and -γ family members.

A common observation for lipases and phospholipases is that they are more active at lipid−water interfaces than in bulk solution, due to the relatively ill-defined phenomenon of "interfacial activation". Two hypothetical explanations for this, the substrate theory and the enzyme theory, have been proposed. In the former, the interfacial conformation and concentration of substrate molecules in lipid aggregates are favorable for enzymic catalysis, thereby leading to faster rates of enzyme-catalyzed reactions. In the latter, the conformation adopted by the enzyme when it interacts with the lipid substrate contributes to greater enzyme activity.

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[1] Abbreviations: PLCβ, β-isoform of phospholipase C; PIP_2, phosphatidylinositol (4,5)-bisphosphate; PC, phosphatidylcholine; PS, phosphatidylserine; IP_3, inositol (1,4,5)-trisphosphate; DG, diacyglycerol; mol %, mole fraction expressed as a percentage; PH, pleckstrin homology.

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Support for the enzyme theory has been presented from studies of members of the family of $\alpha\beta$ hydrolases (Brzozowski *et al.*, 1991), and work with carboxylester lipases in mixed monomolecular films supports the substrate theory (Muderhwa & Brockman, 1992; Smaby *et al.*, 1994). It seems reasonable to suggest that accelerated PLC activity in response to extracellular stimuli will involve G protein- and tyrosine kinase-mediated influences on PLC at both the enzyme and substrate level.

In this study, we have examined the influences of monolayer surface pressure and substrate content on the catalytic activity of PLCβ1 and PLCβ2. The data show that the activity of each enzyme decreased as surface pressure increased, in accordance with Boguslavsky *et al.* (1994), although the effect was not monotonic. Reduced activity was partially attributable to reduced penetration of PLCβ into the monolayer. In studies where the mole fraction of PIP_2 was increased in mixed PS/PIP_2 monolayers, PLCβ activity increased abruptly at relatively low threshold mole fractions of PIP_2. We propose that the lateral packing of adjacent phospholipid molecules and possible formation of domains in the monolayer enriched in PIP_2 may regulate PLCβ activity, in support of the substrate theory of interfacial activation of these enzymes.

EXPERIMENTAL PROCEDURES

Materials. PC was purchased from Sigma, and PS was from Boehringer Mannheim. PIP_2 was purified from Folch extract of bovine brain (Sigma) as described previously (James *et al.*, 1994). [^{33}P]ATP was from Amersham and was used to make [^{33}P]PIP_2 (specific activity approximately 3000 Ci/mmol) using partially-purified PIP kinase (rat brain) as described before (James *et al.*, 1994). Phospholipid stocks were stored as chloroform/methanol solutions or in solvents from suppliers at −80 °C. Recombinant PLCβ1 and PLCβ2 were expressed and purified as described previously (Paterson, A., *et al.*, 1995); 18.2 MΩ water was used for all salt and buffer solutions.

Monolayer Assays. Monolayer PLC assays were performed as described previously (James *et al.*, 1994, 1996) for 15−25 min. Mixed phospholipid monolayers were spread from solvent stock solutions using gas-tight Hamilton syringes over a subphase buffer comprising 10 mM Hepes, pH 7.2, 120 mM KCl, 10 mM NaCl, 2 mM EGTA, 1 mM $MgCl_2$, and 1 μM free Ca^{2+} ions. PLC (20 ng−1 μg) was added to the subphase after 5 min *via* an injection port in the trough. The subphase was stirred continuously to ensure rapid mixing of solutes in the bulk solution. The extent of the reaction was determined by sampling the radioactivity remaining in the monolayer and in an aliquot of the subphase buffer by liquid scintillation spectrometry. Time courses were monitored continuously by measuring the loss of radioactive PIP_2 from the monolayer using an FC-006 remote detector (Bioscan) (approximately 3% efficiency of detection of ^{33}P when suspended 5 mm above the monolayer) as described before (James *et al.*, 1996).

Measurements of Interfacial Enzyme Concentrations. ^{35}S-Labeled PLCβ1 (specific activity ≥500 cpm/ng) was made by expression of recombinant PLCβ1 in Sf9 cultures in the presence of [^{35}S]methionine. Briefly, Sf9 cells (1.2×10^8) were infected with 3.6×10^8 plaque-forming units of recombinant baculovirus encoding rat PLCβ1 and subsequently labeled with 14 mCi of EXPRE^{35}S^{35}S labeling mix (NEN Dupont). ^{35}S-PLCβ1 was purified as described (Paterson *et al.*, 1996). Radiolabeled enzyme was used to measure the concentration of PLC associated with the monolayer under different conditions. ^{35}S-PLCβ1 was added to the subphase buffer beneath unlabeled monolayers as described above for catalytic assays, and radioactivity associated with the interface was measured by aspiration directly into a scintillation vial. Results were corrected for contamination with subphase and used to quantitate interfacial enzyme concentrations. At low surface pressures (≤15 mN/m), radioactivity at the monolayer increased linearly throughout the duration of the experiment, but at higher surface pressures, PLC association with the monolayer reached a plateau after approximately 5 min.

Pressure−Area Isotherms of Phospholipids. Pressure−area isotherms for monolayer phospholipids were performed using a trough milled to 4 mm depth out of Teflon, with a compression surface area of 410 cm^2 (Nima Technology, Coventry, England). Monolayers containing 20 nmol of phospholipid were spread over the same subphase buffer used in PLC assays and subsequently compressed with a computer-controlled Teflon barrier, driven by a direct current motor, at a rate of 100 cm^2 per minute. Isotherms of purified PS (Boehringer Mannheim) and PIP_2 (purified from Folch extract) showed smooth transitions from gaseous to liquid-expanded/liquid-compressed phases, with final collapse pressures of approximately 43 mN/m each. The molecular areas of PS and PIP_2 at 30 mN/m in the presence of 1 μM free Ca^{2+} ions were approximately 80 and 90 Å^2, respectively. Isotherms of mixed monolayers in Figure 5A are shown as pressure against trough area, due to the unknown relative molecular areas of the different phospholipids under these conditions.

Kinetic Model of Phospholipase C Activity. The simplest model by which PLCs are thought to act at lipid interfaces comprises two distinct equilibria, which describe binding of the enzyme to the interface and subsequently binding to substrate within the interface, in an ordered sequential manner, outlined in Scheme 1.

Scheme 1

$$E \underset{k_d}{\overset{k_p}{\rightleftharpoons}} E^* \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E^*S \xrightarrow{k_{cat}} E^* + P$$

In this scheme, enzyme E binds to the lipid interface with a penetration rate k_p, to become interfacial enzyme E*. E* may dissociate to free E dissolved in the bulk solution (with rate k_d) or may bind a substrate molecule S, described by k_1. E*S can then catalyze the production of products P with the rate $k_{cat.}$ In this model, possible diffusional constraints exerted upon the association of PLC with the monolayer, which could contribute to the establishment of the first equilibrium, have been ignored. It has been demonstrated with other lipolytic enzymes [reviewed by Verger (1980)] that the unstirred layer which exists beneath a lipid monolayer is minimized by stirring the subphase and that diffusion of the enzyme through this layer does not contribute significantly to the induction time τ. In our experiments, the subphase was stirred with a Teflon-coated flea at approximately 90 rpm, at which rate the monolayer was

stable. Diffusional limitations have therefore been discounted from the proposed reaction scheme for penetration of the monolayer by PLCβ.

Measurements of interfacial PLCβ1 using radiolabeled enzyme will detect enzyme which has bound to the monolayer, penetrated the monolayer, and that which has secondarily bound PIP_2 substrate, that is, E* + E*S. The induction time, τ, of the PLC-catalyzed reaction, which is the time which elapses between addition of enzyme to the subphase buffer and the onset of measurable catalysis, comprises the separate induction times which arise as a result of the distinct separate equilibria E $\rightleftharpoons$ E* and E* $\rightleftharpoons$ E*S. τ was measured from continuous trace recordings of the loss of radiolabeled PIP_2 from monolayers as a result of PLC activity (see Figure 1), by computer-aided integration of the traces from which decreasing peak heights were detected due to PIP_2 hydrolysis. Induction times were independent of the concentration of enzyme added to the subphase buffer (S. R. James, unpublished data) and were not observed if PLC was added to monolayer reactions before Ca^{2+} which is necessary for PLC catalysis. Thus, no detectable lag in PIP_2 hydrolysis was observed when Ca^{2+} was added 5 min after PLCβ (S. R. James and R. A. Demel, unpublished observations). Therefore, the observed induction times reported here are probably due to the equilibrium in Scheme 1 leading to penetrated enzyme (E*) being significantly limiting when PLCβ is added to a subphase which already contains Ca^{2+}.

RESULTS

Monolayer Surface Pressure and PLCβ Activity. Before the mechanistic basis of activation of PLCβ isoforms by G protein subunits can be understood, the molecular interactions of these enzymes with lipid interfaces and the reciprocal influences these have on each other must be defined. We have previously shown in micellar assays that, as part of their catalytic action, PLCβ isoforms make multiple contacts with lipid substrates, specifically directed to PIP_2 molecules, and that the characteristics of these micelles appear to alter during the reaction such that $\geq$70% of available PIP_2 remains unhydrolyzed (James *et al.*, 1995, 1996). By contrast, PIP_2 hydrolysis in monolayers goes to completion, and the activity of the avian isoform of PLCβ from turkey erythrocytes shows a bell-shaped relationship with increasing surface pressure (James *et al.*, 1994). Figure 1 shows that increasing monolayer surface pressure in PC/PS/PIP_2 monolayers (70:27:3 by molarity) was accompanied by a decline in PIP_2 hydrolysis catalyzed by mammalian PLCβ1 and PLCβ2. Typical time courses for PLCβ1-catalyzed loss of radioactivity from monolayers due to PIP_2 hydrolysis are shown in Figure 1A. As the initial surface pressure was increased from 15.4 to 30.6 mN/m, the rates of reaction taken from the linear portions of these traces were 17.3, 11.5, 5.1, and 1.8% available PIP_2 hydrolyzed per minute per microgram of PLCβ1. The overall relationship between PLCβ activity and increasing surface pressure is shown in Figure 1B. The rate of PLCβ1-catalyzed PIP_2 hydrolysis decreased 3−4-fold between 15 and 25 mN/m, after which activity fell more slowly. By contrast, PLCβ2 activity decreased more sharply, approximately 3-fold between 20 and 23 mN/m, after which activity fell more gradually. The pressure−activity relationship for PLCβ1 shown in Figure 1 is similar but not identical to that reported by Boguslavsky *et al.* (1994). Clearly, monolayer assays of PLC activity reveal subtle differences in the behavior of different β-isoforms which is not the case in assays using detergent mixed micelles (James *et al.*, 1994, 1995).

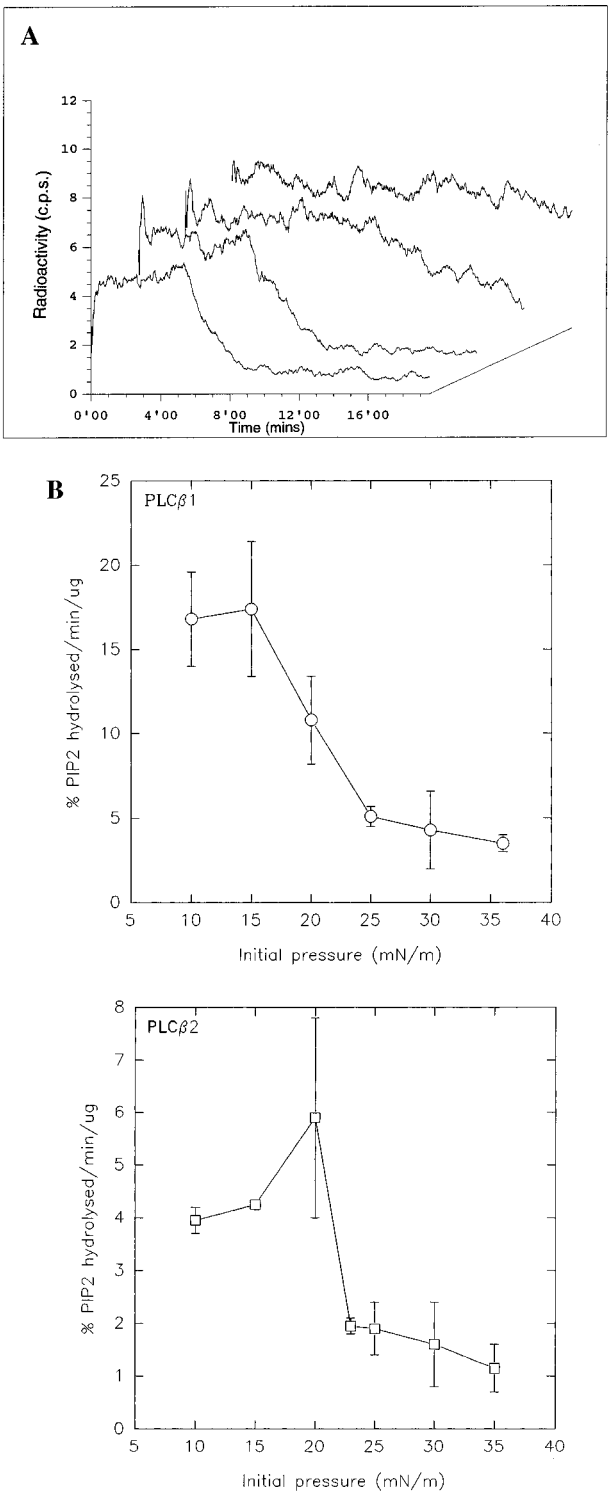

FIGURE 1: Relationship between monolayer surface pressure and PIP_2 hydrolysis by PLCβ1 and PLCβ2. PC/PS/PIP_2 (70:27:3 mol/mol) monolayers containing ^{33}P-labeled PIP_2 were spread as described under Experimental Procedures, and 1 μg of enzyme was added to the subphase after 5 min. Reaction times were 15 min (PLCβ1) and 25 min (PLCβ2). (A) Typical time courses of the loss of radioactive PIP_2 from monolayers catalyzed by PLCβ1. Traces are offset in three dimensions for clarity. Initial surface pressures were 15.4, 20.4, 25.2, and 30.6 mN/m (front to back). (B) Pressure−activity relationships for PLCβ1 (upper panel) and PLCβ2 (lower panel), respectively. Each data point is the mean ± range or SD of duplicate or triplicate determinations, respectively.

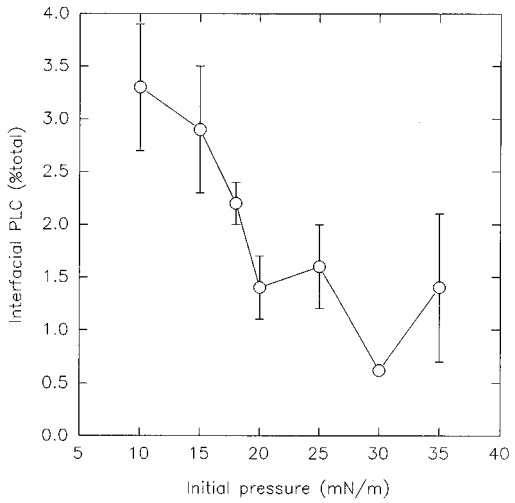

FIGURE 2: Association of ^{35}S-PLCβ1 with PC/PS/PIP$_2$ monolayers (70:27:3 mol/mol) as the surface pressure increased. ^{35}S-PLCβ1 (1.5 ± 0.3 μg, 79 050 ± 14 700 cpm, n = 18) was injected under unlabeled monolayers and subsequent association measured as described under Experimental Procedures.

We have previously examined the way in which PLCβs bind to their lipid substrates, using phospholipid vesicles which were subsequently centrifuged and free and lipid-bound PLC quantitated by activity measurements (James *et al.*, 1995). One way to do similar experiments in monolayers is to use radiolabeled enzyme and monitor increases in monolayer radioactivity due to enzyme binding under different conditions. We made ^{35}S-PLCβ1 of high specific radioactivity for the purpose of quantifying interfacial PLCβ1 in the monolayer.

To determine if the reciprocal relationship between surface pressure and PLCβ activity correlated with decreasing interfacial enzyme concentrations, unlabeled PC/PS/PIP$_2$ monolayers (70:27:3 by molarity) were incubated with ^{35}S-labeled PLCβ1 for similar times and at similar pressures as for catalytic assays. After 15 min, monolayer radioactivity due to bound enzyme was measured. Results in Figure 2 indicate that a maximum of ≤4% of added PLCβ1 (0.3 pmol) associated with the monolayer at the most permissive surface pressures under these conditions, and that PLCβ1 association decreased with increasing surface pressure. When the data in Figure 2 were fitted to a linear equation (not shown), the line intercepted the x-axis at 44 mN/m. This value, which is the exclusion pressure for PLC above which no enzyme penetrates the monolayer, coincides with the collapse pressure of naturally-occurring phospholipids (approximately 43−45 mN/m). Although experiments of this kind are useful in quantifying protein concentrations in the monolayer under different conditions (E* + E*S), they do not distinguish between catalytically active and inactive enzyme in the interface. Determining the proportion of enzyme which is catalytically active requires a different approach involving careful collection of the monolayer and subsequent reconstitution of enzyme activity from it. These data do, however, establish a clear relationship between surface pressure and enzyme associated with the monolayer.

Measurements of the induction time τ between addition of enzyme to the subphase and onset of catalysis were made from continuous trace recordings of the loss of radioactivity from monolayers (Figure 3). Induction times were comparable for PLCβ1 and PLCβ2 at the same initial surface

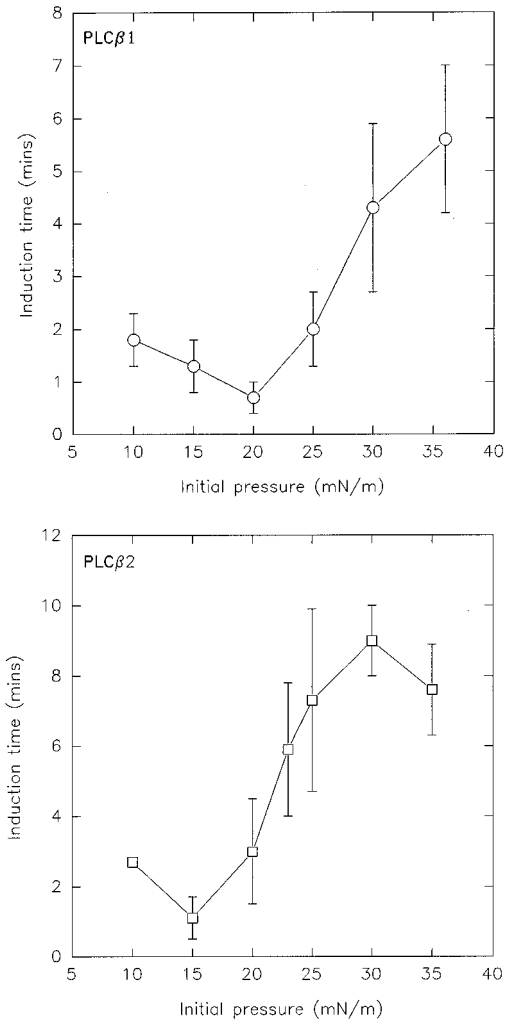

FIGURE 3: Induction times (τ) for PIP$_2$ hydrolysis by PLCβ1 (upper panel) and PLCβ2 (lower panel) in PC/PS/PIP$_2$ (70:27:3 mol/mol) monolayers. Data were derived by computer-aided integration of continuous trace recordings of ^{33}P-PIP$_2$ metabolism in experiments shown in Figure 1.

pressures, and were greater at higher monolayer pressures. Under some conditions, it has been reported that the penetration rate, k_p, is inversely proportional to the τ time (Verger *et al.*, 1973). The data in Figure 3 therefore suggest that increasing surface pressure reduces the penetration rate of both PLCβ1 and PLCβ2 into the monolayer. Thus, under the conditions of fixed monolayer composition and increasing surface pressure, PIP$_2$ hydrolysis by mammalian PLCβ1 and PLCβ2 decreases due to the slower rate with which the enzyme associates with the monolayer. Indeed, if a different assay protocol is adopted, in which enzyme is added prior to Ca^{2+} ions, the onset of hydrolysis is immediate (Boguslavsky *et al.*, 1994; S. R. James and R. A. Demel, unpublished observations), which suggests that the equilibrium E* ⇌ E*S is not limiting. The data in Figure 3 therefore indicate that the lag time between addition of PLCβ to the subphase and onset of catalysis is due to the first equilibrium in Scheme 1 leading to penetrated enzyme E* (described by the rate constant k_p) being limiting.

The relationships in Figure 3 are inverted bell shapes, in which induction times are apparently higher at the lowest surface pressure investigated, 10 mN/m, than at 15−20 mN/m. However, the observed relationships may be artifactual, because the determination of τ times is dependent on the

catalytic action of the enzymes. A surface pressure of 10 mN/m is very low compared to the surface pressures which PLCs are likely to encounter *in vivo*, and it is possible that PLC denatures in the interface at this very low pressure. This would mean that although PLC penetrates the monolayer with the highest penetration rate at 10 mN/m, a significant proportion of the enzyme molecules are catalytically inactive in the monolayer, leading to an artificially extended lag period before catalysis is detected.

Substrate Concentration and PLCβ Activity. Kinetic analyses of PLCβ isoforms in mixed micellar assays have revealed that half-maximal activity is observed with mole fractions of PIP_2 between 0.1 and 0.2 (James *et al.*, 1995). This value is the interfacial Michaelis constant for these enzymes. A lower value was determined for PLCδ in similar assays (approximately 0.05; Cifuentes *et al.*, 1993). Monolayers comprised of PIP_2 with PS to dilute the surface concentration of substrate were utilized in an attempt to derive an equivalent constant in this system. For both PLCβ1 and PLCβ2, PIP_2 hydrolysis was very slow (between 1 and 10 pmol/min) when the mol % was $\leq$17.6. However, when the surface concentration of PIP_2 was increased to 29%, PLCβ1 (Figure 4A) and PLCβ2 (Figure 4B) activity increased quite sharply by 7- and 4-fold, respectively. The catalytic rates of both enzymes increased further as the mol % of PIP_2 was further increased, and no plateau in activity was reached, even at 60 mol % PIP_2, suggesting that enzyme activity remained substrate-limited. These data were not adequately described by the Hill equation, in contrast to similar experiments performed in mixed micellar assays (James *et al.*, 1995), even when the Hill coefficient was increased manually to 3 or 4.

Induction times for the results shown in Figure 4A,B did not alter across the range of PIP_2 mole percentages examined, and were approximately 3 min for each enzyme (Figure 4C). These data therefore suggest that the abrupt increase in PLCβ activity above mole percentages of PIP_2 of 17.9 was not due to enhanced penetration rates of the enzyme into the monolayer. Indeed, measurements of concentrations of interfacial PLCβ1 using ^{35}S-labeled enzyme (Figure 4D) in monolayers at a single initial pressure of 30 mN/m showed that as the mole fraction of PIP2 was increased, enzyme concentrations decreased. Reduced PLCβ1 concentrations at 60 mol % PIP_2 were statistically significant relative to both 2.5 mol % and 0 mol % PIP_2 ($p = 0.023$ and $p = 0.002$, respectively, paired *t*-test). A possible interpretation of the increase in PLC activity above 17.6 mol % PIP_2 (Figure 4A,B) is that the interfacial characteristics of the substrate lead to the enhanced hydrolysis of PIP_2.

Pressure−Area Isotherms of PS/PIP_2 Mixed Monolayers. An attempt to assess the miscibility of PS and PIP_2 in mixed monolayers of differing relative compositions was made, with the goal of precluding the possibility that lateral phase separation of the lipids caused the abrupt increase in PLCβ activity. Pressure−area isotherms of homogeneous PS and PIP_2 monolayers and mixed composition monolayers were constructed, to compare phase transition pressures and collapse pressures. Both PS and PIP_2 monolayers exhibited smooth transitions from gaseous to liquid-expanded phases as compression was applied (Figure 5), although PIP_2 was significantly more expanded than PS. PS monolayers collapsed abruptly at a surface pressure of 44.5 $\pm$ 2.1 mN/m. By contrast, from numerous isotherms, PIP_2 monolayers showed a more protracted loss of liquid-expanded behavior beginning at approximately 35 mN/m and finally collapsing into the subphase between 43 and 45 mN/m. As the proportion of PIP_2 in mixed PS/PIP_2 monolayers increased, isotherms showed gradual increases in average molecular area (Figure 5A,B). Figure 5B shows the average molecular area of mixed monolayers plotted against the mol % of PIP_2. As the mol % of phosphoinositide increased, the average molecular area increased, although a linear relationship was not seen, with some noise between 15 and 30 mol % PIP_2. This may indicate nonhomogeneous distribution of lipids although such an interpretation requires confirmation using more direct techniques.

DISCUSSION

The data presented here with PLCβ1 and PLCβ2, and those published earlier for three other isoforms of PLC (PLCγ1, Boguslavsky *et al.*, 1994; PLCδ1, Rebecchi *et al.*, 1992, Boguslavsky *et al.*, 1994; turkey erythrocyte PLCβ, James *et al.*, 1994), clearly show that PIP_2 hydrolysis in monolayers is surface pressure-dependent, consistent with some element of penetration of lipid interfaces by this family of enzymes. The pressure−activity relationship for turkey erythrocyte PLCβ is strikingly different from that for all other isozymes, showing maximum activity against PIP_2 at 30 mN/m (James *et al.*, 1994), which may indicate a different mechanism of lipid binding by this enzyme in monolayers. However, at surface pressures commonly thought to reside in biological membranes (>30 mN/m), all PLC isoforms are minimally active. Boguslavsky *et al.* (1994) have proposed that PLCs must do work in penetrating monolayers, which increases as the surface pressure rises. Measurements of τ times for PLCβ1 and PLCβ2 suggest that the rate of penetration of monolayers decreases with increasing pressure, as do interfacial enzyme concentrations, consistent with the above proposal.

The induction times for PLCβ activity in monolayers are in stark contrast to the situation in assays using vesicular substrates. When PLCβ activity is measured using unilamellar vesicles containing PIP_2, no lag times are measurable in our assays (James *et al.*, 1996; S. Smith and S. R. James, unpublished observations). Time courses show that IP_3 generation is measurable at the earliest time points and that PLCβ activity plateaus to a low rate when only 10% of available substrate has been utilized. The reasons for this difference are not clear. However, as reviewed by Verger (1980), the ratio of surface area of available substrate to volume is much larger in bulk solution than in monolayers which leads to greater interfacial enzyme concentrations. It is possible that the binding of enzyme to the interface is not limiting in bulk solution reactions, leading to fast induction times which cannot be measured reliably.

It is tempting to speculate that the putative restraining influence of the surface pressure of the cellular plasma membrane on PLC activities is somehow overcome by G protein subunits and tyrosine phosphorylation during receptor activation of the inositol lipid signaling cascade, as was previously proposed by Boguslavsky and colleagues (Boguslavsky *et al.*, 1994). Thus, the association of PLCs with their lipid substrates may be facilitated when the enzymes are activated, shifting the E $\rightleftharpoons$ E* equilibrium to the right. This would be manifest as a reduction in the τ reaction times

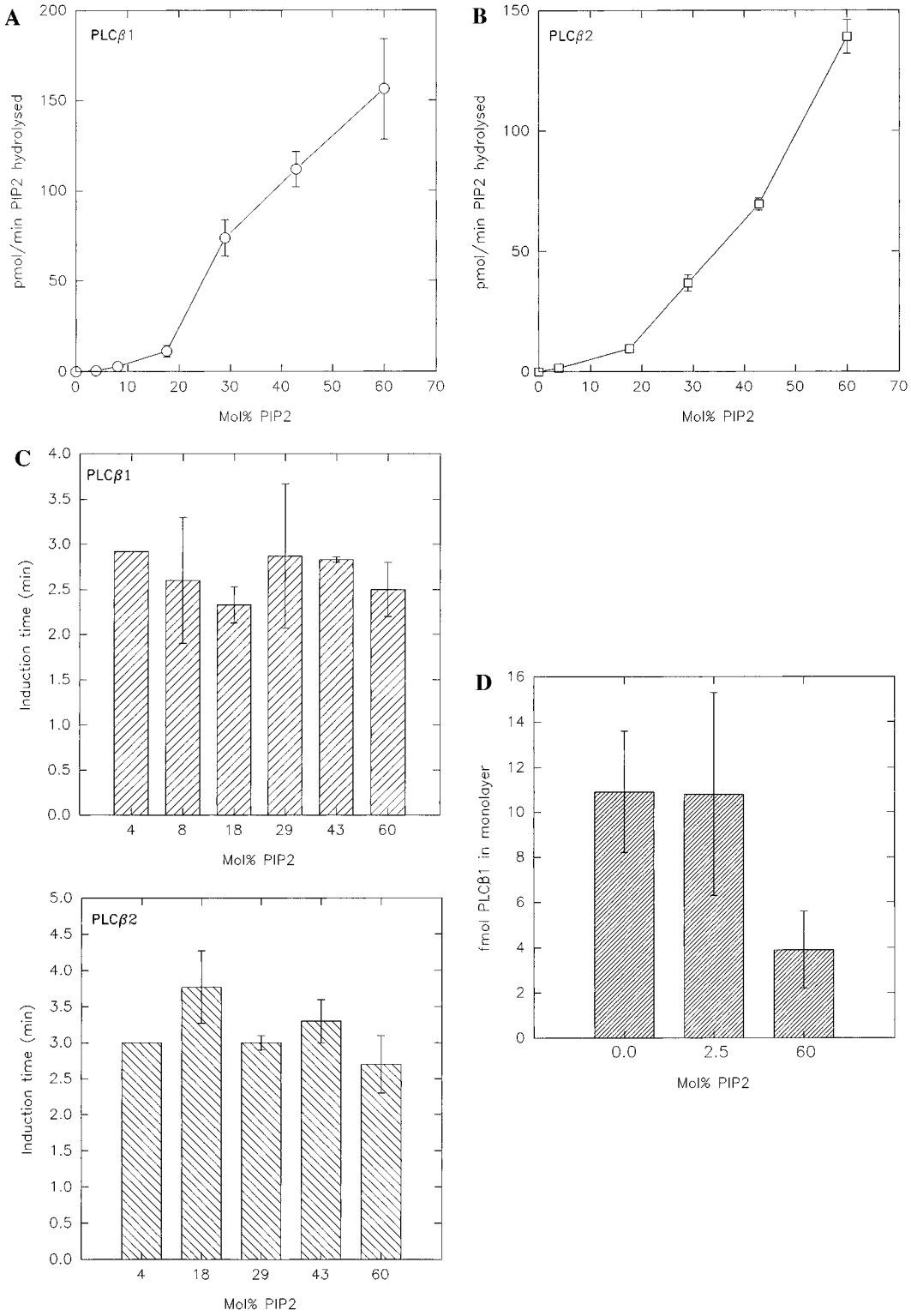

FIGURE 4: Effects of increasing mole fractions of PIP_2 on PLCβ activity. (A) PIP_2 hydrolysis by PLCβ1 (20 ng) in monolayers at 30 mN/m, containing PS and the indicated mol % of PIP_2. Monolayers contained 5.8 nmol of phospholipid at this pressure. Data are the means $\pm$ SD of three separate experiments at each mole fraction. (B) PIP_2 hydrolysis by PLCβ2 (100 ng) in PS/PIP_2 monolayers at initial surface pressures of 30 mN/m. Data are means $\pm$ ranges of duplicate experiments at each mole fraction. (C) Induction times for PLCβ1-catalyzed (top panel) and PLCβ2-catalyzed (bottom panel) PIP_2 hydrolysis in PS/PIP_2 monolayers at 30 mN/m, taken from continuous trace recordings of the experiments shown in panels A and B. (D) Association of ^{35}S-labeled PLCβ1 with monolayers at a single surface pressure of 30 mN/m, as a function of the mole fraction of PIP_2. Experiments contained 17.6 $\pm$ 2.1 ng of PLCβ1 (5890 $\pm$ 905 cpm, $n = 18$). Each bar is data from 6 separate experiments.

(increased penetration rates) contributing to greater overall velocity of the PLC-catalyzed reaction. The greater ability of activated PLCs to hydrolyze PIP_2 relative to nonactivated enzyme may derive from alterations in the conformation of the enzyme which enable it to penetrate membranes at the relatively high surface pressures thought to be present in cellular systems.

The data presented here also show that PLCβ activity is low at surface concentrations of PIP_2 beneath 15−20 mol %. above which activity increases markedly. In similar

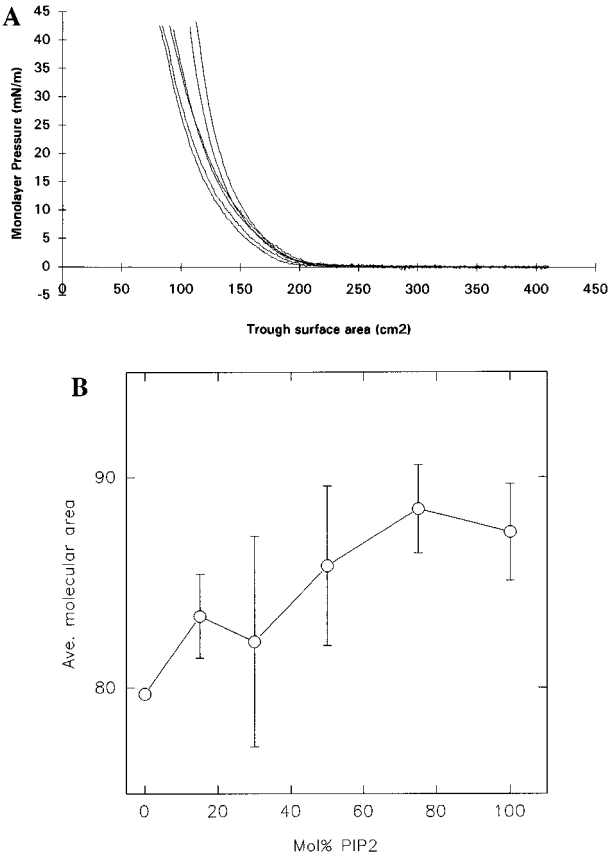

FIGURE 5: (A) Pressure/area isotherms at 25 °C for monolayers containing (from left to right) PS/PIP_2 in molar ratios of 1:0, 0.85:0.15, 0.7:0.3, 0.5:0.5, 0.25:0.75, and 0:1. Each monolayer contained 20 nmol of phospholipid spread over a subphase comprising PLC assay buffer (20 mM Hepes, pH 7.2, 120 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, and 1 μM free Ca^{2+}). The monolayer surface area was 410 cm^2 and was compressed at a rate of 100 cm^2/min. Data are plotted against the trough surface area from 300 cm^2 onward to avoid ambiguities of the precise molecular areas of PS and PIP_2, and are representative of at least three monolayers at each mol % PIP_2. (B) Plots of the average molecular area at 30 mN/m *versus* the mol % PIP_2 taken from isotherms such as those in panel A, repeated 3−4 times at each mol %. Data are means ± SD.

experiments using mixed micelles, the activity of PLCβ1, PLCβ2, and the β-isoform of PLC from turkey erythrocytes was described by the Hill equation (James *et al.*, 1995). If the amino-terminal PH domain of PLCβ isoforms was involved in the penetration of the enzyme into monolayers, it would be reasonable to expect that the overall activity of the enzyme in monolayers would be second order with respect to the mole fraction of PIP_2. However, experiments with radiolabeled enzyme demonstrated that the concentration of the enzyme in the monolayer decreased as the mole fraction of PIP_2 increased and data showing the activity of PLCβ1 and PLCβ2 in monolayers did not fit the Hill equation. This suggests that PLCβ binding to monolayers is not linearly dependent upon substrate mole fraction and that the activity of the enzyme in monolayers is not a simple function of substrate concentration. The association of PLCβ isoforms with phospholipid monolayers therefore reveals characteristics which were not apparent with lamellar structures such as unilamellar vesicles, involving an electrostatic interaction (in addition to putative PIP_2-directed binding) which is not seen in experiments measuring binding to vesicles.

The induction times for PIP_2 hydrolysis in experiments where the mol % of PIP_2 was systematically increased were consistent around 3 min at all PIP_2 mole fractions. This suggests that the equilibrium E ⇌ E*, leading to enzyme which has penetrated the monolayer, is not affected by the increased surface area comprising PIP_2. However, the data also indicate that the rates of binding a second PIP_2 molecule in the interface (k_1) and its hydrolysis to products (k_{cat}) increase as the mol % of PIP_2 exceeds 17.6 mol %. It is therefore feasible that the faster hydrolytic activity of PLCβ at mole percentages of PIP_2 above 18% may be due to the behavior of the lipid facilitating the action of interfacially-bound enzyme, rather than increases in the concentration of enzyme in the monolayer. Although pressure−area isotherms of PS and PIP_2 monolayers were similar, increasing mol % of PIP_2 appeared to alter the characteristics of mixed PS/PIP_2 monolayers such that they were intermediate between pure PS and pure PIP_2 films. It is possible that these intermediate characteristics between 15 and 60 mol % PIP_2 are optimal for PLCβ activity.

Efficient PIP_2 hydrolysis by PLC isoforms is achieved by processive catalysis in which the enzyme binds semi-stably to the lipid interface and hydrolyzes many substrate molecules before its release (Wahl *et al.*, 1992; Cifuentes *et al.*, 1993; James *et al.*, 1995). We propose that in mixed monolayers this may be facilitated by coalescence of PIP_2 molecules into significantly-enriched lateral areas, to which PLC molecules become localized, a hypothesis in line with the substrate hypothesis of interfacial activation of phospholipases. Thus, individual enzyme molecules would encounter many PIP_2 molecules within a small surface area, which may cause the high apparent enzyme activity at low PIP_2 mole fractions. Such lateral domain formation would fall short of phase separations, causing immiscibility of PIP_2 and PS, but would rather exist as enriched PIP_2 domains "dissolved" in nonsubstrate phospholipid.

Reaction products also may have some effect on PLC activity, contributing to these observations. We have been unable to show an effect of DG or IP_3 on PLCβ activity in mixed micellar assays (James *et al.*, 1996). However, IP_3 has been shown to inhibit PIP_2 hydrolysis by preventing binding of PIP_2 by PLCδ (Cifuentes *et al.*, 1994). DG may cause disruptions in monolayer structure which favor PLC activity, but we have no evidence for this. Although DG can promote nonlamellar phases in different phospholipid systems (Seddon & Cevc, 1993), it readily forms monolayers with a collapse pressure of approximately 35 mN/m and molecular area between 75 and 80 $Å^2$ at 30 mN/m (not shown). Thus, it is not clear whether DG would alter the characteristics of the monolayer interface (hydration, packing) in a manner that aids PLC activity.

These data may have significant implications for G protein-mediated activation of PLCβ. Experiments were performed at a variety of surface pressures, and at relatively high pressures (30 mN/m), PLC activity with 3 mol % PIP_2 in the monolayer was almost minimal. The data suggest that only a moderate increase in PIP_2 mol % at these pressures causes a dramatic shift in PLCβ activity toward V_{max}. Thus, it may be that a component of the mechanism of activation of PLCβ by G protein α or $\beta\gamma$ subunits is the localization of enzyme at domains enriched in PIP_2 in the plasma membrane. Experiments looking directly at the effects of these proteins on PLCβ activity will be reported elsewhere.

Our results show that PLCβ activity in monolayers is dependent upon the characteristics of the lipid interface, including surface pressure and surface composition. The data contribute to understanding the positive and negative influences exerted upon these signal transducing enzymes by lipid interfaces, including cell membranes, which is important as a prelude to developing a clear picture of the molecular mechanisms of their activation by G protein α and $\beta\gamma$ subunits.

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