Essential Ca\(^{2+}\)-independent Role of the Group IVA Cytosolic Phospholipase A\(_2\) C2 Domain for Interfacial Activity*†‡§

Received for publication, February 7, 2003, and in revised form, March 31, 2003 Published, JBC Papers in Press, April 2, 2003, DOI 10.1074/jbc.M301386200

David A. Six‡ and Edward A. Dennis§
From the Department of Chemistry and Biochemistry and the School of Medicine, University of California, San Diego, La Jolla, California 92093-0601

The cytosolic Group IVA phospholipase A\(_2\) (GIVAPLA\(_2\)) translocates to intracellular membranes to catalyze the release of lysophospholipids and arachidonic acid. GIVAPLA\(_2\) translocation and subsequent activity is regulated by its Ca\(^{2+}\)-dependent phospholipid binding C2 domain. Phosphatidylinositol 4,5-bisphosphate (PI-4,5-P\(_2\)) also binds with high affinity and specificity to GIVAPLA\(_2\), facilitating membrane binding and activity. Herein, we demonstrate that GIVAPLA\(_2\) possessed full activity in the absence of Ca\(^{2+}\) when PI-4,5-P\(_2\) or phosphatidylinositol 3,4,5-trisphosphate were present. A point mutant, D43N, that is unable to bind Ca\(^{2+}\) also had full activity in the presence of PI-4,5-P\(_2\). However, when GIVAPLA\(_2\) was expressed without its Ca\(^{2+}\)-binding C2 domain (ΔC2), there was no interfacial activity. GIVAPLA\(_2\) and ΔC2 both had activity on monomeric lysophospholipids. ΔC2, but not the C2 domain alone, binds to phosphoinositides (PIP\(_n\)) in the same manner as the full-length GIVAPLA\(_2\), confirming the location of the PIP\(_n\) binding site as the GIVAPLA\(_2\) catalytic domain. Moreover, proposed PIP\(_n\)-binding residues in the catalytic domain (Lys\(_{488}\), Lys\(_{541}\), Lys\(_{543}\), and Lys\(_{544}\)) were confirmed to be essential for PI-4,5-P\(_2\)-dependent activity increases. Exploiting the effects of PI-4,5-P\(_2\), we have discovered that the C2 domain plays a critical role in the interfacial activity of GIVAPLA\(_2\) above and beyond its Ca\(^{2+}\)-dependent phospholipid binding.

The Group IVA phospholipase A\(_2\) (GIVAPLA\(_2\)) plays a central role in intracellular phospholipid hydrolysis. Although it is only one of many different mammalian phospholipase A\(_2\)s (1), it is the rate-limiting provider of lysophospholipid and the free polyunsaturated fatty acids such as arachidonic acid that go on to form platelet-activating factor and eicosanoids, respectively (2–5). These various downstream products are central to many physiological processes as well as many pathological conditions (6, 7).

The activity of GIVAPLA\(_2\) in mammalian cells is regulated by at least two major mechanisms that can act separately or in conjunction with each other. The first is by increasing intracellular Ca\(^{2+}\) concentrations, which leads to the translocation of GIVAPLA\(_2\) from the cytosol to its substrate phospholipids in the Golgi, ER, and nuclear membranes (8–10). This Ca\(^{2+}\)-dependent process is mediated by the C2 domain of GIVAPLA\(_2\), which binds two Ca\(^{2+}\) ions with a low micromolar affinity (11, 12). The increased [Ca\(^{2+}\)] that leads to translocation of the C2 domain to membranes also leads to membrane penetration of several hydrophobic side chains (13–16), which allows the catalytic α/β hydrolase domain to come into contact with its phosphatidylinositol substrate (17). The second major regulatory mechanism for GIVAPLA\(_2\) is through phosphorylation at one or more serines (18–20). It appears that phosphorylation leads to an activation of GIVAPLA\(_2\), by increasing the specific activity of the enzyme (18–20). Besides Ca\(^{2+}\) and phosphorylation, other factors, such as phosphoinositides (PIP\(_n\)), have been implicated in the regulation of GIVAPLA\(_2\) activity.

Early reports by Kojima and co-workers (21) and Leslie and Channon (22) on partially purified rat and mouse GIVAPLA\(_2\), respectively, indicated that several anionic lipids, especially polyphosphoinositides, increased the activity of GIVAPLA\(_2\). We showed with pure, recombinant human protein that GIVAPLA\(_2\) activity is generally enhanced by anionic phospholipids but specifically and more potently enhanced by PIP\(_n\)s, with phosphatidylinositol 4,5-bisphosphate (PI-4,5-P\(_2\)) being optimal (23). We further demonstrated that GIVAPLA\(_2\) binds in a 1:1 stoichiometry to PI-4,5-P\(_2\) with high affinity and specificity (23). In the presence of PI-4,5-P\(_2\) and the absence of Ca\(^{2+}\), GIVAPLA\(_2\) bound to phosphatidylinositol-containing surfaces and was active in vitro (23). Extending this work to cellular systems, we demonstrated that elevated levels of both phosphatidylinositol 4-phosphate (PI-4-P) and PI-4,5-P\(_2\) correlated with and were necessary for GIVAPLA\(_2\)-dependent arachidonate release by lipopolysaccharide-primed, UV light-activated P\(_388\)D1 murine macrophage-like cells (24, 25). Importantly, no change in intracellular [Ca\(^{2+}\)] was detected, further supporting the potential importance of the PIP\(_n\) effect (24).

There have now been several reports of GIVAPLA\(_2\) activity in vivo without any change in the resting levels of intracellular Ca\(^{2+}\) (5, 24–27).

In this study, we expand the understanding of how GIVAPLA\(_2\) may be regulated by Ca\(^{2+}\) and PIP\(_n\)s. We show that GIVAPLA\(_2\) has significant Ca\(^{2+}\)-independent activity in the
presence of many PIP₃s. We have shown that the Asp₄₃ → Asn mutation, which cannot bind Ca²⁺, also has full activity in the presence of PI-4,5-P₂. Whereas binding to Ca²⁺ is not necessary for GIVAPLA₂ activity, we now show that the presence of the C2 domain is required for all interfacial activity. This result demonstrates for the first time a second, novel role of the C2 domain, in that it is required to maintain GIVAPLA₂ in an active conformation or orientation at a membrane interface.

Finally, we have confirmed that the active site domain alone, and not the C2 domain, contains a functional PIP₃ binding site.

This site includes four lysine residues at positions 488, 541, 543, and 544.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine brain PI-4,5-P₂ and PI-4-P were from Roche Applied Science. Pure, native human GIVAPLA₂ (28), pure human serum 228 to alamine GIVAPLA₂ (5228A) (29), anti-Group IVA PLA₂ antibody (30), and a pALTER plasmid (Promega, Madison, WI) with the cDNA of His-tagged Group IVA PLA₂ (31) were generous gifts from Drs. Ruth Kramer and John Sharp (Lilly). Pure, recombinant human GIVAPLA₂ proteins (His₄₉-tagged wild type and the quadrupe Ser to Ala mutant at residues 437, 454, 505, and 727 (32)) were generous gifts from Dr. Michael Gell (University of Washington, Seattle). Pure, recombinant human GIVAPLA₂ proteins with the following single amino acid substitutions (K271Q, K273Q, K281Q, K282Q, and K283Q). The primers and conditions for generating the C2 domain construct are also detailed in the Supplementary Materials.

In order to generate the hydrolysis domain without the C2 domain (ΔC2), the pVL1393-GIVAPLA₂ vector was cut with XhoI as described in Ref. 13. After ligation to reform a single XhoI site, this new plasmid lacked its normal Met start codon; therefore, the next naturally occurring Met at position 134 became the start new codon. Thus, ΔC2 consists of residues 134–758 and contains the same C-terminal His₁₄ tag as the full-length enzyme.

**Group IVA PLA₂ Expression**—The protocols and reagents for generating recombinant GIVAPLA₂ in Spodoptera frugiperda (SF9) insect cells were from Pharmingen (BD Biosciences) unless otherwise indicated. In brief, SF9 insect cells from suspension culture (EX-CELL™ 400 with Gln) were plated and co-transfected with Baculogold linearized baculovirus DNA and baculovirus transfer vectors containing either His GIVAPLA₂, Δ43N, ΔC2, the C2 domain, or the seven other point mutants. In order to be certain to obtain the correct, pure protein, plaque assays were performed to clonally select and amplify one virus that was confirmed to express the active, folded, and correctly sized protein. The plaque assay was performed as indicated (Pharmingen BD Biosciences), but the plaques were more readily visualized with Neutral Red Dye (Sigma-Aldrich Co., St. Louis, MO). After a clonal virus was obtained and amplified, insect cell tissue culture plates (20 cm) were infected at a multiplicity of infection of < 1. Recombinant protein was harvested from the infected SF9 cells that were grown in TMN-FH insect cell medium.

GIVAPLA₂, Construct Purification—SF9 cells that had been infected with recombinant baculoviruses were pelleted and then lysed with Pharmingen’s insect cell lysis buffer on ice for 60 min and then centrifuged for 15 min at 4 °C at 16,000 × g to remove all unbroken cells and debris. This clarified lysate contained substantial levels of recombinant His GIVAPLA₂ (or control XYLE protein), such that the recombinant protein band was easily visible and distinguishable on an SDS-PAGE, followed by Coomassie-stained gel. Activity assays confirmed the high levels of expression.

Pure, recombinant protein was easily obtained from the clarified lysate using His tag affinity purification. The Talon system (Clontech/BD Biosciences) containing a Co²⁺ resin was successfully used according to instructions for batch adsorption purification. The recombinant, tagged proteins were eluted successfully from the Talon resin with imidazole elution buffer but not by low pH elution buffer. The lysate, washes, and all elutions were subject to SDS-PAGE followed by Coomassie staining. Each construct was also subject to a Western blot on Immobilon-P membranes and was easily detected with an anti-His tag antibody (data not shown) as a control for further use of the antibodies. Enzyme activity, which was confirmed in the presence of PIP₃s, was also substantially increased in the presence of PIP₃.

The mixed micelle substrate was prepared as described previously (23, 37). The micelles were initially made up in 20 mM HEPES and 100 mM KCl (200 μM CaCl₂, 1 mg/ml fatty acid free bovine serum albumin (BSA), and 1 mM dithiothreitol. The mixed micelles were composed of 1 mM PAPC (200,000 cpm) and 3 mM Triton X-100 (Microsomes), and were incubated at 30°C for 30 min before addition of the enzyme.

After micelle formation, the mixed micelle substrate was prepared as described previously (23, 37). The micelles were initially made up in 20 mM HEPES and 100 mM KCl (190 μM/assay) to form a cloudy white solution of multimamellar vesicles upon vortexing. The initial substrate buffer did not contain Ca²⁺ to avoid precipitating any phospholipid, especially the PIP₃s, and incorporated unlabeled Triton X-100 (5 μl of 150 μM per assay), resulting in a rapid clearing of the cloudy white vesicles as the clear mixed micelles form at a 2.5-fold higher concentration (2.5 mM PAPC and 7.5 μM Triton X-100) than that desired in the final assay mix. The mixed micelles were allowed to form over 30 min (with occasional vortexing) to ensure that all the phospholipids were released from the glass surface and incorporated into the micelles. The assay buffer (250 μl/assay) each were added to glass test tubes (16 × 125 mm). All of the Ca²⁺ or EGTA was found exclusively in the assay buffer. The tubes had been previously siliconized with Glassclad 18, to
help prevent the enzymes and substrates from adsorbing onto the glass surface. The mixed micelles in the tubes were then gently shaken in a 40 °C water bath for several minutes. The reaction was initiated by the addition of GIVAPLA2 (1 μg in 50 μl per sample of assay buffer that lacked Ca2+ and EGTA) followed by vortexing. This brought the final volume to 500 μl and the desired final concentrations as noted above. After a 60-min incubation, the reaction was quenched, and the fatty acid monomers were extracted using a modified Dole protocol (38) as previously described (23, 37). The final radioactive cpm were doubled in the calculation of specific activity because only 1 of 2 ml of heptane were counted. Background experiments where no enzyme was added were always performed and were subtracted from the data obtained with enzyme.

Standard PI-4,5-P2, PLA2, Activity Assay—This assay was identical to the standard PAPC PLA2 activity assay, with the exception that the mixed micelles contained 1 mol % PI-4,5-P2 (i.e. 0.96 mM PAPC (200,000 cpm), 0.04 mM PI-4,5-P2, and 3 mM Triton X-100) (23). Since PI-4,5-P2 enhances the activity of GIVAPLA2 by up to 120-fold, the amount of enzyme used was dropped to 0.1 μg, and the time was shortened to 10 min. The micelles were prepared in an identical manner to that described above. The enzyme and time of incubation were varied in all assays to achieve ~5% hydrolysis. This low level of hydrolysis ensured that the substrate-containing interface was not significantly perturbed by the hydrolysis products. The specific activities obtained in the standard assays vary, particularly with different sources of the GIVAPLA2 (native or His-tagged enzyme from our laboratory, Lilly, Dr. Gelb, or Dr. Cho); however, the ratio of the activity with PI-4,5-P2 to without PI-4,5-P2 is remarkably consistent at around 100-fold. In all experiments, a matched wild type control is compared with each mutant to control for the source of the enzymes and the tags they may contain.

Memoneric and Micellar Lyso-Phospholipase Activity Assay—The Lyso-PC monomer assay conditions, adapted from previous work (13, 39), were 20 mM HEPES (pH 7.5), 100 mM KCl, 200 μM Ca2+, 1 mg/ml BSA, and 1 mM dithiothreitol in a final volume of 50 μl. The monomer assay contained 4 μM Lyso-PC, which is below the critical micelle concentration of 7 μM (40). The substrate preparation was essentially identical to the above assays, except that the monomers were suspended in assay buffer (400 μl) without any Ca2+ or EGTA. The Ca2+ or EGTA (50 μl) was added directly to the assay tubes before the assay was initiated.

The assay was only begun after confirming the expected cpm per unit volume in the substrate solution. This was important because, in contrast to micelles and vesicles, there were no visual signs of fully solubilized monomers. The Dole procedure was used to extract the fatty acids exactly as described above, except that excess cholesterol palmitic acid (50 μg) was added to the quenched assay tube to enhance the extraction efficiency of the small amounts of pure, radiolabeled palmitic acid product (~0.03 μg). In the micellar form of this assay (above 7 μM Lyso-PC, the substrate concentration was increased up to 1 μM, and the radioactivity was adjusted to (where possible) to 200,000 cpm. For this higher substrate concentration, no unlabeled palmitic acid was needed in the assay workup. Unless otherwise indicated, all results are presented as the mean ± S.D. from a representative experiment with each condition tested in duplicate or triplicate.

Pip. Binding Assay—Pip. GIVAPLA2 binding was determined using Pip Strips from Echelon Research, Inc., and was carried out according to the provided protocols. A small plastic dish (~66 cm2) for each Pip Strip and a rocking platform kept at 4 °C were used for each of the following steps. The strips were blocked for 1 h in 15 ml of 10 mM Tris (pH 8.0), 150 mM NaCl, 0.1% Tween 20, and 3% BSA (TBS-T/BSA). The target protein (5 μg) was incubated with a strip overnight (12–18 h) in 10 ml of TBS-T/BSA. The strip was then washed with the standard washing protocol: three washes with 10 ml of TBS-T/BSA for 10 min. The washed strip was incubated for 30 min in 10 ml of TBS-T/BSA with 2.5 μl of GIVAPLA2-specific primary antibody (as provided by Lilly) or the His tag-specific antibody (0.2 μg/ml) TetraHis antibody from Qiagen). After washing, the strip was incubated for 30 min with 5 μl of the secondary antibody in 10 ml of TBS-T/BSA. For the anti-GIVAPLA2 primary antibody, the secondary antibody stock was a 1 ml solution of Protein A-HRP as provided by Amersham Biosciences. For the TetraHis primary antibody, the secondary antibody was 0.8 ng/ml goat anti-mouse HRP from Jackson ImmunoResearch Laboratories. In the standard washing, the strip was drained, but not dried, and incubated with premixed enhanced chemiluminescence reagents for several hours to coat the strip. The strip was placed between two plastic sheets, exposed to film, and developed to show spots where the target protein had bound.

FIG. 1. Pip. dependent, Ca2+ independent activity of GIVAPLA2 on mixed micelles. The activity of GIVAPLA2 was assayed on mixed micelles composed of Triton X-100 (3 mM), 1-palmitoyl-2-1[14C]arachidonoyl)-PC (0.96 mM, 200,000 cpm), and various Pip.s (0.04 mM, 1 mol %) as noted. The various non-Pip. phospholipids were tested at a higher surface concentration (0.2 mM, 5 mol %). White bars, assays performed with 200 μM Ca2+, gray bars, assays performed with 5 μM EGTA ([Ca2+] = 2 μM), PE, phosphatidylethanolamine; PS, phosphatidylserine; PG, phosphatidylglycerol; PA, phosphatic acid.

RESULTS

Ca2+ independent Activity of GIVAPLA2—In the absence of Ca2+, GIVAPLA2 is known to bind to and be active on lyso-phospholipid micelles (41), membrane interfaces containing PI-4,5-P2 (23), and membranes composed of the nonnatural anionic phospholipid, phosphatidylmethanol (32). We have previously shown that in the presence of Ca2+, Pip.s and Pip. gave larger enhancements of GIVAPLA2 activity than Pip.s, which in turn gave larger enhancements than all other anionic phospholipids tested (23). Herein we have successfully analyzed the activity enhancements of Pip.s on pure, human GIVAPLA2 in the absence of Ca2+. As expected, the presence of 5 mol % phosphatidylethanolamine, phosphatidyserine, phosphatidic acid, and phosphatidylinositol (PI) in PAPC/Triton X-100 mixed micelles did not yield any activity for native GIVAPLA2 in the absence of Ca2+ as seen in Fig. 1. However, in the presence of 1 mol % PI-3-P, PI-4-P, PI-3,4-P2, PI-4,5-P2, or Pip.s, there was significant GIVAPLA2 activity even in the absence of Ca2+ ([Ca2+] < 2 μM, Fig. 1, gray bars). Moreover, the activities with 1 mol % PI-4,5-P2 and Pip.s were similar to one another with and without Ca2+, and were both more than 100-fold higher than the control that lacked Pip.s but contained Ca2+. Importantly, PI-4-P and PI-3,4-P2 also gave very significant activity enhancements in the absence of Ca2+, relative to PAPC alone (with Ca2+). These GIVAPLA2 activities with Ca2+ were only 30 and 16% of the activities with Ca2+, respectively, but they are still far above the undetectable activity of GIVAPLA2 on PAPC without Ca2+ or Pip.s.

PI-4,5-P2 PLA2 Activity Assay with D43N and ΔC2—the addition of EGTA without any exogenous Ca2+ should have reduced the free [Ca2+] to extremely low levels ([Ca2+] < 2 μM). Nevertheless, we tested recombinant Ca2+ binding-deficient mutants of GIVAPLA2 to confirm that the PI-4,5-P2-GIVAPLA2 interaction can unambiguously replace the Ca2+-C2 domain interaction. A conceptual diagram of these various GIVAPLA2 mutant constructs is shown in Fig. 2.

Using the recombinant, pure D43N, ΔC2, and His GIVAPLA2, we measured activity in the PI-4,5-P2 activity assay with and without Ca2+. The results shown in Fig. 3 clearly demonstrate that in the presence of PI-4,5-P2, D43N has the same activity as His GIVAPLA2. As expected from its inability to bind Ca2+, the D43N activity remained the same with or
without Ca\(^{2+}\). The ΔC2 construct, however, did not have any activity with or without Ca\(^{2+}\), in striking contrast to the D43N activity.

**PAPC PLA\(_2\) Activity Assay with D43N and ΔC2**—Given that the activity of D43N is comparable with His GIVAPLA\(_2\) in the presence of PI-4,5-P\(_2\), it was critical to test D43N without PI-4,5-P\(_2\). As shown in Fig. 4, D43N, ΔC2, and a mixture of ΔC2 and the C2 domain all have no significant activity above base line on PAPC/Triton X-100 mixed micelles in the presence of Ca\(^{2+}\). D43N and ΔC2 were not expected to have any activity in these Ca\(^{2+}\)-dependent assay conditions based on previous reports (13, 42). No activity was seen under these conditions for any His GIVAPLA\(_2\) construct in the absence of both PI-4,5-P\(_2\) and Ca\(^{2+}\) (data not shown).

**Micellar and Monomeric Lysophospholipase Activity of D43N, ΔC2, and GIVAPLA\(_2\)**—Previously, we (41) and others (39) had shown that GIVAPLA\(_2\) has Ca\(^{2+}\)-independent Lyso-PLA activity on pure micelles of 1-palmitoyl-Lyso-PC. We measured the activity of D43N and ΔC2 on Lyso-PC micelles in the absence of PI-4,5-P\(_2\). As shown in Fig. 5A, His GIVAPLA\(_2\) and D43N both have high activity on 100 μM Lyso-PC (relative to ΔC2 and background) at 50 and 15 nmol/min/mg enzyme, respectively. For His GIVAPLA\(_2\) and D43N, there was no difference in activity in the presence or absence of Ca\(^{2+}\) (data not shown). The activity of ΔC2 on Lyso-PC micelles was dramatically lower than His GIVAPLA\(_2\) and D43N, indicating that the C2 domain is required for activity on Lyso-PC micelles (Fig. 5A) as well as on PI-4,5-P\(_2\)/PAPC/Triton X-100 mixed micelles (Fig. 3).

The lack of interfacial PLA\(_2\) or Lyso-PLA activity for ΔC2 might have indicated that it was incapable of all catalytic activity. Previous studies had shown that ΔC2 lacked the ability to bind to and hydrolyze membranes with or without Ca\(^{2+}\) in all tested systems (13, 16). It was also shown, however, that both full-length GIVAPLA\(_2\) and the ΔC2 construct possessed Lyso-PLA activity on monomeric (nonaggregated) substrate (13), since the catalytic residues are all located on this domain (Fig. 2). In order to confirm that ΔC2 behaved as previously reported, a monomer assay was utilized with 4 μM Lyso-PC substrate. As shown in Fig. 5B, His GIVAPLA\(_2\), D43N, and ΔC2 all have significant activity on monomeric Lyso-PC substrate (relative to background and the limit of detection), indicating their active sites are folded properly. Compared with their monomer Lyso-PLA activity (Fig. 5B), the activities of His GIVAPLA\(_2\) and D43N on micellar Lyso-PC substrate were dra-
matically higher at 100 μM (Fig. 5A, 35- and 12-fold, respectively), indicating competent interfacial activity. However, whereas ΔC2 showed a steady increase in activity up to the critical micelle concentration (0.5 nmol/min/mg at ~7 μM), there was no further increase up to 100 μM Lyso-PC (Fig. 5, A and B, and data not shown). In fact, monomer activity accounts for all of the activity (0.54 ± 0.04 nmol/min/mg) seen for ΔC2 in Fig. 5A.

The apparent lower activity of ΔC2 versus His GIVAPLA2 and D43N at 4 μM Lyso-PC is consistent with premicellar aggregation induced by these enzymes. Premicellar aggregation would result in the creation of an interface that could be more efficiently hydrolyzed by the interfacially competent GIVAPLA2 and D43N, but not ΔC2. Whereas the monomer activity of ΔC2 is somewhat lower than the other two full-length enzymes, the hydrolysis measured (0.4 nmol/min/mg) was well above the detection limit at these conditions of 0.075 nmol/min/mg. Some questions have been raised about whether the monomeric Lyso-PLA activity of GIVAPLA2 is due to the same active site as the PLA2 activity. To address this concern, we tested the full-length, active site mutant, S228A. The Ser mutant had no monomeric Lyso-PLA activity. Under the conditions tested, an activity as low as 0.1 nmol/min/mg could have been detected for S228A. The lack of all types of activity by S228A confirms that all known GIVAPLA2 catalytic activities depend on Ser228 (29).

**Binding of GIVAPLA2 Constructs to Immobilized Phospholipids**—To determine which domain(s) of GIVAPLA2 binds to PIP₃, an overlay blot was performed using nitrocellulose-immobilized PIP₃ on a PIP Strip. The PIP Strips have 100-pmol spots of various natural and synthetic phospholipids affixed to them. This includes synthetic dipalmitoyl compounds of all possible PIP₃s. The PIP Strips have been used to study the PIP₃-binding interactions of various PIP₂ antibodies (Echelon Research, Inc.) and PIP₂ proteins (43–45), including phospholipase C (46). The standard PIP Strip binding protocol calls for a 12–16-h incubation with 5 μg of target protein. When this was carried out, no binding was detected for GIVAPLA2 (Fig. 6). Theoretically, GIVAPLA2 is able to sequentially hydrolyze the sn-2 fatty acyl chain of a phospholipid, followed by the sn-1 fatty acyl chain of the resulting lysophospholipid (39, 47). If this occurred on the PIP Strips, the polar head groups would have been released from the surface (along with any bound GIVAPLA₂), eliminating the chemiluminescence signal.

In order to address the possible hydrolysis of the PIP₃s from the PIP Strips, we utilized two complementary techniques. First, GIVAPLA2 was preincubated with an excess of an irreversible serine-dependent PLA₂ inhibitor, MAPF. After incubation of MAPF-inhibited GIVAPLA2 with the PIP Strips and probing with anti-GIVAPLA2 antibody, there was significant signal at several PIP₃ spots, as seen in Fig. 6. Second, to rule out any effects of the MAPF, S228A was used. The results for S228A did not differ from those seen with MAPF-treated GIVAPLA2 (data not shown). The location and identity of each spot on the PIP Strips is shown in a schematic diagram in Fig. 6.

The identity of the spots indicated that under these conditions GIVAPLA2 bound PI-3-P, PI-4-P, and phosphatidylinositol 5-phosphate to give a consistently intense signal in every condition tested. Signal was also detected with PI-4,5-P₂, PI-3,4-P₃, phosphatidylinositol 3,5-bisphosphate, and occasionally weak signal with other anionic lipids, PIP₂, PI, phosphatic acid, and phosphatidylserine. No signal was detected for phosphatidylethanolamine, phosphatidylcholine, or inositol 1,3,4,5-tetrakisphosphate. The same results were obtained for His

![Fig. 6](image_url)

**Fig. 6. Binding of GIVAPLA₂ and various mutants to PIP₃s in a protein-phospholipid overlay blot.** Binding of various GIVAPLA₂ constructs was tested in protein-phospholipid overlay blots (PIP Strips). The PIP Strips were prespotted with 100 pmol of the indicated phospholipids. Each strip was incubated with 5 μg of GIVAPLA₂, MAPF-treated GIVAPLA₂, ΔC2, or the C2 domain and probed with an anti-GIVAPLA₂ antibody or anti-His tag antibody followed by an HRP-conjugated secondary antibody. The location of each phospholipid is shown in the schematic diagram. The blots correspond to the specific proteins as indicated under each blot. PE, phosphatidylethanolamine; PS, phosphatidylserine; FG, phosphatidylglycerol; PA, phosphatic acid; PI-5-P, phosphatidylinositol 5-phosphate; IP₃, inositol 1,3,4,5-tetrakisphosphate.

GIVAPLA2 with an anti-GIVAPLA2 antibody or the TetraHis antibody (data not shown).

To test which domain was able to bind to PIP₃, ΔC2 and the C2 domain were preincubated with MAPF, incubated with PIP Strips, and probed with the TetraHis antibody. The results clearly indicated that ΔC2, but not the C2 domain, showed the same signal as the MAPF-treated GIVAPLA2 (Fig. 6). In addition, D43N was tested in the same manner and found to match the results seen for the MAPF-treated GIVAPLA2 (data not shown). Although it was crucial for native and His GIVAPLA2, MAPF was not necessary and did not interfere with the results for ΔC2 and the C2 domain. No effect of Ca²⁺ was seen for any construct tested.

The PIP Strip assay was used primarily to compare the ability of different GIVAPLA₂ constructs to bind to PIP₃. For this qualitative comparison among the various constructs, this assay was efficient and straightforward. However, it cannot be used as a quantitative measure of strength of binding or specificity of binding for any protein. There are several reasons for this limitation, including the nonphysiological density of negative charges from the various PIP₃s (especially PIP₃) in the pure spots and the improbable 30-Å² surface area for each polar head group. This surface density calculation would strongly suggest that the phospholipids on the PIP Strips were not arranged as a canonical monolayer on the PIP Strips.

Another factor to consider is the effect of the nonbinding
portions of each protein. Many PIP₃-binding proteins, such as pleckstrin homology domains and antibodies, generally have single phospholipid binding sites in various surface-exposed loops (48, 49). Some of these proteins like pleckstrin homology domains also bind tightly to the soluble head groups, such as inositol 1,4,5-trisphosphate (50). GIVAPLA₂ is different, because it does not bind inositol 1,4,5-trisphosphate, but instead binds to PIP₃s only in an interface (23). Moreover, unlike pleckstrin homology domains, GIVAPLA₂ has at least three membrane binding attachment points: the active site, the C2 domain, and the PIP₂ binding site. When GIVAPLA₂ binds to the PIP Strips, it is conceivable that one or both of the other binding sites are brought in close proximity to the surface, raising the possibilities for steric or electrostatic repulsion. These possibilities might be exacerbated on the PIP Strips by the lack of lateral mobility of the PIP₃s and would be most significant with PIP₃.

Confirmation of the Key Residues for the PIP₃ Interaction—Recent work of Das and Cho (33) indicated using mutagenesis that several basic residues were directly involved in the binding and activity enhancements from PI-4,5-P₃. The two mutants, K488E and K541A/K543A/K544A, were assayed on phospholipid vesicles under conditions where GIVAPLA₂ activity with 5 mol % PI-4,5-P₃ was up to 3.5-fold higher than without (33). Both K488E and K541A/K543A/K544A had increased basal activity (4- and 2-fold, respectively) (33). Interestingly, both of these mutants had no increase in activity with up to 5 mol % PI-4,5-P₃ but rather each had a slight decrease (33). Because of the small enhancements from PI-4,5-P₃ in these assay conditions, complicated by the mutants’ enhanced basal activity, we undertook to confirm the results in assay conditions that maximize the level of enhancement from PI-4,5-P₃ (100-fold or greater) (23).

We tested the basal and PI-4,5-P₃-stimulated activity of K488E and K541A/K543A/K544A in the standard PAPC and standard PI-4,5-P₃ assays. Our results showed that the basal activity increased ~4-fold for both (Fig. 7), which qualitatively agrees with the previous report (33). The results in the presence of only 1 mol % PI-4,5-P₃ showed that K488E and K541A/K543A/K544A activities increased only 15–60%, compared with ~50-fold for the WT control (Fig. 7). These results qualitatively match those seen previously (33). Thus, we have confirmed that these residues appear to be crucial for the GIVAPLA₂-PIP₃ interaction.

Ruling Out Proposed PIP₃-binding Residues—In a previous report (23), we hypothesized that the PIP₃-binding site of GIVAPLA₂ might be located at a string of basic residues between amino acids 271 and 283. We have now mutated all of those basic side chains to remove the charges or functional groups. The mutations included K271Q, K273Q, R274Q, S278A, K281Q, K282Q, and K283Q. The resulting mutant proteins had no defects in basal or PI-4,5-P₃-enhanced activity (data not shown) and thus gave around the same 100-fold increase with 1 mol % PI-4,5-P₃ as seen for WT GIVAPLA₂ in the mixed micelle system (23). The results are consistent with results for a triple mutant K271A/K273A/R274A that was recently shown to have no effects on the basal or PI-4,5-P₃-stimulated activity (33).

Investigating Effects of GIVAPLA₂ Phosphorylation on PIP₃ Activation—To rule out any complex interaction between PIP₃ effects and GIVAPLA₂ phosphorylation, we tested a quadruple mutant, S437A/S455A/S505A/S727A, that was not phosphorylated versus a matched His GIVAPLA₂ control that was partially phosphorylated naturally by the expression system (19). We found that in the standard PAPC assay, the quadruple mutants had about 25% of the specific activity of the control His GIVAPLA₂ (Fig. 8). This 4-fold higher activity of the phosphorylated His enzyme versus the quadruple mutant is consistent with the 3–4-fold higher activity previously seen between phosphorylated GIVAPLA₂ versus less phosphorylated GIVAPLA₂ (18, 19, 51). We also found that in the standard PI-4,5-P₃ assay, the quadruple mutant had about 25% of the specific activity of the control His GIVAPLA₂ (Fig. 8). Therefore, the control His GIVAPLA₂ and the quadruple mutant activities were both stimulated by around 100-fold in the presence of 1 mol % PI-4,5-P₃. This indicated no positive or negative interaction between PIP₃-stimulation and GIVAPLA₂ phosphorylation at these four residues, which are normally partially phosphorylated during their cellular expression (36). One other phosphorylation site (Ser⁵¹⁵) was recently identified (20). This fifth potential phosphorylation target was not phosphorylated in the His GIVAPLA₂ control used in Fig. 8, based on previous detailed research of the same protein (36) and thus is also apparently uninvolved in the effects of PI-4,5-P₃. Importantly, these results suggest that phosphorylation and levels or availability of PIP₃s can coordinate the increase of the activity of GIVAPLA₂ (Fig. 8, S228A activity without PI-4,5-P₃ versus GIVAPLA₂ activity with PI-4,5-P₃).

Basal and PI-4,5-P₃-enhanced GIVAPLA₂ Activity Highly Depend on the Quality of the Interface—The high Lyso-PLA activity of GIVAPLA₂ is a useful feature of GIVAPLA₂ that we have more fully explored. In Triton X-100 mixed micelles, the large GIVAPLA₂ activity enhancements from PI-4,5-P₃ were seen when the substrate was either PAPC or Lyso-PC, as shown in Fig. 9A. For GIVAPLA₂, the Lyso-PLA activity on PI-4,5-P₃/Lyso-PC/Triton X-100 mixed micelles was Ca²⁺-inde-
1. Just as for the PLA2 activity, the Lyso-PLA activity without Ca2+ contrast to the Lyso-PC micelle system that showed no Ca2+-dependent activity. In this system, the substrate was 1-palmitoyl-2-(1-[14C]arachidonoyl)-PC (1 mM (white bars) or 0.96 mM (gray bars), 200,000 cpm). For Lyso-PLA activity (right four bars), the substrate was 1-(1-[14C]palmitoyl)-Lyso-PC (1 mM (white bars) or 0.96 mM (gray bars), 200,000 cpm). The nonsubstrate phospholipid, PI-4,5-P2, was incorporated into half of the micelles (0.04 mM, 1 mol %, gray bars). The left two bars for each substrate represent assays performed with 200 μM Ca2+, whereas the right two bars represent assays performed with 500 μM EGTA ([Ca2+] < 2 mM). B, the activity of GIVAPLA2 was assayed on pure micelles composed of 1-(1-[14C]palmitoyl)-Lyso-PC. In the white bars are Lyso-PC micelles (1 mM, 200,000 cpm), and in the gray bars the Lyso-PC micelles (0.96 mM, 200,000 cpm) were supplemented with PI-4,5-P2 (0.4 mM, 4 mol %). The left two bars represent assays performed with 200 μM Ca2+, whereas the right two bars represent assays performed with 500 μM EGTA ([Ca2+] < 2 mM).

The addition of 3 mM Triton X-100 to 1 mM Lyso-PC should have had a surface dilution effect on the activity of GIVAPLA2, reducing the activity 4-fold (56, 57). Indeed, in the presence of Ca2+ and absence of PI-4,5-P2, the activity decreases by about 6.5-fold with the dilution of Triton X-100 (Fig. 9, B versus A, white bars). However, when PI-4,5-P2 was incorporated into the Lyso-PC micelles, the expected 4-fold decrease from Triton X-100 dilution was instead a 4-fold increase (Fig. 9, B versus A, gray bars). This indicates that in the presence of Ca2+, the PI-4,5-P2 effects on GIVAPLA2 Lyso-PLA activity depend on the quality of the interface.

The same surface dilution effects on Lyso-PLA activity should be seen in the absence of Ca2+ (Fig. 9, A and B, gray bars). However, without PI-4,5-P2, the 4-fold dilution with Triton X-100 gives a 26-fold reduction of activity. This indicates that in the absence of Ca2+, Lyso-PLA activity also strongly depends on the quality of the interface. Finally, in the presence of PI-4,5-P2 with no Ca2+, the dilution by Triton X-100 gives a 1.4-fold increase in contrast to the anticipated 4-fold decrease. These results confirm that the quality of the interface is also important for Ca2+-independent, PI-4,5-P2-dependent activity. Together, these results indicate that the nature or quality of the interface, be it detergent mixed micelles, lysophospholipid micelles, or small unilamellar vesicles, can dramatically impact the activity of GIVAPLA2 as well as the effects of Ca2+ and PI-4,5-P2 on its activity. By taking advantage of the effects of Ca2+, PI-4,5-P2, and the quality of interface, a specific assay for GIVAPLA2 was developed (58) that can distinguish this enzyme from all known mammalian PLA2s. The specific assay is particularly useful to distinguish GIVAPLA2 activity in samples of crude tissue homogenates or cellular preparations from mouse, rat, and human sources (58).
GIVAPLA₂ is normally found evenly distributed throughout the cytosol, whereas its substrate phospholipids are in the intracellular membranes such as ER, Golgi, and nuclear envelope (10). It is well accepted that the translocation of GIVAPLA₂ to its substrate membranes can be regulated by intracellular [Ca²⁺] (see Ref. 10 and references therein). Whereas the effects of Ca²⁺ are mediated through the C2 domain, we have now shown that the C2 domain of GIVAPLA₂ is apparently required for Ca²⁺-independent interfacial activity. This suggests a novel second role for the C2 domain in the Ca²⁺-independent activation of the catalytic domain. This second role may also be important for Ca²⁺-dependent interfacial activity but would be obscured by the primary, Ca²⁺-dependent membrane binding role of the C2 domain.

Recently, evidence has accumulated for the translocation and activation of GIVAPLA₂ without a corresponding rise in intracellular [Ca²⁺] (5, 24–27). Along with our previous results (23), the results presented herein further strengthen the notion that PI-4,5-P₂, and possibly other PIP₃s, may have an analogous role to Ca²⁺ in increasing the membrane affinity of GIVAPLA₂. This membrane affinity increase would facilitate increased activity by bringing enzyme and substrate together.

Nonplasma membrane PIP₃s are synthesized in situ (e.g. nuclear envelope, Golgi, and ER membranes) and have been implicated in a wide variety of functions separate from the plasma membrane (59–62). The Ca²⁺-independent activity for GIVAPLA₂ seen in vitro at 1 mol % PI-4,5-P₂ is a physiologically relevant surface concentration for many cell membranes (63). More recently, significant levels of PI-4,5-P₂ have been visually identified in various intracellular membranes such as Golgi, ER, and cytosolic nuclear envelope in astrocytoma and squamous carcinoma cells (61) and at the cytosolic perinuclear membranes of HEK293 cells (33). In the HEK293 cells, the perinuclear IP₁-4,5-P₂ matched the localization seen separately for GIVAPLA₂ in the same cells (33). Co-transfection of a PI-4,5-P₂-binding protein and GIVAPLA₂ partly reduced the GIVAPLA₂-dependent arachidonic acid release (33), indicating that there may indeed be a physiological interaction between GIVAPLA₂ and PI-4,5-P₂.

Herein we report that GIVAPLA₂ can have its highest activity on PI-4,5-P₂ or PIP₃-containing mixed micelles with or without Ca²⁺. Along with PI-4,5-P₂ (23) and Lyso-PC (39, 41), PIP₃ is shown to be a third physiologically relevant lipid that can lead to full Ca²⁺-independent GIVAPLA₂ activity in vitro. However, PI-4,5-P₂, as the much more abundant precursor of PIP₃ (63, 64), is probably more relevant than PIP₃. In addition to PI-4,5-P₂ and PIP₃, PI-3,4-P₂ and PI-4-P also gave significant, but not maximal, Ca²⁺-independent activity. Since PI-4-P is the most abundant of the PIP₃ species (24, 63), it is possible that it too could significantly contribute to the activity of GIVAPLA₂ in vivo. The up-regulation of PI-4-P levels, and subsequently PI-4,5-P₂ levels, was observed in murine P388D₁ macrophage-like cells primed by lipopolysaccharide and activated by UV light (24). Under these conditions, GIVAPLA₂ specifically acted to release arachidonic acid without any changes in intracellular [Ca²⁺] (24). The increased levels of PIP₃ seen in the P388D₁ cells may have been generated at the intracellular membranes to which GIVAPLA₂ targets (10), as one or more PI kinases have been observed to localize to those membranes (60, 65).

The full activity of D43N in PI-4,5-P₂ mixed micelles and high activity toward Lyso-PC micelles unambiguously confirmed that native and His GIVAPLA₂ can have full activity in the absence of Ca²⁺. Whereas Ca²⁺ may be dispensable for GIVAPLA₂ activity, the C2 domain is not. The ΔC2 construct did not have any PLA₂ or Lyso-PLA activity on interfacial substrates with or without Ca²⁺, PI-4,5-P₂, or both. The monomer Lyso-PLA activity of ΔC2 indicated that the catalytic site was functional but lacked any activity on aggregated substrates. In an attempt to rescue the defect in ΔC2, free C2 domain was added, creating a 1:1 ratio between the C2 domain and ΔC2. This mixture did not have any activity (with or without PI-4,5-P₂), meaning that the two separate domains did not interact with each other to form an interfacially competent enzyme. These results indicate for the first time that an intact C2 domain is required for GIVAPLA₂ interfacial activity regardless of its capacity to bind Ca²⁺.

PIP Strip binding assays were employed to determine whether the C2 domain or the ΔC2 construct (or both) contained the PIP₃ binding site. The PIP₃-binding pattern seen in the PIP Strip assay for the MAEP-inhibited native GIVAPLA₂, His GIVAPLA₂, S228A, and D43N all matched ΔC2, whereas the C2 domain showed no PIP₃ binding. This suggested that the active site domain, but not the C2 domain, binds PIP₃s. These results complement the recent finding that a specific GIVAPLA₂ PIP₃ binding site is located on the active site domain (33). We have confirmed these results with our own assay systems and concluded that the PI-4,5-P₂ binding site residues probably include Lys₄₈₈, Lys₄₆₁, Lys₄₄₃, and Lys₄₄₄, which are indeed located in the ΔC2 construct (33). Previous results suggest that the catalytic domain may also bind to phosphatidylinositol methanol vesicles in a Ca²⁺-dependent manner (32), perhaps through the PIP₃ binding site. Interestingly, these four residues are identical in all vertebrate GIVAPLA₂ orthologs but not in its paralogs, GIVBPLA₂ or GIVCPLA₂. We have found that these two paralogs are not activated by PI-4,5-P₂, in contrast to GIVAPLA₂ (66). The location of the PIP₃ binding site on the GIVAPLA₂ catalytic domain contrasts to the recently identified PI-4,5-P₂ binding site in the C2 domain of protein kinase Cα (67).

Although the C2 domain may not be required for PIP₃ binding, it seems to be necessary for a catalytically competent interfacial enzyme, perhaps by facilitating an interdomain conformational activation or active site orientation. One other possibility is that various groups of C2 domain residues are separately involved in PIP₃ binding, Lyso-PC binding, and the traditional Ca²⁺-dependent membrane binding. This seems less likely given the presence of the key PIP₃-binding residues on the catalytic domain and the ability of the catalytic domain itself to bind to PIP₃s on PIP Strips. The C2 domain has previously been shown to possess some Ca²⁺-independent membrane affinity (32, 68), which, while weak, could help explain the critical role of the C2 domain in interfacial activity.

Several others reports have shown that PIP₃s, including PI-4,5-P₂, enhance the activity of GIVAPLA₂ by less than 10-fold (21, 22, 33, 55). At first glance, this seems to contradict our reports that PI-4,5-P₂ enhances the activity of GIVAPLA₂ in large unilamellar vesicles composed of PAPC by 20-fold (1 mol %) or 55-fold (3 mol %) and in PAPC/Triton X-100 mixed micelles by up to 120-fold (1 mol %) (23). A likely explanation is that when PI-4,5-P₂ is added to assay systems that have low activity, the enhancing effects are striking, as in Triton X-100 mixed micelles (23). When PI-4,5-P₂ is added to assay systems that already have high activity, the enhancing effects of PI-4,5-P₂ may appear muted as in Lyso-PC vesicles (Fig. 9B) and small unilamellar vesicles (21, 22, 33, 55). Nevertheless, despite the high basal levels of activity seen in these reported assays, the additions of small amounts of PI-4,5-P₂ led to reliable, although modest, 4–6-fold activity enhancements as in Fig. 9B and Refs. 22, 33, and 55.

Interestingly, the enhancing effects of PI-4,5-P₂ are not mim-
icked by the soluble head group, inositol 1,4,5-trisphosphate (23), indicating a membrane-dependent binding interaction. The PIP2-binding interaction also seems to induce an apparent activation by unknown means, perhaps through a specific orientation or conformational change. This putative PIP2-dependent activation would be in addition to the increased membrane affinity of GIVAPLA2 seen in the presence of PIP2,4,5-P3 (23).

This suggests that in addition to the analogous role of PIP2 to Ca2+ (membrane binding), there is a second analogous role of PIP2 to phosphorylation (activation or a higher specific affinity). Whether a PIP2-dependent apparent activation is related to the newly discovered structural requirement of the C2 domain for interfacial activity is an interesting question. The putative structural requirement of the C2 domain and apparent activation of GIVAPLA2 by PIP2,4,5-P3, as indicated in this study and other studies (17, 33) will require significant dynamics and structural studies to more fully elucidate the molecular regulation of GIVAPLA2 activity and apparent activation.

Acknowledgments—We are grateful to Drs. Ruth Kramer and John Sharp for the GIVAPLA2 proteins, antibodies, and DNA construct. We thank the skillful help of Wonhwa Cho for providing samples of GIVAPLA2 WT, K488E, and K454A/K544A proteins. We are grateful to Dr. Michael Gelb for providing a sample of GIVAPLA2 WT and S437A/S505A/S727A mutants. We are grateful to Dr. Ching-Shih Chen for providing the PI-PLC-I, PI-PLC-II, and PI-PLC-III used in these experiments. We thank Dr. Marilyn Martin and Raymond Deenius for many fruitful discussions and Drs. Wonhwa Cho, Michelle Winestead, and Tina Johnson for helpful suggestions during the preparation of the manuscript.

References