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Essential Ca^{2+}-independent Role of the Group IVA Cytosolic Phospholipase A_2 C2 Domain for Interfacial Activity*[S]

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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$_n$s) 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$_2$)[1] plays a central role in intracellular phospholipid hydrolysis. Although it is only one of many different mammalian phospholipase A_2s (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 phosphatidylcholine 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$_n$s), 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$_2$ both bound to phosphatidylcholine-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 P388D$_1$ 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

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[1] The abbreviations used are: GIVAPLA$_2$, the wild type (749-amino acid) Group IVA phospholipase A_2 (cytosolic phospholipase A_2 α); PLA$_2$, phospholipase A_2; BSA, fatty acid-free bovine serum albumin; ΔC2, α/β hydrolase domain of GIVAPLA$_2$ (amino acids 134–756, including C-terminal His tag); His GIVAPLA$_2$, GIVAPLA$_2$ with a C-terminal extension of one Tyr and six His residues; HRP, horseradish peroxidase; Lyso-PC, 1-palmitoyl-L-lyso-3-phosphatidylcholine; Lyso-PLA, lysophospholipase; MAFP, methyl arachidonyl fluorophosphonate; PAPC, 1-palmitoyl-2-arachidonoyl-phosphatidylcholine; PI, phosphatidylinositol; PIP, PI monophosphate; PIP$_2$, PI bisphosphate; PIP$_3$, PI 3,4,5-trisphosphate; PI-4,5-P$_2$, PI 4,5-bisphosphate; PI-4-P, PI 4-phosphate; PI-3-P, PI 3-phosphate; PI-3,4-P$_2$, PI 3,4-bisphosphate; PIP$_n$, phosphoinositide; TBS-T/BSA, Tris-buffered saline-Tween 20/BSA solution; WT, wild type.

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presence of many PIP$_n$s. We have shown that the Asp^{43} → Asn mutant that cannot bind Ca^{2+} also has full activity in the presence of PI-4,5-P$_2$. Whereas binding to Ca^{2+} is not necessary for GIVAPLA$_2$ 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 GIVAPLA$_2$ 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 PIP$_n$ binding site. This site includes four lysine residues at positions 488, 541, 543, and 544.

EXPERIMENTAL PROCEDURES

Materials—Bovine brain PI-4,5-P$_2$ and PI-4-P were from Roche Applied Science. Pure, native human GIVAPLA$_2$ (28), pure human serine 228 to alanine GIVAPLA$_2$ (S228A) (29), anti-Group IVA PLA$_2$ antibody (30), and a pALTER plasmid (Promega, Madison, WI) with the cDNA of His-tagged Group IVA PLA$_2$ (31) were generous gifts from Drs. Ruth Kramer and John Sharp (Lilly). Pure, recombinant human GIVAPLA$_2$ proteins (His$_6$-tagged wild type and the quadruple Ser to Ala mutant at residues 437, 454, 505, and 727 (32)) were generous gifts from Dr. Michael Gelb (University of Washington, Seattle). Pure, recombinant human GIVAPLA$_2$ proteins (His$_6$-tagged wild type and the two mutants, K488E and K541A/K543A/K544A (33)) were generous gifts of Dr. Wonhwa Cho (University of Illinois, Chicago). Radiolabeled L-α-1-palmitoyl-2-([1-^{14}C]arachidonoyl) phosphatidylcholine (PAPC) and 1-[1-^{14}C]palmitoyl-L-Lyso-3-phosphatidylcholine (Lyso-PC) were provided by PerkinElmer Life Sciences. Dipalmitoyl PI-3-P, PI-3,4-P$_2$, and phosphatidylinositol 3,4,5-trisphosphate (PIP$_3$) were a generous gift from Dr. Ching-Shih Chen (The Ohio State University, Columbus, OH). All other cold phospholipids were from Avanti Polar Lipids Inc. (Alabaster, AL). PIP Strips™ were from Echelon Research, Inc. (Salt Lake City, UT). Glassclad 18 silanizing agent was from United Chemical Technologies, Inc. (Bristol, PA). Biosafe II liquid scintillation mixture was from RPI Corp. (Mount Prospect, IL). Oligonucleotide primers were from Proligo (La Jolla, CA). *Pfu*Turbo DNA polymerase was from Stratagene (La Jolla, CA). *Eco*RI, *Xba*I, and *Dpn*I endonucleases were from Invitrogen. *Bgl*II was from Amersham Biosciences. *Nde*I was from New England Biolabs (Beverly, MA). Baculogold baculovirus system (including the pVL1393 and pAcHLT-A plasmids), TNM-FH insect cell media, and Talon Co^{2+} affinity resin were from BD Biosciences (San Diego, CA). EX-CELL™ 400 (with Gln) protein-free insect cell medium was from JRH Biosciences (Lenexa, KS). Methyl arachidonoyl fluorophosphonate (MAFP) was from Cayman Chemical Co. (Ann Arbor, MI). Neutral Red dye solution, Triton X-100, Tween 20, buffers, and other salts were from Sigma. Goat anti-mouse horseradish peroxidase (HRP) was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). TetraHis antibody was from Qiagen (Valencia, CA). Enhanced Chemiluminescence kits and Protein A-HRP were from Amersham Biosciences. Immobilon-P membrane was purchased from Millipore Corp. (Bedford, MA).

Group IVA PLA$_2$ Cloning and Mutagenesis—A pALTER vector containing the cDNA of human GIVAPLA$_2$ with a C-terminal His tag extension (abbreviated as His GIVAPLA$_2$ to differentiate it from the 749-amino acid native GIVAPLA$_2$) was transferred into a baculovirus transfer vector pVL1393 in the proper orientation by double digesting the pALTER-His-GIVAPLA$_2$ with *Eco*RI and *Bgl*II, isolating the GIVAPLA$_2$ insert, and ligating it into the precut pVL1393 plasmid. GIVAPLA$_2$ has been almost exclusively expressed in insect cell expression systems in very good yield (28, 34–36). This plasmid was used for protein expression (see below).

Mutagenesis was carried out in the pALTER vector before transfer to an expression vector. The bidentate Ca^{2+}-binding ligand, Asp^{43}, was mutated into an Asn residue (D43N) using the QuikChange™ site-directed mutagenesis protocol (Stratagene) with matched mutagenesis primers (see Supplementary Materials for complete sequences and conditions). A putative PIP$_n$-binding site proposed based on striking biochemical similarities and loose sequence homology to pleckstrin homology domains (23) was probed with seven other mutants made with the same method as above. The seven mutants were K271Q, K273Q, R274Q, S278A, K281Q, K282Q, and K283Q. The primers and conditions for generating these seven mutants are detailed in the Supplementary Materials. All mutations were confirmed by DNA sequencing.

In order to generate the isolated C2 domain, two different mutant primers were used to amplify the C2 domain cDNA, add a new stop codon, and create *Nde*I and *Bgl*II restriction sites to allow it to be directionally subcloned into a His tag-containing vector, pAcHLT-A. The final C2 domain construct contained amino acids 12–140 of GIVAPLA$_2$ along with an N-terminal His$_6$ tag and thrombin cleavage site. The primers and conditions for generating the C2 domain construct are also detailed in the Supplementary Materials.

In order to generate the hydrolase domain without the C2 domain (ΔC2), the pVL1393-GIVAPLA$_2$ vector was cut with *Xba*I as described in Ref. 13. After ligation to reform a single *Xba*I site, this new plasmid lacked its normal Met start codon; therefore, the next naturally occurring Met at position 134 became the new start codon. Thus, ΔC2 consists of residues 134–756 and contains the same C-terminal His$_6$ tag as the full-length enzyme.

Group IVA PLA$_2$ Expression—The protocols and reagents for generating recombinant GIVAPLA$_2$ 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 GIVAPLA$_2$, D43N, 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 solution as described in a supplemental protocol (Clontech BD Biosciences). 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 TNM-FH insect cell medium.

GIVAPLA$_2$ 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 GIVAPLA$_2$ (or control XYLE protein), such that the recombinant protein band was easily visible and distinguishable on an SDS-PAGE, 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 Co^{2+} resin was successfully used according to instructions for batch adsorption purification. The recombinant, tagged proteins were found to elute successfully from the Talon resin with imidazole elution buffer but not by low pH elution buffer. The lysate, all 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 that antibody. The gels and blots indicated that the recombinant proteins were essentially pure and at a high concentration after elution by imidazole. The pure, recombinant proteins in imidazole elution buffer had full activity based on native control protein, such that all GIVAPLA$_2$-related proteins were used directly from the concentrated elute or after storage at −20 °C in the imidazole elution buffer supplemented with glycerol.

Standard PAPC PLA$_2$ Activity Assay—For the basal specific activity of GIVAPLA$_2$ (23), assays were performed in buffer composed of 20 mM HEPES at pH 7.7, 100 mM KCl, 200 μM CaCl$_2$, 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 in a final volume of 500 μl.

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 μl/assay) to form a cloudy white solution of multilamellar vesicles upon vortexing. The initial substrate buffer did not contain Ca^{2+} to avoid precipitating any phospholipid, especially the PIP$_n$s. After resuspension of the phospholipids, Triton X-100 was added (10 μl of 150 mM 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 mM 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.

After micelle formation, the mixed micelles (200 μl/assay) and the assay buffer (250 μl/assay) each were added to glass test tubes (16 × 125 mm). All of the Ca^{2+} or EGTA was found exclusively in the assay buffer. The tubes had been previously siliconized with Glassclad 18, to

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help prevent the enzymes and substrates from adsorbing onto the glass surface. The mixed micelles (450 μl/assay) in the tubes were then gently shaken in a 40 °C water bath for several minutes. The reaction was initiated by the addition of $GIVAPLA_2$ (1 μg in 50 μl per sample of assay buffer that lacked Ca^{2+} 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 acids 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-P_2 PLA_2 Activity Assay—This assay was identical to the standard PAPC PLA_2 activity assay, with the exception that the mixed micelles contained 1 mol % PI-4,5-P_2 (*i.e.* 0.96 mM PAPC (200,000 cpm), 0.04 mM PI-4,5-P_2, and 3 mM Triton X-100) (23). Since PI-4,5-P_2 enhances the activity of $GIVAPLA_2$ 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 amount of 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 $GIVAPLA_2$ (native or His-tagged enzyme from our laboratory, Lilly, Dr. Gelb, or Dr. Cho); however, the ratio of the activity with PI-4,5-P_2 to without PI-4,5-P_2 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.

Monomeric and Micellar Lysophospholipase 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 Ca^{2+}, 1 mg/ml BSA, and 1 mM dithiothreitol in a final volume of 500 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 resuspended in assay buffer (400 μl/assay) without any Ca^{2+} or EGTA. The Ca^{2+} 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 cold 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 mM, and the radioactivity was adjusted (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_n Binding Assay—PIP_n-$GIVAPLA_2$ binding was determined using PIP Strips from Echelon Research, Inc., and was carried out according to the provided protocols. A small plastic dish (~66 cm^2) 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 (12–16 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 $GIVAPLA_2$-specific primary antibody (as provided by Lilly) or the His tag-specific antibody (0.2 μg/μl TetraHis antibody from Qiagen). After the standard washing, the strip was incubated for 30 min with 2.5 μl of the secondary antibody in 10 ml of TBS-T/BSA. For the anti-$GIVAPLA_2$ 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 mg/ml goat anti-mouse HRP from Jackson ImmunoResearch Laboratories, Inc. After the standard washing, the strip was drained, but not dried, and incubated with premixed enhanced chemiluminescence reagents for several seconds 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.

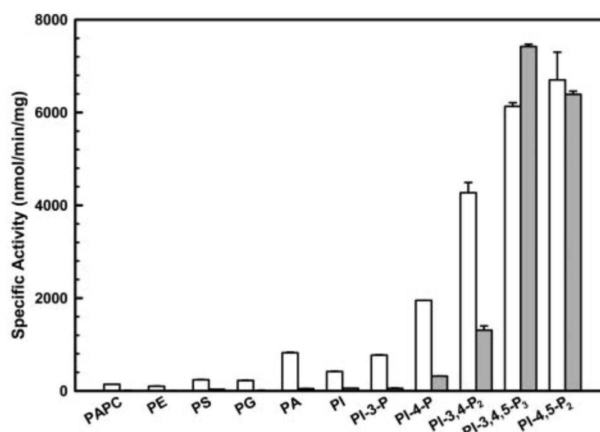

FIG. 1. **PIP_n-dependent, Ca^{2+}-independent activity of $GIVAPLA_2$ on mixed micelles.** The activity of $GIVAPLA_2$ was assayed on mixed micelles composed of Triton X-100 (3 mM), 1-palmitoyl-2-(1-[^{14}C]arachidonoyl)-PC (0.96 mM, 200,000 cpm), and various PIP_ns (0.04 mM, 1 mol %) as noted. The various non-PIP_n phospholipids were tested at a higher surface concentration (0.2 mM, 5 mol %). *White bars*, assays performed with 200 μM Ca^{2+}; *gray bars*, assays performed with 500 μM EGTA ([Ca^{2+}] < 2 nM). *PE*, phosphatidylethanolamine; *PS*, phosphatidylserine; *PG*, phosphatidylglycerol; *PA*, phosphatidic acid.

RESULTS

Ca^{2+}-independent Activity of $GIVAPLA_2$—In the absence of Ca^{2+}, $GIVAPLA_2$ is known to bind to and be active on lysophospholipid micelles (41), membrane interfaces containing PI-4,5-P_2 (23), and membranes composed of the nonnatural anionic phospholipid, phosphatidylmethanol (32). We have previously shown that in the presence of Ca^{2+}, PIP_2s and PIP_3 gave larger enhancements of $GIVAPLA_2$ activity than PIPs, which in turn gave larger enhancements than all other anionic phospholipids tested (23). Herein we have successfully analyzed the activity enhancements of PIP_ns on pure, human $GIVAPLA_2$ in the absence of Ca^{2+}. As expected, the presence of 5 mol % phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, phosphatidic acid, and phosphatidylinositol (PI) in PAPC/Triton X-100 mixed micelles did not yield any activity for native $GIVAPLA_2$ in the absence of Ca^{2+} as seen in Fig. 1. However, in the presence of 1 mol % PI-3-P, PI-4-P, PI-3,4-P_2, PI-4,5-P_2, or PIP_3, there was significant $GIVAPLA_2$ activity even in the absence of Ca^{2+} ([Ca^{2+}] < 2 nM, Fig. 1, *gray bars*). Moreover, the activities with 1 mol % PI-4,5-P_2 and PIP_3 were similar to one another with and without Ca^{2+} and were both more than 100-fold higher than the control that lacked PIP_ns but contained Ca^{2+}. Importantly, PI-4-P and PI-3,4-P_2 also gave very significant activity enhancements in the absence of Ca^{2+}, relative to PAPC alone (with Ca^{2+}). These $GIVAPLA_2$ activities without Ca^{2+} were only 30 and 16% of the activities with Ca^{2+}, respectively, but they are still far above the undetectable activity of $GIVAPLA_2$ on PAPC without Ca^{2+} or PIP_ns.

PI-4,5-P_2 PLA_2 Activity Assay with D43N and ΔC2—The addition of EGTA without any exogenous Ca^{2+} should have reduced the free [Ca^{2+}] to extremely low levels ([Ca^{2+}] < 2 nM). Nevertheless, we tested recombinant Ca^{2+} binding-deficient mutants of $GIVAPLA_2$ to confirm that the PI-4,5-P_2-$GIVAPLA_2$ interaction can unambiguously replace the Ca^{2+}-C2 domain interaction. A conceptual diagram of these various $GIVAPLA_2$ mutant constructs is shown in Fig. 2.

Using the recombinant, pure D43N, ΔC2, and His $GIVAPLA_2$, we measured activity in the PI-4,5-P_2 activity assay with and without Ca^{2+}. The results shown in Fig. 3 clearly demonstrate that in the presence of PI-4,5-P_2, D43N has the same activity as His $GIVAPLA_2$. As expected from its inability to bind Ca^{2+}, the D43N activity remained the same with or

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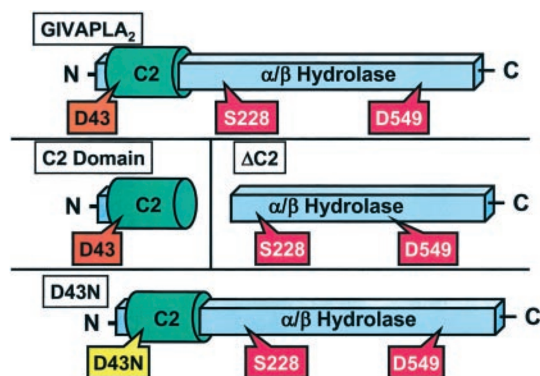

FIG. 2. **Comparison of His GIVAPLA$_2$ and major deletion and mutant constructs.** His GIVAPLA$_2$ is shown in a linear schematic with the N-terminal C2 domain containing a critical calcium ligand (Asp[43]) in *orange* and the C-terminal α/β hydrolase domain containing the active site dyad (Ser[228] and Asp[549]) in *red*. Three other constructs are also shown: the independent C2 domain, the independent α/β hydrolase domain (ΔC2), and the full-length single site mutant D43N.

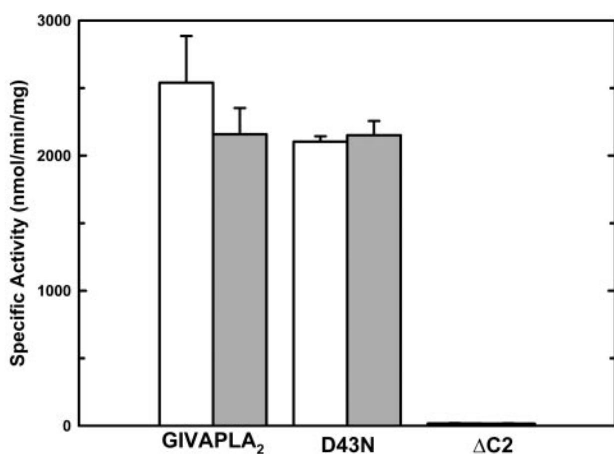

FIG. 3. **PI-4,5-P$_2$-enhanced activity of GIVAPLA$_2$, D43N, and ΔC2 on mixed micelles with and without Ca^{2+}.** The PI-4,5-P$_2$ activities of His GIVAPLA$_2$ and its mutants, D43N and ΔC2, were tested on PI-4,5-P$_2$-containing mixed micelles composed of Triton X-100 (3 mM), 1-palmitoyl-2-(1-[^{14}C]arachidonoyl)-PC (0.96 mM, 200,000 cpm), and PI-4,5-P$_2$ (0.04 mM, 1 mol %). *White bars*, assays performed with 200 μM Ca^{2+}; *gray bars*, assays performed with 500 μM EGTA ([Ca^{2+}] < 2 nM).

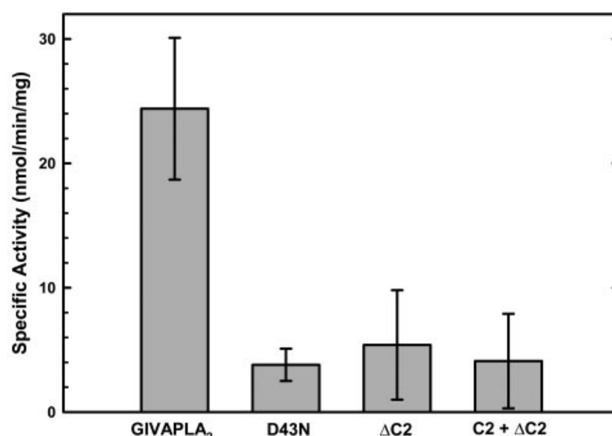

FIG. 4. **Basal activity of GIVAPLA$_2$, D43N, and ΔC2 on mixed micelles in the absence of PI-4,5-P$_2$.** The basal activities of His GIVAPLA$_2$ and its mutants, D43N and ΔC2, were tested on mixed micelles composed of Triton X-100 (3 mM) and 1-palmitoyl-2-(1-[^{14}C]arachidonoyl)-PC (1 mM, 200,000 cpm) in the presence of 200 μM Ca^{2+}.

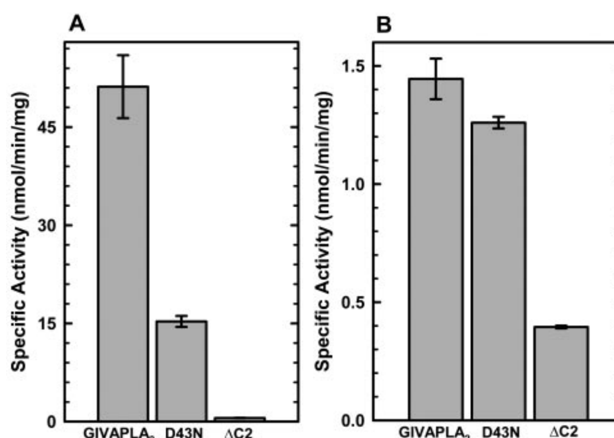

FIG. 5. **Lysophospholipase activity of GIVAPLA$_2$, D43N, and ΔC2 in the absence of PI-4,5-P$_2$ on lysophospholipid micelles or monomers.** *A*, the Lyso-PLA activities of His GIVAPLA$_2$ and its mutants, D43N and ΔC2, were tested on 1-(1-[^{14}C]palmitoyl)-Lyso-PC micelles (100 μM, 200,000 cpm). *B*, the Lyso-PLA activities of His GIVAPLA$_2$ and its mutants, D43N and ΔC2, were tested on 1-(1-[^{14}C]palmitoyl)-Lyso-PC monomers (4 μM, 240,000 cpm).

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. 5*A*, 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. 5*A*) 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. 5*B*, 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. 5*B*), the activities of His GIVAPLA$_2$ and D43N on micellar Lyso-PC substrate were dra-

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matically higher at 100 μM (Fig. 5*A*, 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. 5*A*.

The apparent lower activity of ΔC2 *versus* His GIVAPLA$_2$ 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 GIVAPLA$_2$ 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 GIVAPLA$_2$ is due to the same active site as the PLA$_2$ 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 GIVAPLA$_2$ catalytic activities depend on Ser^{228} (29).

Binding of GIVAPLA$_2$ Constructs to Immobilized Phospholipids—To determine which domain(s) of GIVAPLA$_2$ binds to PIP$_n$s, an overlay blot was performed using nitrocellulose-immobilized PIP$_n$s 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 PIP$_n$s. The PIP Strips have been used to study the PIP$_n$ binding interactions of various PIP$_n$ antibodies (Echelon Research, Inc.) and PIP$_n$ 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 GIVAPLA$_2$ (Fig. 6). Theoretically, GIVAPLA$_2$ 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 GIVAPLA$_2$), eliminating the chemiluminescence signal.

In order to address the possible hydrolysis of the PIP$_n$s from the PIP Strips, we utilized two complementary techniques. First, GIVAPLA$_2$ was preincubated with an excess of an irreversible serine-dependent PLA$_2$ inhibitor, MAFP. After incubation of MAFP-inhibited GIVAPLA$_2$ with the PIP Strips and probing with anti-GIVAPLA$_2$ antibody, there was significant signal at several PIP$_n$ spots, as seen in Fig. 6. Second, to rule out any effects of the MAFP, S228A was used. The results for S228A did not differ from those seen with MAFP-treated GIVAPLA$_2$ (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 GIVAPLA$_2$ 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-P$_2$, PI-3,4-P$_2$, phosphatidylinositol 3,5-bisphosphate, and occasionally weak signal with other anionic lipids, PIP$_3$, PI, phosphatidic acid, and phosphatidylserine. No signal was detected for phosphatidylethanolamine, phosphatidylcholine, or inositol 1,3,4,5-tetrakisphosphate. The same results were obtained for His GIVAPLA$_2$ with an anti-GIVAPLA$_2$ antibody or the TetraHis antibody (data not shown).

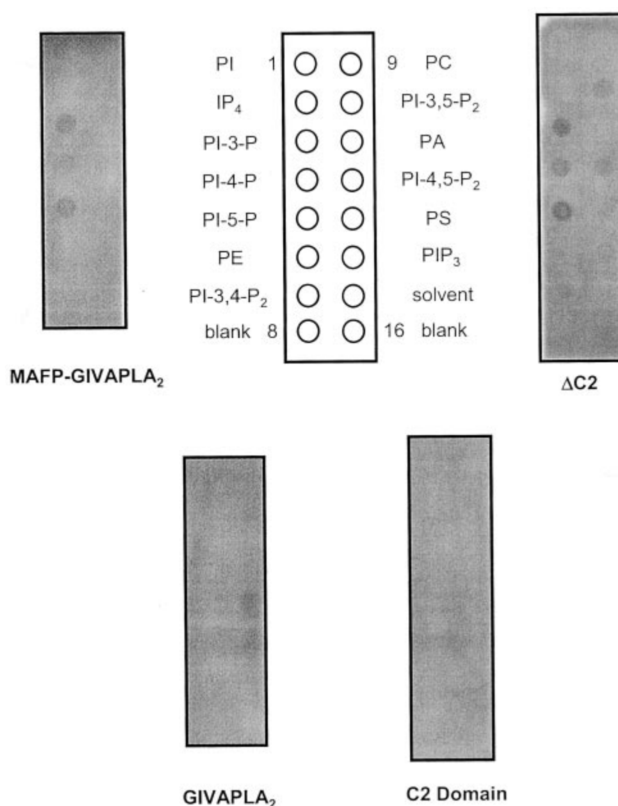

FIG. 6. **Binding of GIVAPLA$_2$ and various mutants to PIP$_n$s in a protein-phospholipid overlay blot.** Binding of various GIVAPLA$_2$ constructs was tested in protein-phospholipid overlay blots (PIP Strips). The PIP Strips were prespotted with 100 pmol of the indicated phospholipids. Each strip was incubated with 5 μg of GIVAPLA$_2$, MAFP-treated GIVAPLA$_2$, ΔC2, or the C2 domain and probed with an anti-GIVAPLA$_2$ antibody or anti-His tag antibody followed by an HRP-conjugated secondary antibody. The location of each phospholipid is as shown on the *schematic diagram*. The blots correspond to the specific proteins as indicated *under* each blot. *PE*, phosphatidylethanolamine; *PS*, phosphatidylserine; *PG*, phosphatidylglycerol; *PA*, phosphatidic acid; PI-5-P, phosphatidylinositol 5-phosphate; IP$_4$, inositol 1,3,4,5-tetrakisphosphate.

To test which domain was able to bind to PIP$_n$s, ΔC2 and the C2 domain were preincubated with MAFP, 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 MAFP-treated GIVAPLA$_2$ (Fig. 6). In addition, D43N was tested in the same manner and found to match the results seen for the MAFP-treated GIVAPLA$_2$ (data not shown). Although it was crucial for native and His GIVAPLA$_2$, MAFP was not necessary and did not interfere with the results for ΔC2 and the C2 domain. No effect of Ca^{2+} was seen for any construct tested.

The PIP Strip assay was used primarily to compare the ability of different GIVAPLA$_2$ constructs to bind to PIP$_n$s. 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 PIP$_n$s (especially PIP$_3$) in the pure spots and the improbable 30-Å^2 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

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portions of each protein. Many PIP$_n$-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). GIVAPLA$_2$ is different, because it does not bind inositol 1,4,5-trisphosphate, but instead binds to PIP$_n$s only in an interface (23). Moreover, unlike pleckstrin homology domains, GIVAPLA$_2$ has at least three membrane binding attachment points: the active site, the C2 domain, and the PIP$_n$ binding site. When GIVAPLA$_2$ 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 PIP$_n$s and would be most significant with PIP$_3$.

Confirmation of the Key Residues for the PIP$_n$ 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-P$_2$. The two mutants, K488E and K541A/K543A/K544A, were assayed on phospholipid vesicles under conditions where GIVAPLA$_2$ activity with 5 mol % PI-4,5-P$_2$ 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-P$_2$ but rather each had a slight decrease (33). Because of the small enhancements from PI-4,5-P$_2$ 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-P$_2$ (100-fold or greater) (23).

We tested the basal and PIP$_2$-stimulated activity of K488E and K541A/K543A/K544A in the standard PAPC and standard PI-4,5-P$_2$ 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-P$_2$ 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 GIVAPLA$_2$-PIP$_2$ interaction.

Ruling Out Proposed PIP$_n$-binding Residues—In a previous report (23), we hypothesized that the PIP$_n$-binding site of GIVAPLA$_2$ 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-P$_2$-enhanced activity (data not shown) and thus gave around the same 100-fold increase with 1 mol % PI-4,5-P$_2$ as seen for WT GIVAPLA$_2$ 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-P$_2$-stimulated activity (33).

Investigating Effects of GIVAPLA$_2$ Phosphorylation on PIP$_n$ Activation—To rule out any complex interaction between PIP$_n$ effects and GIVAPLA$_2$ phosphorylation, we tested a quadruple mutant, S437A/S455A/S505A/S727A, that was not phosphorylated *versus* a matched His GIVAPLA$_2$ 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

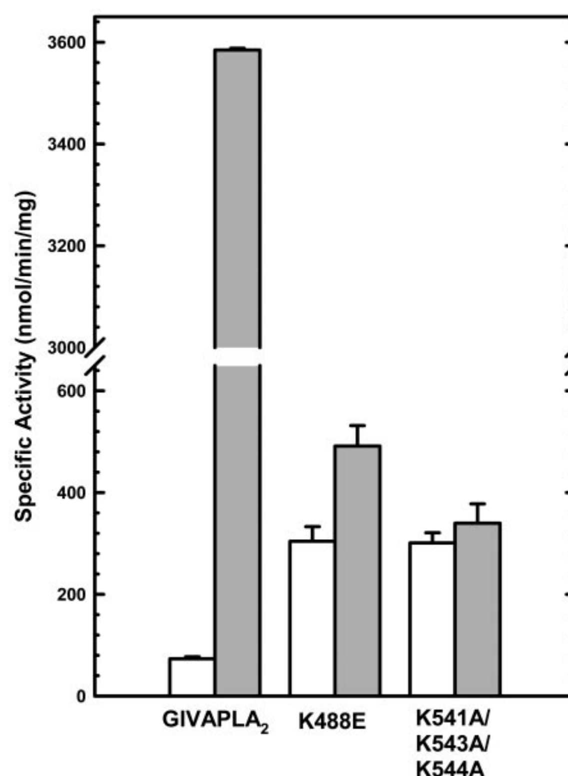

FIG. 7. **Activity of GIVAPLA$_2$ and various mutants with and without PI-4,5-P$_2$.** The basal activity of His GIVAPLA$_2$ and the mutants K488E and K541A/K543A/K544A were assayed on mixed micelles composed of Triton X-100 (3 mM) and 1-palmitoyl-2-(1-[^{14}C]arachidonoyl)-PC (1 mM, 200,000 cpm) as shown in *white bars*. The PI-4,5-P$_2$ enhanced activity was assayed with PI-4,5-P$_2$-containing mixed micelles composed of Triton X-100 (3 mM), 1-palmitoyl-2-(1-[^{14}C]arachidonoyl)-PC (0.96 mM, 200,000 cpm), and PI-4,5-P$_2$ (0.04 mM, 1 mol %) as shown in *gray bars*.

His GIVAPLA$_2$ (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 GIVAPLA$_2$ *versus* less phosphorylated GIVAPLA$_2$ (18, 19, 51). We also found that in the standard PI-4,5-P$_2$ assay, the quadruple mutant had about 25% of the specific activity of the control His GIVAPLA$_2$ (Fig. 8). Therefore, the control His GIVAPLA$_2$ and the quadruple mutant activities were both stimulated by around 100-fold in the presence of 1 mol % PI-4,5-P$_2$. This indicated no positive or negative interaction between PIP$_n$-stimulation and GIVAPLA$_2$ phosphorylation state at these four residues, which are normally partially phosphorylated during their cellular expression (36). One other phosphorylation site (Ser^{515}) was recently identified (20). This fifth potential phosphorylation target was not phosphorylated in the His GIVAPLA$_2$ 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-P$_2$. Importantly, these results suggest that phosphorylation and levels or availability of PIP$_n$s can coordinately increase the activity of GIVAPLA$_2$ (Fig. 8, S228A activity without PI-4,5-P$_2$ *versus* GIVAPLA$_2$ activity with PI-4,5-P$_2$).

Basal and PI-4,5-P$_2$-enhanced GIVAPLA$_2$ Activity Highly Depend on the Quality of the Interface—The high Lyso-PLA activity of GIVAPLA$_2$ is a useful feature of GIVAPLA$_2$ that we have more fully explored. In Triton X-100 mixed micelles, the large GIVAPLA$_2$ activity enhancements from PI-4,5-P$_2$ were seen when the substrate was either PAPC or Lyso-PC, as shown in Fig. 9*A*. For GIVAPLA$_2$, the Lyso-PLA activity on PI-4,5-P$_2$/Lyso-PC/Triton X-100 mixed micelles was Ca^{2+}-inde-

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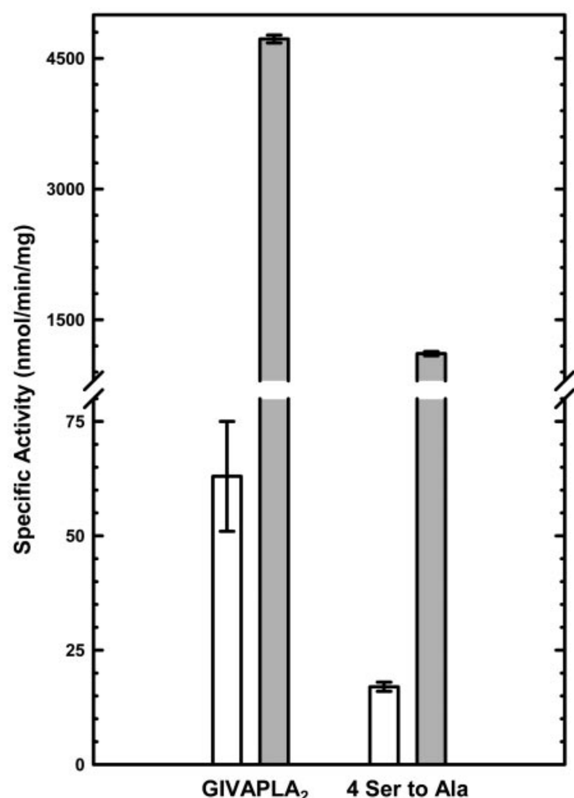

FIG. 8. **Activity of GIVAPLA$_2$ and a quadruple serine to alanine mutant with and without PI-4,5-P$_2$.** The basal activities of His GIVAPLA$_2$ and the quadruple mutant, S437A/S455A/S505A/S727A, were assayed on mixed micelles composed of Triton X-100 (3 mM), 1-palmitoyl-2-(1-[^{14}C]arachidonoyl)-PC (1 mM, 200,000 cpm) as shown in *white bars*. The PI-4,5-P$_2$-enhanced activities were assayed with PI-4,5-P$_2$-containing mixed micelles composed of Triton X-100 (3 mM), 1-palmitoyl-2-(1-[^{14}C]arachidonoyl)-PC (0.96 mM, 200,000 cpm), and PI-4,5-P$_2$ (0.04 mM, 1 mol %) as shown in *gray bars*.

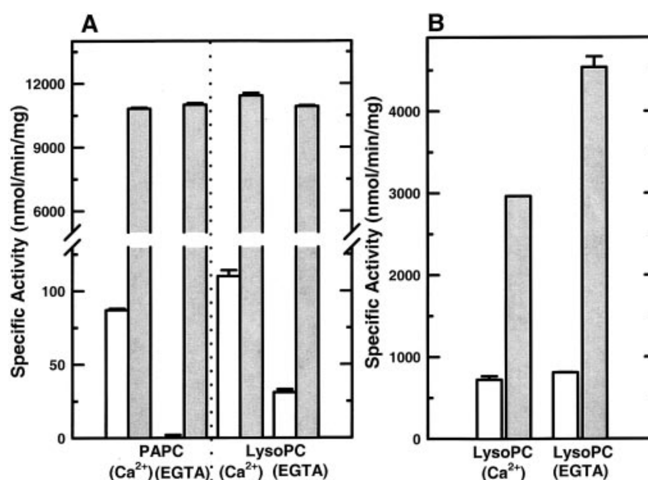

FIG. 9. **Phospholipase A$_2$ and lysophospholipase activity of WT GIVAPLA$_2$ in mixed micelles or pure micelles with and without Ca^{2+} and PI-4,5-P$_2$.** *A*, the activity of GIVAPLA$_2$ was assayed on mixed micelles composed of Triton X-100 (3 mM) and phospholipid (1 mM). For PLA$_2$ activity (*left four bars*), the substrate was 1-palmitoyl-2-(1-[^{14}C]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-[^{14}C]palmitoyl)-Lyso-PC (1 mM (*white bars*) or 0.96 mM (*gray bars*), 200,000 cpm). The nonsubstrate phospholipid, PI-4,5-P$_2$, 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 Ca^{2+}, whereas the *right two bars* for each substrate represent assays performed with 500 μM EGTA ([Ca^{2+}] < 2 nM). *B*, the activity of GIVAPLA$_2$ was assayed on pure micelles composed of 1-(1-[^{14}C]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-P$_2$ (0.4 mM, 4 mol %). The *left two bars* represent assays performed with 200 μM Ca^{2+}, whereas the *right two bars* represent assays performed with 500 μM EGTA ([Ca^{2+}] < 2 nM).

pendent, mirroring that seen for PLA$_2$ activity (Fig. 9*A* and Fig. 1). Just as for the PLA$_2$ activity, the Lyso-PLA activity without PI-4,5-P$_2$ was significantly Ca^{2+}-dependent, although there was still significant Ca^{2+}-independent activity (Fig. 9*A* and see also Ref. 41).

The nature and composition of the vesicles, micelles, monolayers, or monomers that contain substrate phospholipids critically control the specific activity of PLA$_2$s (52–54). We have looked at this quality of interface issue by comparing the hydrolysis of the same substrate in two different interfaces. We monitored the GIVAPLA$_2$ Lyso-PLA activity on Lyso-PC/Triton X-100 mixed micelles (Fig. 9*A*) and pure Lyso-PC micelles (Fig. 9*B*). When the Lyso-PLA assays were prepared without Triton X-100, the 1 mM Lyso-PC substrate formed pure micelles. The ability of GIVAPLA$_2$ to hydrolyze micelles of pure lysophospholipids is known to be Ca^{2+}-independent (Fig. 9*B* and see also Refs. 39 and 41). When these micelles were supplemented with PI-4,5-P$_2$ (4 μM, 4 mol %), there was a similar increase in GIVAPLA$_2$ activity in the absence or presence of Ca^{2+}, as shown in Fig. 9*B*. These increases were in the 3–6-fold range, much like those previously reported for PI-4,5-P$_2$ addition in the small unilamellar vesicle substrate systems (33, 55). This 3–6-fold increase is in contrast to the Lyso-PC/Triton X-100 mixed micelle system that gave greater than a 100-fold increase in activity in the presence of 1 mol % PI-4,5-P$_2$ (Fig. 9*A*). The mixed micelle system in the absence of PI-4,5-P$_2$ was significantly Ca^{2+}-dependent (Fig. 9*A*, *middle* and *right white bars* and see also Ref. 41), in contrast to the Lyso-PC micelle system that showed no Ca^{2+} dependence (Fig. 9*A*).

The addition of 3 mM Triton X-100 to 1 mM Lyso-PC should have had a surface dilution effect on the activity of GIVAPLA$_2$, reducing the activity 4-fold (56, 57). Indeed, in the presence of Ca^{2+} and absence of PI-4,5-P$_2$, 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-P$_2$ 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 Ca^{2+}, the PI-4,5-P$_2$ effects on GIVAPLA$_2$ 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 Ca^{2+} (Fig. 9, *A* and *B*, *gray bars*). However, without PI-4,5-P$_2$, the 4-fold dilution with Triton X-100 gives a 26-fold reduction of activity. This indicates that in the absence of Ca^{2+}, Lyso-PLA activity also strongly depends on the quality of the interface. Finally, in the presence of PI-4,5-P$_2$ with no Ca^{2+}, 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 Ca^{2+}-independent, PI-4,5-P$_2$-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 GIVAPLA$_2$ as well as the effects of Ca^{2+} and PI-4,5-P$_2$ on its activity. By taking advantage of the effects of Ca^{2+}, PI-4,5-P$_2$, and the quality of interface, a specific assay for GIVAPLA$_2$ was developed (58) that can distinguish this enzyme from all known mammalian PLA$_2$s. The specific assay is particularly useful to distinguish GIVAPLA$_2$ activity in samples of crude tissue homogenates or cellular preparations from mouse, rat, and human sources (58).

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DISCUSSION

GIVAPLA$_2$ 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 GIVAPLA$_2$ to its substrate membranes can be regulated by intracellular [Ca^{2+}] (see Ref. 10 and references therein). Whereas the effects of Ca^{2+} are mediated through the C2 domain, we have now shown that the C2 domain of GIVAPLA$_2$ is apparently required for Ca^{2+}-independent interfacial activity. This suggests a novel second role for the C2 domain in the Ca^{2+}-independent activation of the catalytic domain. This second role may also be important for Ca^{2+}-dependent interfacial activity but would be obscured by the primary, Ca^{2+}-dependent membrane binding role of the C2 domain.

Recently, evidence has accumulated for the translocation and activation of GIVAPLA$_2$ without a corresponding rise in intracellular [Ca^{2+}] (5, 24–27). Along with our previous results (23), the results presented herein further strengthen the notion that PI-4,5-P$_2$, and possibly other PIP$_n$s, may have an analogous role to Ca^{2+} in increasing the membrane affinity of GIVAPLA$_2$. This membrane affinity increase would facilitate increased activity by bringing enzyme and substrate together.

Nonplasma membrane PIP$_n$s 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 Ca^{2+}-independent activity for GIVAPLA$_2$ seen *in vitro* at 1 mol % PI-4,5-P$_2$ is a physiologically relevant surface concentration for many cell membranes (63). More recently, significant levels of PI-4,5-P$_2$ 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 PI-4,5-P$_2$ matched the localization seen separately for GIVAPLA$_2$ in the same cells (33). Co-transfection of a PI-4,5-P$_2$-binding protein and GIVAPLA$_2$ partly reduced the GIVAPLA$_2$-dependent arachidonic acid release (33), indicating that there may indeed be a physiological interaction between GIVAPLA$_2$ and PI-4,5-P$_2$.

Herein we report that GIVAPLA$_2$ can have its highest activity on PI-4,5-P$_2$- or PIP$_3$-containing mixed micelles with or without Ca^{2+}. Along with PI-4,5-P$_2$ (23) and Lyso-PC (39, 41), PIP$_3$ is shown to be a third physiologically relevant lipid that can lead to full Ca^{2+}-independent GIVAPLA$_2$ activity *in vitro*. However, PI-4,5-P$_2$, as the much more abundant precursor of PIP$_3$ (63, 64), is probably more relevant than PIP$_3$. In addition to PI-4,5-P$_2$ and PIP$_3$, PI-3,4-P$_2$ and PI-4-P also gave significant, but not maximal, Ca^{2+}-independent activity. Since PI-4-P is the most abundant of the PIP$_n$ species (24, 63), it is possible that it too could significantly contribute to the activity of GIVAPLA$_2$ *in vivo*. The up-regulation of PI-4-P levels, and subsequently PI-4,5-P$_2$ levels, was observed in murine P388D$_1$ macrophage-like cells primed by lipopolysaccharide and activated by UV light (24). Under these conditions, GIVAPLA$_2$ specifically acted to release arachidonic acid without any changes in intracellular [Ca^{2+}] (24). The increased levels of PIP$_n$ seen in the P388D$_1$ cells may have been generated at the intracellular membranes to which GIVAPLA$_2$ 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-P$_2$ mixed micelles and high activity toward Lyso-PC micelles unambiguously confirmed that native and His GIVAPLA$_2$ can have full activity in the absence of Ca^{2+}. Whereas Ca^{2+} may be dispensable for GIVAPLA$_2$ activity, the C2 domain is not. The ΔC2 construct did not have any PLA$_2$ or Lyso-PLA activity on interfacial substrates with or without Ca^{2+}, PI-4,5-P$_2$, 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-P$_2$), 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 GIVAPLA$_2$ interfacial activity regardless of its capacity to bind Ca^{2+}.

PIP Strip binding assays were employed to determine whether the C2 domain or the ΔC2 construct (or both) contained the PIP$_n$ binding site. The PIP$_n$-binding pattern seen in the PIP Strip assay for the MAFP-inhibited native GIVAPLA$_2$, His GIVAPLA$_2$, S228A, and D43N all matched ΔC2, whereas the C2 domain showed no PIP$_n$ binding. This suggested that the active site domain, but not the C2 domain, binds PIP$_n$s. These results complement the recent finding that a specific GIVAPLA$_2$ PIP$_n$ 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-P$_2$ binding site residues probably include Lys^{488}, Lys^{541}, Lys^{543}, and Lys^{544}, which are indeed located in the ΔC2 construct (33). Previous results suggest that the catalytic domain may also bind to phosphatidylmethanol vesicles in a Ca^{2+}-dependent manner (32), perhaps through the PIP$_n$ binding site. Interestingly, these four residues are identical in all vertebrate GIVAPLA$_2$ orthologs but not in its paralogs, GIVBPLA$_2$ or GIVCPLA$_2$. We have found that these two paralogs are not activated by PI-4,5-P$_2$, in contrast to GIVAPLA$_2$ (66). The location of the PIP$_n$ binding site on the GIVAPLA$_2$ catalytic domain contrasts to the recently identified PI-4,5-P$_2$ binding site in the C2 domain of protein kinase Cα (67).

Although the C2 domain may not be required for PIP$_n$ 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 PIP$_n$ binding, Lyso-PC binding, and the traditional Ca^{2+}-dependent membrane binding. This seems less likely given the presence of the key PIP$_n$-binding residues on the catalytic domain and the ability of the catalytic domain itself to bind to PIP$_n$s on PIP Strips. The C2 domain has previously been shown to possess some Ca^{2+}-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 PIP$_n$s, including PI-4,5-P$_2$, enhance the activity of GIVAPLA$_2$ by less than 10-fold (21, 22, 33, 55). At first glance, this seems to contradict our reports that PI-4,5-P$_2$ enhances the activity of GIVAPLA$_2$ 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-P$_2$ 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-P$_2$ is added to assay systems that already have high activity, the enhancing effects of PI-4,5-P$_2$ may appear muted as in Lyso-PC micelles (Fig. 9*B*) 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-P$_2$ led to reliable, although modest, 4–6-fold activity enhancements as in Fig. 9*B* and Refs. 22, 33, and 55.

Interestingly, the enhancing effects of PI-4,5-P$_2$ are not mim-

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icked by the soluble head group, inositol 1,4,5-trisphosphate (23), indicating a membrane-dependent binding interaction. The PIP$_n$-binding interaction also seems to induce an apparent activation by unknown means, perhaps through a specific orientation or conformational change. This putative PIP$_n$-dependent activation would be in addition to the increased membrane affinity of GIVAPLA$_2$ seen in the presence of PI-4,5-P$_2$ (23). This suggests that in addition to the analogous role of PIP$_n$s to Ca^{2+} (membrane binding), there is a second analogous role of PIP$_n$s to phosphorylation (activation or a higher specific activity). Whether a PIP$_n$-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 GIVAPLA$_2$ by PI-4,5-P$_2$ indicated in this study and other studies (17, 33) will require significant dynamics and structural studies to more fully elucidate the molecular regulation of GIVAPLA$_2$ activity and apparent activation.

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Essential Ca^{2+}-independent Role of the Group IVA Cytosolic Phospholipase A_2 C2 Domain for Interfacial Activity

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