Identification of Hydrophobic Interactions between Proteins and Lipids: Free Fatty Acids Activate Phospholipase C δ1 via Allosterism[†]

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ABSTRACT: Lipids are well recognized ligands that bind to proteins in a specific manner and regulate their function. Most attention has been placed on the headgroup of phospholipids, and little is known about the role of the acyl chains in mediating any effects of lipids on proteins. In this report, free fatty acids (FFA) were found to bind and activate phospholipase C δ1(PLC δ1). The unsaturated FFA arachidonic acid (AA) and oleic acid were able to stimulate PLC δ1 up to 30-fold in a dose-dependent manner. The saturated FFA stearic acid and palmitic acid were less efficacious than unsaturated FFA, activating the enzyme up to 8-fold. The mechanism of activation appears to be due to a change in K_m for substrate; 50 μM arachidonate reduced the K_m for the soluble PLC substrate diC$_4$PI from 1.7 ± 0.6 mM to 0.24 ± 0.04 mM (7-fold reduction). V_{max} was not significantly altered. PLC δ1 bound to sucrose-loaded vesicles that contained AA in a concentration-dependent manner. A fragment of PLC δ1 that encompasses the EF-hand domain also bound to micelles containing AA using nondenaturing PAGE. This same fragment also inhibited AA activation of PLC δ1 in a competition assay. These results suggest that the function of the EF-hand domain of PLC δ1 is to bind lipid and to allosterically regulate catalysis. These results also suggest that esterified and nonesterified fatty acids can bind to and regulate protein function, identifying a functional role for hydrophobic interactions between lipids and proteins.

While the general structure of phospholipids is known, very little is known about how that structure dictates function. The data from our laboratory and others clearly show that a single phospholipid like phosphatidylinositol 4,5-bisphosphate (PIP$_2$)[1] has dramatic regulatory properties (*1*−*3*). PIP$_2$ is not, however, a single molecular species. The compositional and positional variations in the fatty acids create great structural heterogeneity. Common fatty acids include stearate 18:0, oleic 18:1, myristate 14:0, palmitate 16:0, linoleate 18:2, linolenate 18:3, and archidonate 20:4. While we are not challenging the importance of the lipid headgroup, we report here that specific interactions do occur between the acyl chains and proteins, leading to allosterism and significant regulation of protein function.

A growing body of evidence suggests that acyl chains, either free (as fatty acids) or as part of phospholipids, can regulate protein function including phospholipase C (PLC) activity. A number of reports have shown that biological amphiphiles such as bile acids and free fatty acids (FFAs) activate PLCs (*4*−*6*). Unsaturated fatty acids such as arachidonic acid (AA) are generated by PLA2 activity and invokes an inflammation cascade (*7*). Involvement of PLCs in FFA-induced calcium mobilization has been implicated in the literature (*8*, *9*). It has been shown that FFAs such as AA can activate PLC γ isozymes (*10*).

We have used PLC δ1 as a model to investigate the molecular basis for noncovalent protein−lipid interactions. The mammalian phospholipase C family consists of five subfamilies that differ in structure, mode of regulation, and function: β, δ, γ, ϵ, and ζ (*11*, *12*). Commonly these isoforms all cleave the polar headgroup from inositol phospholipids to generate second messengers inositol 1,4,5-trisphophate (IP$_3$) and diacylglycerol (DAG). IP$_3$ is a potent mobilizer of intracellular calcium from the endoplasmic reticulum, and DAG is an activator of protein kinase C (PKC). We and others have defined the pleckstrin homology (PH) domain of this molecule as both a targeting and an allosteric (effect on K_s and V_{max}) regulatory domain (*2*, *3*, *13*). PIP$_2$ is the only agonist that we have been able to identify for the PH domain of PLC δ1, although we have described several competitive antagonists. We and others have also examined the C2 domain and described the coordinate action of calcium and phosphatidylserine (PS) in regulating interfacial binding and catalysis (effect on K_m)

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[1] Abbreviations PLC δ1, phospholipase C δ1; PI, phosphatidylinositol; PIP$_2$, phosphatidylinositol 4,5-bisphosphate; PC, phosphatidylcholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DiC$_4$-PI, 1,2-dibutanoyl-*sn*-glycero-3-phosphoinositol; IP$_3$, inositol 1,4,5-trisphophate; DAG, diacylglycerol; cIP, cyclic inositol-1-phosphate; PS, phosphatidylserine; PH domain, pleckstrin homology domain; DDM, dodecyl maltoside; AA, arachidonic acid or arachidonate; SA, stearic acid or stearate; OA, oleic acid or oleate; PA, palmitic acid or palmitate; FFA, free fatty acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; GST, glutathione S-transferase; CMC, critical micelle concentration.

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(*14*, *15*). In the course of these studies, several pieces of data suggested that the acyl chains of phospholipids play a role in protein activation. Foremost among these was the fact that the headgroup of PIP_2, IP_3, could not activate the enzyme (*3*). In this paper, we examine the ability of FFAs to activate PLC δ1.

EXPERIMENTAL PROCEDURES

Materials. Phosphatidylinositol (PI), PIP_2, phosphatidylcholine (PC), and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) were purchased from Avanti Polar Lipids. Fatty acids were from Sigma. ^{3}H-PI and ^{3}H-PC were from Perkin-Elmer Life Sciences. 1,2-Dibutanoyl-*sn*-glycero-3-phosphoinositol (diC_4PI) was purchased from Echelon Biosciences. The expression constructs for PLC δ1(Δ1−132) and PLC δ1(Δ1−210) were generated by the polymerase chain reaction (PCR) using the bacterial expression plasmid pRSETAplc (*2*). The EF-hand domain fragment was generated by the PCR method using the wild-type construct as a template. Instead of using pRSETA as an expression vector, pGEX-2T was used and fused to glutathione S-transferase (GST).

Expression and Purification of PLC δ1. BL21(DE3) *Escherichia coli* were transformed with wild-type/deletion mutant PLC δ1 pRSETA constructs. The culture was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside at 20 °C overnight. The cells were collected and frozen at −80 °C. The cell pellet was resuspended with 50 mM sodium phosphate, pH 8.0, 0.1 M NaCl, and 0.1% Tween-20 plus protease inhibitor cocktail. The suspension was sonicated and centrifuged at 13000*g* at 4 °C for 45 min. The resulting supernatant was applied to appropriate amount of Ni−NTA resin. The resin was washed several times with the suspension buffer and then several times more with 50 mM sodium phosphate, pH 8.0, 0.1M NaCl, 0.1% Tween-20, and 15 mM imidazole. Then the protein was eluted with 50 mM sodium phosphate, pH 8.0, 0.1 M NaCl, 0.1% Tween-20 and 100 mM imidazole. The active fraction was identified using a PI hydrolysis assay. The protein concentration of the fraction was determined by Bradford assay. The EF-hand domain fragment fused to GST was expressed as described above. The fusion protein was purified in a manner similar to the method described by Ellis et al. (*16*) without further separation with the anion exchanger resin.

PLC δ1 Activity Assay. Assays using dodecyl maltoside (DDM)/PI mixed micelles were performed in a manner similar to those described by Cifuentes et al. (*17*). The indicated amount of PI containing ^{3}H−PI and fatty acid in chloroform/methanol (2:1) was dried under argon. The lipids were solubilized in a solution of 0.1 M NaCl, 50 mM HEPES, pH 7.3, and DDM. The mixtures were vortexed and sonicated briefly. Then acetylated BSA was added to 50 μg/mL, and EGTA was added to 1 mM. After the appropriate amount of enzyme was added, reactions were initiated by adding $CaCl_2$ to the total concentration of 2.8 mM. Activity assays were performed at 30 °C. Reactions were stopped by adding chloroform/methanol/HCl (100:100:0.6), followed by 1 N HCl containing 5 mM EDTA. The aqueous and organic phases were separated by centrifugation. The aqueous phases were counted by liquid scintillation.

Specific activities of the enzymes were determined by fitting the data points from each assay (typically 4−6 points) with linear regression. The duration of these assays was 4−10 min where no more than 25% of substrate was hydrolyzed and the rate of the hydrolysis was linear. The PI/deoxycholate assays were carried out according to the method described in ref *3*.

^{31}P NMR Assays of PLC δ1 Activity. The specific activity of PLC δ1 was measured using ^{31}P NMR (202.3 MHz) spectroscopy as described previously using a Varian INOVA 500 spectrometer (*18*). All substrates were solubilized in 50 mM HEPES, pH 7.3, with 0.1 M NaCl and 50 μg/mL BSA. The amount of enzyme added in each kinetic run was adjusted to carry out less than 20% hydrolysis within the reaction time (typically 0.25−1 h). Specific activity of the enzyme toward diC_4PI was determined using a fixed time point assay. The reaction (200 μL total volume containing various concentrations of PI and either 250 μM DDM or 200 μM DDM and 50 μM AA) was incubated for 15−30 min at 34 °C and then quenched with 200 μL of chloroform. The aqueous layer was separated from the chloroform layer by centrifugation for 5 min at 14 000 rpm in an Eppendorf microcentrifuge. I-1-P, the only product observed in this reaction, in the aqueous layer was quantified by ^{31}P NMR spectroscopy. For cIP hydrolysis, the reaction was also monitored at 34 °C after the addition of enzyme by acquiring ^{31}P NMR spectra over the time course of 1−2 h. In both assays, PI cleavage and cIP hydrolysis rates were measured from the integrated intensity of I-1-P resonances using the ^{31}P resonance of added glucose-6-phosphate (1 mM) as an internal reference. The NMR ^{31}P activity assays initially followed the reaction for several hours under conditions where <25% of the substrate was cleaved. After establishing the regime where kinetics were linear, fixed time points were analyzed. This was necessary to quantify substrate cleavage at low substrate concentrations.

*PLC δ1 Activity Assay with SUV***.** The indicated amount of PC, PI containing ^{3}H−PI, and fatty acid in chloroform/methanol (2:1) was dried under argon. The lipids were suspended with 90 mM NaCl and 45 mM HEPES, pH 7.3. After vigorous vortexing, the suspension was incubated at room temperature for 1 h. After three freeze-and-thaw cycles, the suspension was sonicated on ice. Then acetylated BSA was added to 50 μg/mL, and EGTA was added to 1 mM. Activity assays were performed at 37 °C. The assays and analysis of the data were carried out in the same manner as with the mixed micelle assays described above.

Protein−Lipid Vesicle Binding Assay. The indicated amount of DMPC and fatty acids dissolved in chloroform/methanol (2:1) were dried under argon. Sucrose and HEPES, pH 7.3, were added to 170 and 20 mM, respectively. After vigorous vortexing, the suspension was incubated at room temperature for 1 h. After three freeze-and-thaw cycles, the suspension was extruded 11 times through a 100-nm membrane. The resulting large unilamellar vesicles were diluted with 0.2 M KCl and 20 mM HEPES, pH 7.3, and collected by centrifugation. The vesicles were resuspended in 0.1 M KCl and 20 mM HEPES, pH 7.3. This vesicle suspension was serially diluted with the same buffer as the resuspension buffer, and 2.7 μg of PLC δ1 was added to the vesicles. After 30-min incubation at room temperature, protein/lipid mixtures were centrifuged at 100000*g* at 4 °C for 30 min. The supernatants were removed, and the pellets were redissolved in SDS sample buffer. The protein bound

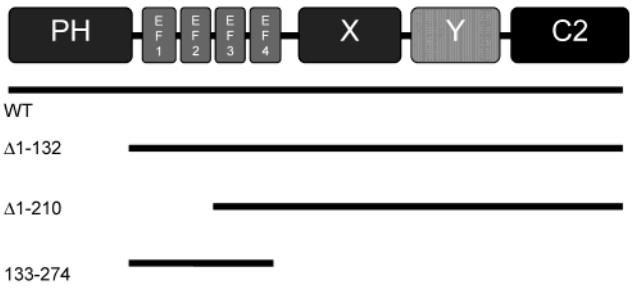

FIGURE 1: Schematic representation of deletion mutants used. The expression constructs for PLC δ1(Δ1−132) and PLC δ1(Δ1−210) were generated by the polymerase chain reaction (PCR) using the bacterial expression plasmid pRSETAplc as a templete (*2*). The EF-hand domain fragment 133−274 was also generated by the PCR method using the wild-type construct as a template.

to the vesicles was analyzed by SDS−PAGE on 10% polyacrylamide gels followed by Coomassie staining. Pelleting efficiency was determined by adding a trace amount of ^{3}H−PC to the lipids.

Nondenaturing Polyacrylamide Gel Electrophoresis (*Nondenaturing PAGE*). The wild-type PLC δ1 and EF-hand fragment (133−274) were added to 0.1 M NaCl, 50 mM HEPES, pH 7.3, and 16% glycerol containing 0.92 mM DDM (no AA) or 200 μM AA suspended with 0.72 mM DDM (+AA). The polyacrylamide gels (6% acrylamide) were polymerized in 50 mM Tris/acetate, pH 7.5, and the same buffer was used as a running buffer. The electrophoreisis was performed at 15 mA at room temperature. The gels were visualized by Coomassie staining.

RESULTS

Effects of Different Free Fatty Acids on PLC δ1s. We examined effects of four different FFAs on PLC activity. The saturated FFAs SA (18:0) and PA (16:0) as well as the unsaturated FFAs AA (20:4) and OA (18:1) were added into detergent mixed micelles (Figure 2). The indicated amount of the FFAs was incorporated as described under Experimental Procedures. The total concentration of DDM, fatty acid, and substrate were fixed at 250 μM, and the substrate concentration was constant at 3 μM. Both saturated and unsaturated FFA containing micelles were able to robustly activate wild-type PLC δ1. In general, the unsaturated fatty acids were much more efficacious than their saturated counterparts, being able to activate the enzyme up to 20−30-fold toward 3 μM PI substrate, while the saturated fatty acids were only able to maximally activate the enzyme 5−10-fold (Figure 2A−D). Activation was dose-dependent, with the largest fold activation seen at the higher 50 μM concentration of FFA (Figure 2E−H).

To locate the region of the fatty acid binding site(s), we created two deletion mutants, PLC δ1(Δ1−132) and PLC δ1(Δ1−210) (Figure 1), and then compared the effects of FFAs on the deletion mutants with those on the wild-type PLC δ1 (Figure 2). When PI hydrolysis rates for the purified proteins determined in a sodium deoxycholate/PI mixed micelles (330 μM PI + 0.1% sodium deoxycholate) were compared, there was no significant difference in specific activities of wild-type and PLC δ1(Δ1−132), although there was some mild reduction seen with PLC δ1(Δ1−210). The specific activities were 47.4 μmol min^{-1} mg^{-1} for the wild-type, 55.6 μmol min^{-1} mg^{-1} for PLC δ1(Δ1−132), and 42.9 μmol min^{-1} mg^{-1} for PLC δ1(Δ1−210). Activity assays with the truncation mutants were performed in the exact same manner as those for the wild-type enzyme. The two mutants were also significantly activated by the fatty acids in a dose-dependent manner, although there was some reduction in the extent of activation when compared with the fold activation of the wild-type enzyme (Figure 2A−D). PLC δ1(Δ1−132), which lacks the entire PH domain was activated up to 30- and 12-fold by 50 μM AA and 50 μM OA, respectively. PLC δ1(Δ1−210), which lacks the PH domain and the N-terminal half of the EF-hand domain (EF1 and EF2) was activated up to 17- and 9.5-fold by 50 μM AA and 50 μM OA, respectively. The deletion mutants were activated less than 4-fold by the saturated fatty acids even at 50 μM. The trend observed for the wild-type enzyme held true for the deletion mutants (i.e., the unsaturated fatty acids were more potent than the saturated fatty acids). Further deletion from the N-terminus (greater than 210) of PLC δ1 was impossible due to the complete loss of its enzymatic activity (data not shown), which is consistent with the findings by other investigators (*16*).

Effects of Alcohol Derivatives on PLC δ1. Our previous study implicated that there was a moderate increase (less than 3-fold increase) in PIP_2 hydrolysis when anionic nonsubstrate phospholipids such as phosphatidic acid were incorporated into detergent mixed micelles (*14*). A FFA molecule is comprised of a fatty acyl chain and a carboxyl group. The intrinsic pK_a value of the membranous OA was calculated as 7.5−7.8 according to Kramer et al. (*19*) We assessed the possibility of such effects using the same assays employed above by incorporating arachidonyl alcohol or stearyl alcohol instead of the acid counterparts. The stearyl alcohol derivative led to modest activation of both wild-type and deletion mutant enzymes (<4-fold), slightly less than that seen with the SA, while arachidonyl alcohol was inhibitory to the PI hydrolysis of both wild-type and deletion mutant enzymes (Figure 3). This result suggests that charge is also an important factor for enzyme activation although these derivatives had some effect on the PLC activity.

Effect of Free Fatty Acid Is on K_m. To identify the mechanism by which FFAs activate PLC δ1, PI hydrolysis rates were measured with increasing concentrations of substrate in the absence or presence of 25 μM AA (Figure 4). The concentration of DDM was varied so that the total concentration of DDM, AA, and substrate fell between 230 and 260 μM (variance of only ~13%). The DDM concentration was kept above its critical micellar concentration (CMC) (~176 μM) to solubilize both PI substrate and fatty acid activator (*20*). AA shifted the PI hydrolysis curves for both wild-type and PLC δ1(Δ1−132) to the left without changing the maximum velocities (2.4 and 1.1 μmol min^{-1} mg^{-1}, respectively). $S_{(0.5)}$ for wild-type PLC δ1 decreased from 36.3 to 11.8 μM and that of PLC δ1(Δ1−132) decreased from 38.7 to 10.8 μM where $S_{(0.5)}$ was defined as the substrate concentration for the half-maximal activity. Cooperative behavior was observed in the curves, which may be indicative of interfacial effects (Figure 4). For both of the enzymes, more than a 3-fold reduction in $S_{(0.5)}$ was observed. AA clearly has an effect on PLC δ1 binding to aggregated substrates.

It is sometimes difficult to distinguish effects caused by translocation of an enzyme to a vesicle or micelle or by a

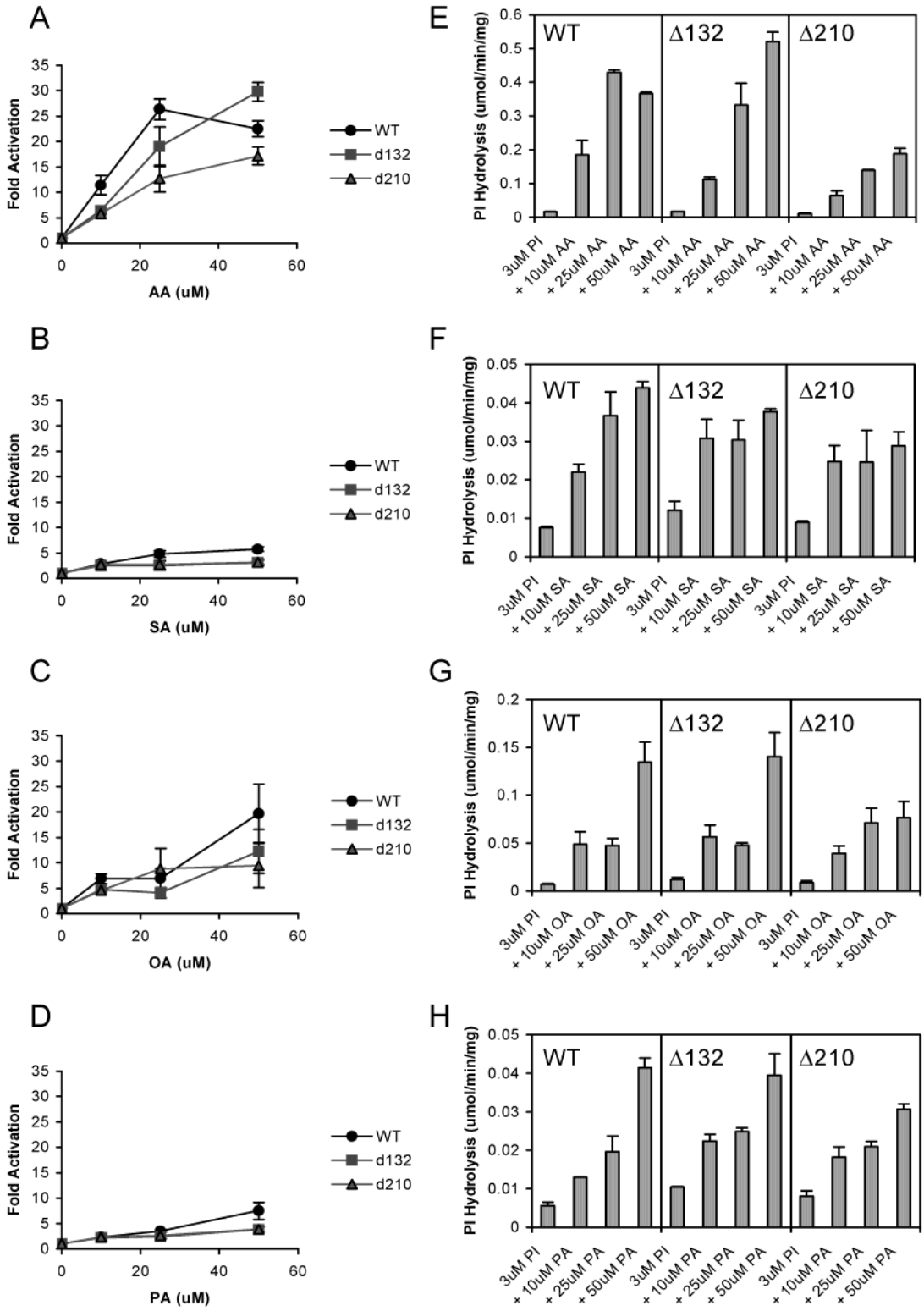

FIGURE 2: Dose-dependent effects of different free fatty acids on wild-type PLC δ1 and deletion mutants (Δ1−132 and Δ1−210). (Panels A−D) PI hydrolysis rate at three different concentrations of free fatty acids are expressed as fold activation. AA, arachidonic acid; SA, stearic acid; OA, oleic acid; PA, palmitic acid. (Panels E−H) PI hydrolysis rates at three different concentrations of free fatty acids are expressed as specific activity. The total concentration of DDM, fatty acid, and substrate were fixed at 250 μM, and the substrate concentration was constant at 3 μM. Error bars represent the standard deviation of two experiments.

true change in substrate binding. To circumvent this problem, a simpler approach was taken using water-soluble substrates. The short-chain phosphatidyl inositol diC$_4$PI was used as substrate for the phosphotransferase step and the cyclic intermediate cIP for the phosphodiesterase reaction to assess the effect of AA activation on both k_{cat} and K_m (*18*). Since AA must be solubilized by detergent, the control measures the activity of PLC δ toward diC$_4$PI (CMC >100 mM) in the presence of detergent 250 μM DDM. For diC$_4$PI, K_m = 1.7 ± 0.6 mM and V_{max} = 5.7 ± 0.9 μmol min^{-1} mg^{-1} when analyzed with simple Michaelis−Menten kinetics. Furthermore, only I-1-P (and no cIP) was detected as the phosphor-

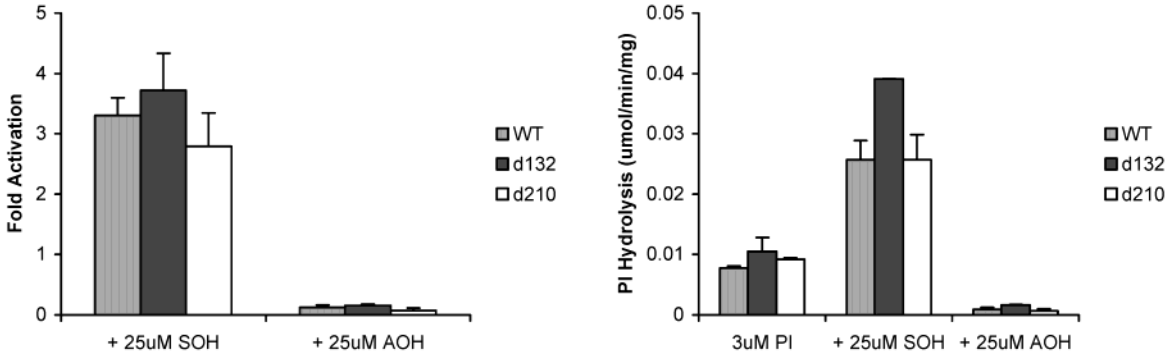

FIGURE 3: Effects of alcohol derivatives on wild-type and deletion mutants. The activity assays were performed in the same manner as in the experiments shown in Figure 2. Stearyl alcohol (SOH) moderately activated hydrolysis of PI by both wild-type and deletion mutants while arachidonyl alcohol (AOH) was inhibitory.

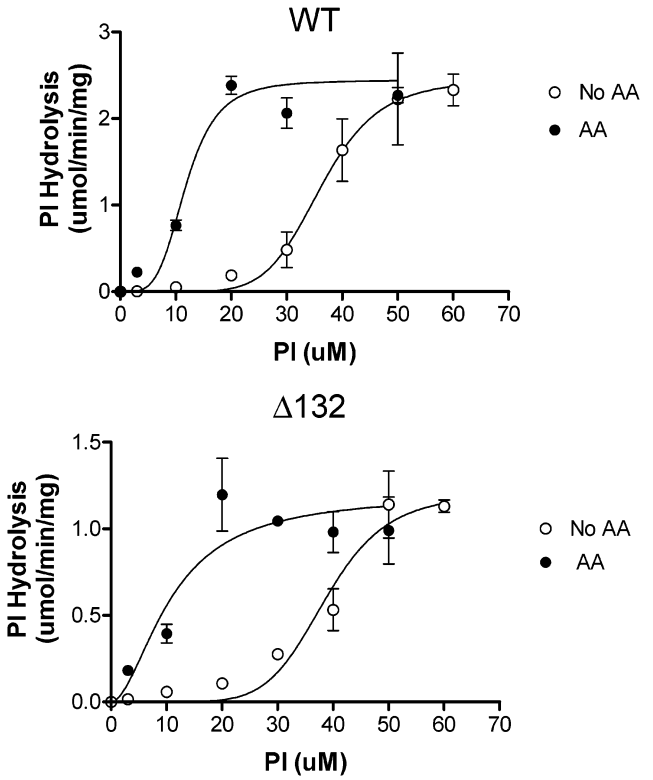

FIGURE 4: Arachidonic acid shifts the substrate dependency curve to the left without changing the maximum velocity. PI hydrolysis rates were measured with increasing concentrations of substrate in the absence (open circles) or presence (filled circles) of 25 μM AA. $S_{(0.5)}$ for wild-type PLC δ1 decreased from 36.3 to 11.8 μM (upper panel) and that of PLC δ1(Δ1−132) decreased from 38.7 to 10.8 μM (lower panel). The concentration of DDM was varied so that the total concentration of DDM, AA, and substrate fell between 230 and 260 μM (~13%). $S_{(0.5)}$ was defined as the substrate concentration for the half-maximal activity. Each point was generated from at least two experiments. Error bars represent the standard error and are shown when larger than the symbol.

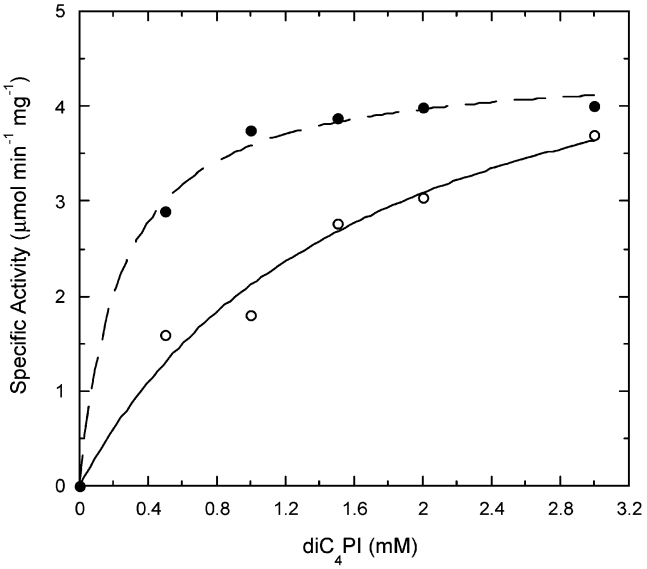

FIGURE 5: Effect of arachidonic acid on PLC δ1 cleavage of soluble substrates. The rates of soluble PI (DiC_4PI) hydrolysis in the presence (filled circles) of AA solubilized with 200 μM DDM were compared with 250 μM DDM in the absence (open circles) of AA. Total detergent and lipid concentration was fixed and 250 μM. There was a significant decrease in K_m (see text). Many of the assays with soluble substrates were done in duplicate; the errors in determining specific activities via ^{31}P NMR assays were $\leq$10%. Thus, the activation of diC_4PI cleavage observed is well out of the range of errors for this technique.

ylated product. When cleavage of the same substrate was carried out with 50 μM AA solubilized by 200 μM DDM, there was little activation at high diC_4PI concentrations but substantial activation below 1 mM (Figure 5). Calculation of kinetic parameters indicated that K_m had decreased to 0.24 $\pm$ 0.04 mM (7-fold reduction) while V_{max} was not significantly altered (4.4 $\pm$ 0.1 μmol min^{-1} mg^{-1}). For this substrate, AA binding to the PLC δ1 clearly enhances substrate binding.

The catalytic second step, the hydrolysis of cyclic inositol-1-phosphate (cIP) to I-1-P, was also examined to see if AA binding to the enzyme alters this step. As shown in Table 1, the hydrolysis of cIP is enhanced by the detergent itself. A 2-fold increase in activity toward 10 mM cIP with 230 versus 115 μM DDM was observed. The total amphiphile concentration (DDM + AA) was constant for one set of experiments to enable us to compare the results. Over an AA concentration range of 25−75 μM, there was no enhanced cIP cleavage (Table 1). The concentrations of cIP used (5 and 10 mM) were well below the K_m for this substrate (*18*). AA binds to PLC δ1 and alters its affinity for PI substrate but has no effect on cIP binding or hydrolysis.

PLC δ1 Can Be Activated by Free Fatty Acids Incorporated into Vesicles. FFAs incorporated into micelles stimulated PLC δ1 activity. We performed activity assays using PC vesicles to see if this activation by FFA is also feasible in the bilayer membrane, which is more relevant to biological membranes. The PC vesicles containing AA or SA as well

Table 1. Specific Activities of PLC δ1 toward CIP in the Presence of Dodecyl Maltoside and Arachidonic Acid[a]

cIP (mM)	AA (μM)	DDM (μM)	specific activity (μmol min^{-1} mg^{-1})	(+ AA)/(−AA)
5	0	115	0.030	
5	25	90	0.029	0.97
10	0	115	0.106	
10	25	90	0.108	1.02
10	0	230	0.194	
10	25	205	0.179	0.92
10	50	180	0.196	
10	75	155	0.155	0.79

[a] The catalytic second step, the hydrolysis of cIP, was measured as described under Experimental Procedures. Many of the assays with cIP were done in duplicate; the errors in determining specific activities via ^{31}P NMR assays were ≤10%.

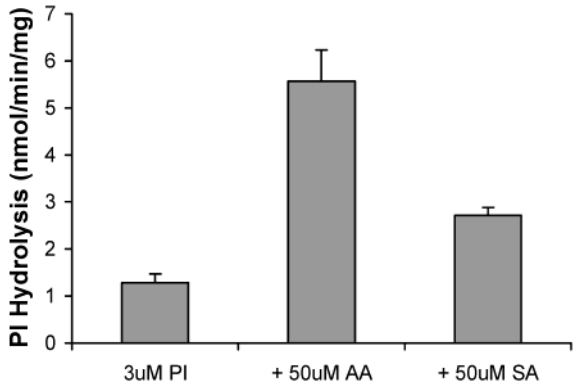

FIGURE 6: PLC δ1 is activated by fatty acid containing vesicles. The PC vesicles containing AA or SA as well as PC vesicles containing no fatty acid were prepared according to the method described under Experimental Procedures. The concentration of PC plus fatty acid was kept at 300 μM, and the substrate concentration was 3 μM. PLC δ1 was activated by AA containing vesicles and SA containing vesicles. Error bars represent the standard deviation of three experiments.

as PC vesicles containing no fatty acid were prepared according to the method described under Experimental Procedures. As seen in Figure 6, there was a significant difference between with and without FFAs (more than 4- and 2-fold activation with AA and SA, respectively), although the magnitude was less than that of the miceller system. Also the specificity toward fatty acids was the same as the miceller system, that is, AA was more potent than SA.

PLC δ1 Binds to Fatty Acid Containing Vesicles. To assess if enzyme binding to fatty acid correlated with activation, we performed a vesicle ultracentrifugation assay. Sucrose loaded unilamellar vesicles were prepared with AA. Wild-type PLC δ1 bound to AA containing vesicles in a concentration-dependent manner (Figure 7A). PLC δ1 bound much better (2−3-fold more bound) to vesicles containing AA than to those without it. The pelleting efficiencies for the vesicles (PC only, 20% AA and 20% SA) in Figure 7B were determined using ^{3}H-labeled PC, and they were 66.6%, 45.5%, and 21.3%, respectively. The PC vesicles pelleted better than the AA containing vesicles. Therefore the difference in the protein amount between the two vesicles reflects a difference in true binding to the incorporated fatty acid. Although PLC δ1 also bound to vesicles that contained SA, the low pelleting efficiency of SA containing vesicles precluded making comparisons with AA containing vesicles.

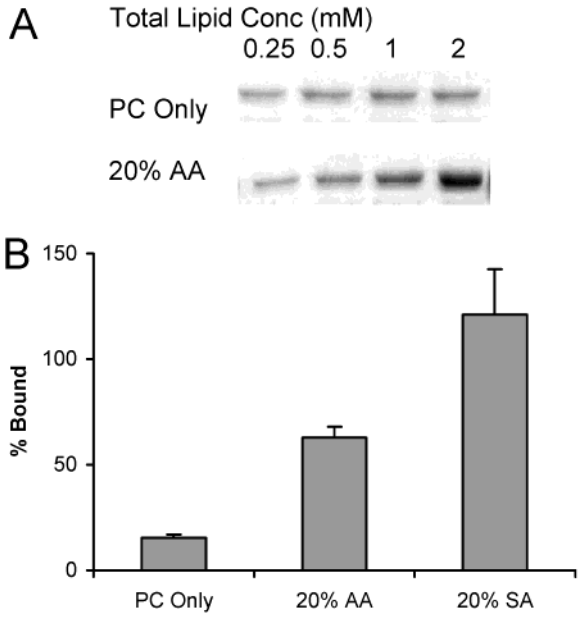

FIGURE 7: PLC δ1 binds to fatty acid containing vesicles. (Panel A) PLC δ1 protein recovered from the pellets containing AA vesicles increased as the lipid concentration increased. PLC δ1 was visualized with Coomassie staining. (Panel B) PLC δ1 bound to both AA containing vesicles and SA containing vesicles. The total lipid concentration was 2 mM. The pelleting efficiency for each vesicle was determined using ^{3}H-labeled PC, and they were 66.6%, 45.5%, and 21.3%, respectively. Error bars represent the standard deviation of at least three experiments.

AA Causes Mobility Shifts in Nondenaturing PAGE. Since PLC δ1(Δ1−132) was activated by FFA via the same allosterism (i.e., decrease in $S_{(0.5)}$), the site(s) of interaction with FFA that leads to the activation of this enzyme must reside within the rest of the molecule. Further deletion from the N-terminus of PLC δ1 could narrow down the site(s) of FFA binding. However, deletions of more than 210 amino acids cause loss of enzymatic activity. Therefore, it is difficult to assess the effect of FFA on PLC activity for deletion mutants that are greater than residue 210. There are numerous examples that demonstrate interactions between lipids and EF-hand motifs (*21*−*26*). Thus it is reasonable to hypothesize that the EF-hand domain of PLCδ1 is able to bind AA. To test this hypothesis, we created a protein fragment encompassing most of the EF-hand domain PLC δ1(133−274) (Figure 1). The protein fragment was added to a sample buffer containing either DDM (as control) or AA solubilized with DDM. The mobility shift was observed in the sample containing AA (Figure 8A). Since a protein migrates on the basis of its net charge, size, and shape, the mobility shift must reflect a change in at least one of these factors. The same experiments were performed with bovine serum albumin (BSA) as a control. BSA is known to bind fatty acids and AA caused a mobility shift of this protein (Figure 8A). Thus, the EF-hand domain fragment PLC δ1(133−274) changes its mobility on nondenaturing gels in the presence of AA, suggesting that these motifs are sufficient to bind fatty acid. The use of nondenaturing gel electrophoresis to detect ligand binding has been used with many proteins including the EGF receptor (*27*). The mobility shift occurs even in the absence of calcium. The mobility shift upon binding to AA occurred to the wild-type enzyme, while the enzyme barely entered the gel without AA under these

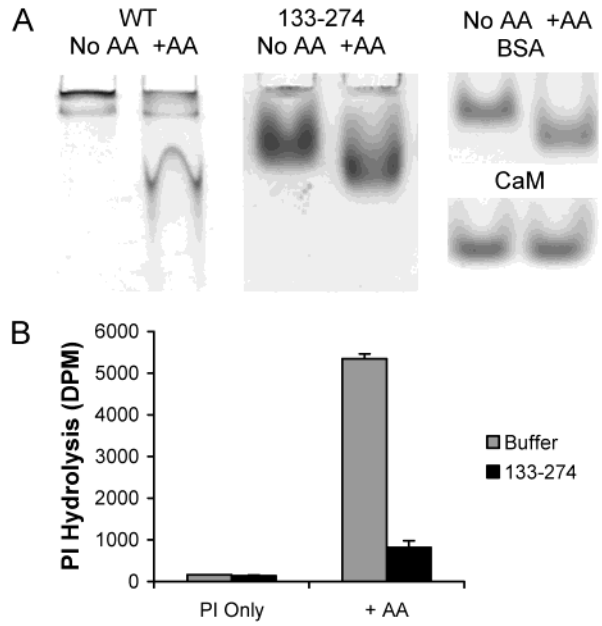

FIGURE 8: Arachidonic acid causes mobility shifts in nondenaturing PAGE. (Panel A) AA caused mobility shifts in nondenaturing PAGE. The wild-type PLC δ1 and EF-hand fragment (133−274) were added to 0.1 M NaCl, 50 mM HEPES, pH 7.3, and 16% glycerol containing 0.92 mM DDM (no AA) or 200 μM AA suspended with 0.72 mM DDM (+AA). The same experiments were performed with bovine serum albumin (BSA) and calmodulin (CaM) as a control. BSA is known to bind fatty acids, and AA caused mobility shift of this protein. (Panel B) The EF-hand fragment (133−274) inhibited the AA activation. PI hydrolysis of the wild-type PLC δ1 was measured in the absence (gray bar) or presence (black bar) of the EF-hand fragment. PI concentrations for the competition assay were 2.7 μM. AA was added to 22.5 μM. The EF-hand fragment was added to 13.8 μM. The assays were preformed in a fashion similar to the experiments shown in Figure 2. The reaction was stopped at 5 min. The data shown here are the means of duplicate determinations and representative of three similar experiments.

conditions. Neither calmodulin (Figure 8A) nor PLCδ1(Δ1−294) (data not shown) exhibited any mobility shift by AA in nondenaturing PAGE under the same conditions used in the experiment shown in Figure 8. PLCδ1(Δ1−294) is catalytically inactive, so we cannot rule out that this is a misfolded protein. The fact that calmodulin does not bind indicates that not all EF-hand domains bind FFA and suggests specificity. Nondenaturing gel electrophoresis with the purified protein fragment suggests that the EF-hand domain constitutes a site for fatty acid binding. In the crystal structure of PLC δ1, the three resolved EF hands interact closely with the C2 domain (*42*). While this domain might not adopt a similar structure when expressed independent of the rest of the protein, analysis using circular dichroism spectroscopy indicates that the isolated domain is well-folded and predominantly helical as monitored by the magnitude of the molar ellipticity and double minima at 208 and 222 nm (unpublished data). A high helical content is consistent with the structure of the EF-hand observed in the crystal structure of the enzyme (*42*). The protein also appears to be monomeric as judged by preliminary NMR studies of the recombinant fragment, which show well-dispersed ^{1}H resonances with relatively narrow line widths.

The EF-Hand Domain Fragment PLC δ1(133−274) Inhibits AA Activation of PLC δ1. Competition assays were performed using the EF-hand fragment (133−274) to see if the fragment by binding AA would inhibit the activation caused by AA (Figure 8B). The fragment did not significantly affect PI hydrolysis in the absence of AA (less than 15% reduction), while it remarkably reduced PI hydrolysis in the presence of AA by 85%. The substrate mixtures were initially buffered with 0.9 mM EGTA. Then calcium was added to 2.8 mM so that the free calcium concentration was above 1.8 mM, far in excess of the molarity of the added fragment. The total calcium concentration was raised to ensure that the free calcium concentration was not reduced if the fragment were to bind calcium. Since the fragment did not reduce basal PI hydrolysis, adequate free calcium was available to the enzyme in the presence of the fragment.

DISCUSSION

The point of this paper is to show that AA binding to PLC δ1 can affect its efficiency. The mixed micelle assay system was chosen as one that does not have other phospholipids in it that could modulate the PLC activity. We and others have found that there is no such thing as a neutral diluent phospholipid. On the contrary, the detergent DDM appears to be a truly neutral diluent. Hence, our decision to perform the bulk of the assays using detergent mixed micelles.

We examined whether FFAs could regulate the activity of PLC δ1. The saturated FFAs SA (18:0) and PA (16:0) as well as the unsaturated FFAs AA (20:4) and OA (18:1) were incorporated into detergent mixed micelles. Because the solubility of the free acid is extremely low, it is unlikely that significant soluble FFA is present. The CMC for AA in the presence of calcium has been calculated to be 6.5 μM (*28*). We are well above that concentration so that the FFA used in our assays should be aggregated. Both saturated and unsaturated FFA containing micelles were able to robustly activate wild-type PLC δ1 as well as two truncation mutants PLC δ1(Δ1−132) and PLC δ1(Δ1−132) that lacked the PH domain. In general, the unsaturated fatty acids were much more efficacious than their saturated counterparts, being able to activate the enzyme up to 20−30-fold, while the saturated fatty acids were only able to maximally activate the enzyme 5−10-fold (Figure 2A−D). The increased efficacy of unsaturated fatty acids compared to saturated fatty acids seems to be a common paradigm. Unsaturated fatty acids such as linolenic, linoleic, oleic, and AA can activate protein kinase C, while saturated fatty acids have little effect (*29*). Manning and Sun reported that PLC activity stimulated by deoxycholate was inhibited by OA and AA (*4*). This might suggest that deoxycholate and FFAs share a common activation mechanism and that deoxycholate might even be more potent than OA or AA. This notion is also supported by the fact that truncated PLC δ1 lacking the PH domain can be activated by FFAs as well as bile acids such as deoxycholate in mixed micelles (*16*).

Activation of PLC δ1 by fatty acids was dose-dependent with the largest fold activation seen at the higher 50 μM concentration of FFA (Figure 2 E-H). This may be higher than the physiological concentration since AA is readily metabolized to other signaling molecules in vivo. While it is difficult to determine the free AA concentration, some reports have estimated cellular AA concentrations to be at

or higher than 50 μM. Using a mass per volume calculation, the concentration of esterified AA has been calculated to be approximately 5 mM (*30*). Since this represents an average over the whole cell, it is likely that the concentration is even higher in some compartments such as the plasma membrane. If only 1% of this esterified AA is released via the activation of phospholipase A2, then the concentration of free AA would be 50 μM. In fact, in some cell types such as platelets, the percent release is much higher on the order of 10% (*31*). In another study, the unesterified AA concentration in pancreatic islets was estimated to be 38−75 uM after stimulation with glucose (*32*, *33*).

Incorporation of the alcohol derivative of AA into detergent mixed micelles did not lead to activation; however, there was some inhibition (Figure 3). The stearyl alcohol derivative led to modest activation, slightly less than that seen with the free SA. This result suggests that charge is also important for enzyme activation. Charge has also been shown to be necessary for the binding of FFA to S100A8 and S100A9 proteins (*23*). Charge by itself is not sufficient, since SA and AA have markedly different effects on PLC δ1 yet the same charge. The hydrophobic interactions seem necessary for activation but may not be sufficient for binding since these interactions are generally weak. The ionic interactions between the protein and the carboxyl group of the FFA or the hydrogen bonding between the protein and the alcohol derivative may be necessary for tight association.

Activity assays were also performed using PC vesicles to see if activation by FFA also occurs in bilayers, a system more akin to biological membranes. Hydrolysis in vesicles containing FFA was greater than in vesicles containing PC alone (more than 4- and 2-fold activation with AA and SA, respectively), although the magnitude was less than that seen in the miceller system. Moreover, the specificity toward fatty acids was the same as that of the miceller system; AA was more potent than SA. These results indicate that the enzyme has some contact with the hydrophobic moiety of the lipids even in the bilayer system. A previous study using a lipid monolayer suggests that penetration of PLC δ1 is shallow (*34*). The poor penetration of PLC δ1 into bilayers may explain why we see more robust activation of PLC δ1 in micelles as compared to bilayers. Micelles are more easily penetrated than bilayers since they are looser more fluid structures. Our finding on the fatty acid specificity is also consistent with the results obtained by Irvine et al. (*6*), which showed that addition of AA or OA stimulated the hydrolysis of membrane phosphatidylinositol by the brain supernatant containing PLC. Moreover, it has been reported that PLA2 activities alter the local membrane structure that can modulate protein binding/activity (*35*). We postulate that these local events in vivo might play a role in helping PLC δ1 penetrate bilayers. Not all proteins regulated by FFA are membrane associated. Some fatty acid binding proteins are in fact soluble (*36*). While we believe that the FFA concentration is extremely low based on the CMC for FFA, soluble proteins are able to bind FFA and be regulated by them. Therefore, bilayer penetration is not necessary for these proteins.

Where is the site of interaction between PLC δ1 and the acyl chains? There are numerous potential sites in the protein. PLC δ1 contains several well-characterized protein motifs including an N-terminal PH domain, four EF-hand motifs, the catalytic TIM barrel, and a C2 domain. Three of these motifs in PLC δ1 have previously been shown to bind to lipid: the catalytic center, the PH domain, and the C2 domain. In addition, there is a ridge of hydrophobic amino acids (Leu-320, Tyr-358, Phe-360, Leu-529, and Trp-555) surrounding the catalytic site that are postulated to insert into the lipid bilayer and could potentially interact with the acyl groups of phospholipids (*37*). The fact that the two N-terminal truncation mutants were still activated by the fatty acids suggests that domains other than the PH domain are responsible for activation. Nondenaturing gel electrophoresis with a purified protein fragment encompassing most of the EF-hand domain from PLC δ1(acids 133−274) suggests that the EF-hand domain constitutes a site for fatty acid binding. The EF-hand domain construct changes its mobility on nondenaturing gels in the presence of AA, suggesting that these domains are sufficient to bind fatty acid (Figure 8). This fragment also markedly inhibits the ability of AA to stimulate intact PLC δ1 in a competition assay (Figure 8B) (*16*). In the crystal structure, the EF-hand domain closely interacts with the C2 domain, which is also indispensable to the enzymatic activity (*16*, *42*). The isolated EF-hand domain may not be exactly in the same fold as in the holo enzyme due to the lack of its interaction with the C2 domain. Despite this concern, judging from the CD spectrum (unpublished data), the isolated domain was well-folded and predominantly helical, which is consistent with the crystal structure (*42*). Since it is extremely difficult to determine the stoichiometry of FFA binding to this fragment or to intact PLC δ1, we cannot be sure that this is the only site for FFA binding. There are a number of EF-hand motif containing proteins that are involved in lipid binding. Several examples of such proteins are recoverin (*38*, *39*), calcineurin B (*40*), and calmyrin (*21*) that are myristoylated. There are also EF-hand containing proteins that are not myristoylated but can still bind to lipids. Examples include S100A8/S100A9 (*22*, *23*) and diacylglycerol kinase α (*24*, *25*). It has also been reported that *Arabidopsis* protein kinase, which also contains EF-hand motifs, is activated by lipids even when the N-teminal myristoylation motifs were deleted (*26*, *41*). The S100 family proteins are compelling examples since this protein is largely made up of two EF-hand motifs flanked by largely hydrophobic regions at the ends of a central hinge region, and the S100A8 and S100A9 proteins bind AA. These proteins are regulated by both calcium and lipids and are important for calcium signaling. Thus it is reasonable to hypothesize the EF-hand domain of PLC δ1 is able to bind AA, although the catalytic and C2 domains cannot be completely ruled out from the candidates for the FFA binding.

Several groups have attempted to elucidate the function of the EF-hand domain of PLCs. All of the mammalian PLCs including the newly identified ζ isozymes have four EF-hand motifs that consist of four loop−helix−loop−helix motifs arrayed in two lobes (each containing two EF-hands). The overall structure of this region in PLC δ1 closely resembles that of calmodulin. While the first two EF-hand motifs of PLC δ1 have residues that are able to bind calcium, there is no evidence that they do. The EF-hand domain did not bind calcium or calcium analogues when the crystals of PLC δ1were soaked (*42*). Isothermal titration calorimetry performed for calcium binding to PH domain truncation mutants of PLC δ1 demonstrated a stoichiometry of four. This is consistent with a single calcium binding to the

catalytic site, three to the C2 domain, and none to the EF-hand motifs (*43*). Yamamoto et al. demonstrated that the affinity of the fragment containing the PH and EF-hand domains of PLC δ1 increases to PIP_2, while the affinity to IP_3 decreases in a calcium-dependent manner, suggesting that calcium may be able to bind to the EF-hand motifs (*44*). Unlike mammalian PLCs, higher plant PLCs such as that of *Arabidopsis* lack the entire PH domain and the first two EF-hand motifs, suggesting that the second lobe (EF-hand 3 and 4) may be more critical to enzyme function (*45*, *46*). Consistent with this notion is the fact that deletion of the last two EF-hand motifs in PLC δ1 results in a loss of phosphodiesterase activity (*16*, *45*). Essen et al. (*42*) note that the second lobe of the EF-hand domain (i.e., EF3 and EF4) is probably important for the stability of the whole enzyme. The structure of EF3 and EF4 are similar to the calcium-saturated state of calmodulin, although there is no apparent bound calcium in the loops of these domains in PLC δ1. Despite all these studies, the function of the EF-hand motifs in PLC is not known (*11*, *12*).

Our data implicate the entire EF-hand domain (motifs 1−4) in mediating the effects of FFA. This conclusion is supported by the binding of fragment 133−274 (EF-hand motifs 1−4) to AA containing micelles and the ability of this fragment to inhibit AA activation of the holoenzyme (Figure 8). Truncation mutant PLC δ1(Δ1−132) contains all four EF-hand motifs. This mutant responds to FFA like the wild-type enzyme (Figure 2). Truncation mutant PLC δ1(Δ1−210) contains EF-hand motif 3 and 4 but not 1 and 2. This mutant is still activated by FFA, suggesting that EF-hand motifs 3 and 4 are capable of mediating the effects of FFA. Since deletion mutant PLC δ1(Δ1−210) is not as responsive to FFA as PLC δ1(Δ1−132), we postulate that EF-hand motifs 1 and 2 may also play a role in mediating the effects of FFA. An alternative hypothesis to explain the differences between PLC δ1(Δ1−132) and PLC δ1(Δ1−210) would be that EF-hand motifs 3 and 4 may not exist in the same conformation in PLC δ1(Δ1−210) as in the larger PLC δ1(Δ1−132) truncation mutant. Further studies will attempt to define the roles of each of the four EF-hand motifs. The fact that both wild-type and PLC δ1(133−274) bind to AA in the absence of calcium and that binding is not modified by the addition of millimolar calcium (data not shown) is consistent with current data suggesting that the EF-hand domain does not bind calcium. Data garnered thus far suggest that calcium is not required nor does it affect FFA regulation of PLC δ1. The definitive determination of the EF-hand region of PLC δ1 as the region mediating FFA allosterism awaits additional studies.

To assess if enzyme bound to AA, we performed a vesicle ultracentrifugation assay (*47*). This assay is considered by many to be the gold standard for detecting protein lipid interactions. Wild-type PLC δ1 bound to sucrose loaded unilamellar vesicles containing AA in a concentration-dependent manner (Figure 7A). PLC δ1 binding was specific for AA since the enzyme bound much better (2−3-fold more bound) to vesicles containing AA than to those without it. Likewise, FFA binding to the EF-hand motifs of PLC δ1 seems to be specific, since FFA did not bind to the EF-hand domain of other proteins such as calmodulin (Figure 8A). PLC δ1 also bound to vesicles that contained SA. Presumably, while charge is playing the major role in driving the binding of FFA to PLC δ1, it is not sufficient to induce activation.

Since PLC δ1(Δ1−210) was still activated by the fatty acids, EF3 and EF4 are likely involved in fatty acid binding. EF3 and EF4 are composed of amphipathic helices and could accommodate a hydrophobic moiety such as an acyl chain. Interactions between proteins with amphipathic moieties and fatty acids/acyl groups have been implicated in several reports (*48*, *49*). The amphipathic "lid" covering the active site of lipoprotein lipase plays an important role in mediating its selectivity toward substrates, water-soluble or long-chain fatty acid triglycerides (*49*). It is tempting to speculate that this region, the second lobe of the EF-hand domain mediates fatty acid binding. The interactions between protein and lipid are likely weak. The PLC δ1(133−274) fragment did not bind well to AA containing vesicles. We suspect that the interaction was too weak to withstand the forces generated during the ultracentrifugation step. The fragment did bind well to AA containing micelles as shown by nondenaturing gel electrophoresis. We speculate that another domain in PLC δ1 such as the PH domain may be necessary to target this enzyme to more tightly packed bilayers. It has been shown that PLC δ1 which lacks the entire PH domain translocates less well than the full-length enzyme to bilayer membranes (*50*). The PH domain plays a major role in membrane targeting of this and other enzymes. Once tethered to the membrane, the EF-hand domain might be able to interact with fatty acids since the local concentration of fatty acids around the EF-hand domain increases. This point has to be demonstrated in future studies using bilayer systems. It seems that the binding of AA to the holoprotein is more complex, and it is hard to separate binding due to AA from interactions of other domains in the protein with the bilayer (Figure 7). Although we do not know to what degree the EF-hand domain interacts with more tightly packed bilayers, the position of the EF-hand domain adjacent to the C2 domain integrated with the catalytic core of PLC δ1, which makes it reasonable for allosteric effects to occur between these functional domains.

To identify the mechanism by which FFAs such as AA activate PLC δ1, we examined the effect of AA on substrate dependency. As is shown in Figure 4, the addition of 25 μM AA to the detergent mixed micelles caused a marked leftward shift in the substrate dependency curve. The maximum rate of hydrolysis did not appear to change, but the $S_{(0.5)}$ decreased from 36.3 to 11.8 μM. This observation is similar to the one made by Sekiya et al. on PLC γ1 activation by AHNAK in the presence of AA (*10*). AA clearly has an effect on PLC δ1 binding to aggregated substrates. Does it also affect the catalytic properties of the enzyme? This is sometimes difficult to deconvolute with a vesicle or micelle assay system (*51*, *52*). A simpler approach is to use water-soluble substrates to assess the kinetic parameters for the two-step catalysis of substrate by this family of enzyme. The short-chain phosphatidyl inositol diC_4PI was used as substrate for the phosphotransferase step and the intermediate cIP for the phosphodiesterase reaction to assess the effect of AA activation on both k_{cat} and K_m (*18*). Any activation observed must reflect a direct effect of AA on PLC δ1. There was little activation at high diC_4PI concentrations but substantial activation below 1 mM. Calculation of kinetic parameters indicated that K_m had

decreased to 0.24 ± 0.04 mM (7-fold reduction) while V_{max} was not significantly altered. For this substrate, AA binding to the PLC δ1 clearly enhances substrate binding as evidenced by the change in K_m. If binding of the soluble short-chain PI is enhanced, it is likely that binding of a long-chain PI substrate will also be enhanced in a similar fashion.

The catalytic second step, the hydrolysis of cIP to I-1-P, was also examined to see if AA binding to the enzyme alters this step. As shown in Table 1, the hydrolysis of cIP was enhanced by the detergent DDM (compare the 2-fold increased activity toward 10 mM cIP with 230 versus 115 μM DDM). As long as the total amphiphile concentration (DDM + AA) is constant, any specific effects of AA can be measured. The concentrations of cIP used (5 and 10 mM) were well below the K_m for this substrate (*18*), so that if AA binding to the enzyme altered its conformation for the hydrolysis step, we should see an activation of cIP hydrolysis that reflects either a lowered K_m or an increased V_{max}. Over an AA concentration range of 25−75 μM, there was no enhanced cIP cleavage. AA binds to PLC δ1 and alters its affinity for PI substrate but has no effect on cIP binding or hydrolysis. These results indicate that AA does not merely act as a generic detergent, which has been shown to affect the cIP hydrolysis (*18*). While it is not known if AA binding to the enzyme enhances binding of PI via its substrate acyl chains or glycerol backbone, it does demonstrate that AA is an allosteric activator of PLC δ1.

Could AA mediated activation of PLC δ1 be due to effects other than allosterism? Could FFA have some effects on substrate that cause enhanced activity? We believe that our experiments definitively rule out these possibilities. If AA mediated activation of PLC δ1 was due solely to targeting of the enzyme to the detergent mixed micelles, we would not expect a change in K_m, but a change in the interfacial binding constant K values. It is also unlikely that FFA is affecting substrate availability, since detergent mixed micelles are extremely fluid. However, the most convincing argument for allosterism comes for the kinetics determined using the monomeric substrate diC_4PI. DiC_4PI is truly monomeric under our conditions. Although the K_m is relatively high for this soluble substrate, ~1 mM, the CMC is likely to be much higher: ~300 mM (similar to diC_4PC). No substrate micelles should appear until the concentration reaches the CMC. In our experiments, the concentration of substrate was 2 orders of magnitude (100-fold) below the CMC. If the enzyme did act on micellar substrate, the shape of the activity versus substrate curve would be different from that observed in our experiments. Figure 5 demonstrates a plateau in the curve of activity vs diC_4PI concentration that would not occur on micellar substrate. In the latter case, the v versus S curve would be expected to keep increasing with substrate concentration until enough micellized diC_4PI was present to saturate the enzyme. Furthermore, the amount of detergent plus fatty acid (250 μM) was kept constant in these assays. The 7-fold drop in K_m was observed with 50 μM AA/200μM DDM versus 250 μM DDM. Does some of the monomeric substrate get incorporated into the FFA detergent mixed micelles? Our experimental evidence suggests otherwise. This small amount of detergent has no effect on NMR parameters of mM diC_4-phospholipids, demonstrating that there is no detectable partitioning of soluble substrate into the micelles. The use of detergent mixed micelles to deliver the FFA to the enzyme does not cause any nonspecific effects since the diC_4PI substrate remains monomeric and soluble.

FFAs are increasingly being recognized as bioactive molecules. While most of the effects of AA are attributed to its conversion by oxygenases to prostaglandins and leukotrienes, direct effects have been described (*30*). For example, AA is a ligand for a novel family of G protein coupled receptor termed GPR40 (*53*). AA will also activate PPAR receptors and NADPH oxidase (*30*). FFAs play an important role in diabetes by inhibiting insulin-stimulated glucose uptake and glycogen synthesis (*54*). Most obese individuals have elevated circulating plasma levels of FFAs. Many of these individuals develop insulin resistance and type-2 diabetes. The mechanism involves several aspects of insulin signaling including FFA inhibition of insulin induced protein kinase B (Akt) activation and activation of PKC. Unsaturated or cis-fatty acids (OA and AA) have been demonstrated to directly activate protein kinase C (PKC) (*55, 56*). The mechanism appears to be due to an allosteric effect that increases V_{max}. Another mechanism for the increase in PKC activity seen in diabetic patients could be explained by our findings of PLC activation by FFA, since a product of PLC is DAG, a potent activator of many isoforms of PKC. PLCs have been found to be involved in the AA stimulated respiratory burst and calcium mobilization in human neutrophils (*8*). PLC activity increased after AA stimulation, and a PLC inhibitor blocked the respiratory burst induced by AA. The mechanism of PLC activation by AA was not addressed by this paper; however, it suggests that FFA can directly or indirectly activate poly-phosphoinositide hydrolysis.

We postulate that both FFAs as well as esterified fatty acids serve as important ligands for proteins. It has been shown that brain PIP_2 or PI from animal sources such as liver are able to induce dimerization and higher oligomerization of synuclein than PI from plant (*57*). PIP_2 and PI from animal sources have a higher content of AA than PI from plant. The authors go on to show that AA can also cause oligomerization. The ability of esterifed fatty acids to activate PLC δ1 awaits further study.

Our findings demonstrate that FFA can bind and regulate protein function in a specific manner. The EF-hand domain of PLC δ1 appears to represent a site for FFA binding. Further studies utilizing site-directed mutagenesis should better elucidate the structural determinants mediating FFA binding and activation of PLC δ1.

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