Supplemental Information

Role of phosphorylation and basic residues in the catalytic domain of cytosolic phospholipase $A_2\alpha$ in regulating interfacial kinetics and binding and cellular function

Purification of cPLA$_2\alpha$. A spinner flask culture of 950 ml of Sf9 cells (10^6 cells/ml) was infected with 13.5 ml of standard P3 viral stock (~10^9 pfu/ml). Cells were collected 72 h post infection by centrifugation at 2,600xg at 4 °C, and the cell pellet was stored at -80 °C. Cells were homogenized with a Potter homogenizer on ice in 25-30 ml of 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Pefabloc, 1 mM benzamidine, 50 μg/ml leupeptin, 10 μM pepstatin A, and 5 μg/ml aprotinin. The lysate was centrifuged at 100,000xg for 40 min at 4 °C. The supernatant was loaded on a column containing 50 ml of Q-Sepharose Fast Flow (GE Biosciences) pre-equilibrated with Q-Sepharose buffer (25 mM Tris-HCl, pH 7.5, 100 mM NaCl) at 4 °C. The column was washed at 1.5 ml/min with Q-Sepharose buffer until the OD280 nm reaches a steady minimum (about 2-3 column volumes). The column was developed with a 400 ml gradient of Q-Sepharose buffer containing 100-700 mM NaCl at 1.5 ml/min, collecting 6 ml fractions. cPLA$_2\alpha$ elutes at about 350 mM NaCl. Fractions containing cPLA$_2\alpha$ (detected with a spectrofluorimetric assay (1) and by SDS-PAGE) were pooled, and the sample was mixed with 3-4 ml bed of Ni-NTA-Agarose (Qiagen Inc.) that was pre-equilibrated with Ni-Agarose buffer (25 mM Tris-HCl, pH 7.5, 0.7 M NaCl, 1 mM imidazole). The mixture was tumbled at room temperature for 40 min, and the gel was loaded into a small column. The column was washed with Ni-Agarose buffer for 70 min at 30 ml/h at room temperature. The column was developed with a 40 ml of a gradient of 1-200 mM imidazole in Ni-Agarose buffer at room temperature at 30 ml/h, and 1 ml fractions were collected. The fraction tubes were pre-loaded with 0.5 ml of Ni-Agarose buffer plus 6 mM 2-mercaptoethanol. The cPLA$_2\alpha$-containing fractions were inspected for enzyme purity by SDS-PAGE. Pure cPLA$_2\alpha$-containing fractions were pooled, and the sample was concentrated using an Amicon Ultra-15 ultrafilter (Cat. UFC903008). The enzyme was exchanged into 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2 mM DTT by 4 cycles of dilution and ultrafiltration. The enzyme was concentrated to 1-2 mg/ml (cPLA$_2\alpha$ concentrations were determined from the absorbance at 280 nm (ε_{280} = 0.827 mg^{-1} mL^{-1} cm^{-1}, (1)). Finally, glycerol was added to 30% (v/v) prior to enzyme storage at -20 °C.

Preparation of cPLA$_2\alpha$-PAP. Purified cPLA$_2\alpha$ (see above) was brought into 25 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2 mM DTT by 3 dilution/ultrafiltration cycles using an Amicon Ultra-15 ultrafiltration device at 4 °C. The enzyme was concentrated to 1-2 mg/ml, and ethylene glycol was added to give 30% (v/v). An aliquot of cPLA$_2\alpha$ was mixed with an equal volume of spin-column purified potato acid phosphatase (see below). Ten to twenty Units of phosphatase was used per mg of cPLA$_2\alpha$. Leupeptin and aprotinin were added to give 40 μg/ml each, and the mixture was incubated for 1.5 h at 30 °C. The sample was microfuged to pellet any precipitate, and the supernatant was applied to a Poros HQ column (4.6x100 mm, Poros Inc.) using

solvent A (50 mM Tris-HCl, pH 8.0) and solvent B (solvent A with 1 M NaCl). The column was pre-equilibrated with 12.5% B. After sample loading, the following gradient was run at 0.5 ml/min at room temperature: 0-5 min, 12.5% B; 5-5.2 min 12.5-25% B, 5.2-7 min, 25% B, 7-25 min, 25-100% B. cPLA$_2\alpha$-PAP eluted at 76% B in A and was confirmed using a spectrofluorimetric assay (1). The appropriate fractions were combined, and the sample was concentrated to ~200 µl using an Amicon Ultra-15 device. The cPLA$_2\alpha$ solution was submitted to chromatography on a Superdex-200 size exclusion column (16x70 mm GE Biosciences) pre-equilibrated with 25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 2 mM DTT at 4 °C at a flow rate of 1 ml/min at room temperature. cPLA$_2\alpha$-containing fractions (detected by fluorimetric assay) were combined, and the sample was concentrated as above. Glycerol was added to 30% (v/v) prior to storage at -20 °C.

To prepare the phosphatase, a 2 ml Zebra spin column (Pierce Cat. 89891) was equilibrated with 200 mM MES, pH 6.5, 2 mM DTT, 4 mM $MgCl_2$ according to the manufacturer's instructions. A 210 µl aliquot of potato acid phosphatase (ammonium sulfate suspension, Sigma Cat. P-0157, 50 units, see below) was diluted with 210 µl of 50 mM MES pH 6.5, 100 mM NaCl, 4 mM $MgCl_2$, 1 mM PMSF, 50 µg/ml TLCK, and 100 µg/ml TPCK (protease inhibitors added fresh from frozen stock solutions). The mixture was incubated for 30 min at room temperature and then submitted to spin-column chromatography according to the manufacturer's instructions. The phosphatase was stored on ice, and an aliquot was submitted to an assay using 7.6 mM *p*-nitro-phenylphosphate (Sigma) in 40 mM sodium citrate, pH 4.8. After 10 min at 37 °C, the mixture was quenched by adding 4 volumes of 100 mM NaOH, and the absorbance at 410 nm was read (ε for *p*-nitrophenolate = 18,300 M^{-1} cm^{-1}). One Unit of phosphatase is defined as the amount that produces 1 µmole of product per min at 37 °C.

Phosphoamino analysis of cPLA$_2\alpha$-PAP. A solution containing 50 µg of cPLA$_2\alpha$ or cPLA$_2\alpha$-PAP was dialyzed against 50 mM HEPES, pH 8.0. Both samples were frozen and shipped on dry ice to the Protein Chemistry Laboratory (Texas A&M Univ., http://pcl.calabreso.com). Dialyzed cPLA$_2\alpha$ or cPLA$_2\alpha$-PAP (7 µg) was hydrolyzed using vapor phase HCl at 110 °C for 2 hr (instead of the normal 24 hr) (triplicate samples). In a side-by-side reaction, 5 nmol of authentic phosphoserine was submitted to the same treatment. Amino acid analysis for serine and phosphoserine showed that 52.9 ± 0.2 % of the phosphoserine was hydrolyzed to serine under these conditions. If it is assumed that the same percentage of phosphoserine from the protein sample is hydrolyzed to serine, it is found that cPLA$_2\alpha$ has 1.8 ± 0.1% percent of its serine as phosphoserine (or 1.0 moles phosphoserine per mole of cPLA$_2\alpha$ on average). After phosphatase treatment of cPLA$_2\alpha$ as described above, no phosphoserine was detected. We previously showed by phosphoamino analysis of radiophosphorylated cPLA$_2\alpha$ expressed in Sf9 cells that all phosphates are on serine (2). Thus it is concluded that essentially all of the serine phosphates are removed from cPLA$_2\alpha$ by phosphatase treatment.

Preparation of cPLA$_2\alpha$-505P. cPLA$_2\alpha$-PAP (10 µg, prepared as described above) was placed in a polypropylene microfuge tube followed by 15 µL of 40 mM HEPES, pH 7.4,

20 mM $MgCl_2$, 2 mM DTT (from a frozen stock), and 200 μM ATP (from a frozen stock). The sample was slit into two tubes, activated recombinant MAPK (100 Units, Calbiochem Cat. 45850) was added to one tube and the same volume of buffer to the other (total volume of 15 μl in each tube). The tubes were incubated at 30 °C for 90 min. To evaluate the extent of phosphorylation at Ser-505, an aliquot of cPLA$_2$α-505P and cPLA$_2$α-PAP (~1 μg each) were analyzed on a 10% polyacrylamide gel (83 x 73 mm, 1 mm thick), which was stained with Coomassie blue. The gel was run at 125 V until the dye front reached the end of the stacking gel and then at 200 V until 45 min after the dye front ran off the end of the running gel. An adjacent gel lane was loaded with cPLA$_2$α-PAP, and a complete gel shift after MAPK treatment (slower moving band) was assessed by inspection of the stained gel. The extent of Ser-505 phosphorylation was also assessed by monitoring the increase in enzymatic activity measured with the fluorimetic assay with the umbelifferyl ester of γ-linolenic acid, Triton X-100, and dioleoyl phosphatidylmethanol (1). In this assay, cPLA$_2$α-PAP showed a maximum increase of activity of 2-fold when treated with an increasing amount of MAPK. cPLA$_2$α-PAP was used within 24 hr (stored at 4 °C).

Preparation of cPLA$_2$α-515P. cPLA$_2$α-PAP (1.25 μg, prepared as described above) in 44 μl of 40 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 30 μg/ml calmodulin (Sigma Cat. P-2277), and 50 μM γ-[^{32}P]ATP (400 Ci/mol) was placed in a polypropylene microfuge tube. Half of the mixture (22 μL) was transferred to a second tube, and 1 μl of CaMKII solution (75 Units, Calbiochem Cat. 208706) in CaMKII buffer (20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50% glycerol) was added. To the remaining tube was added 1 μl of CaMKII buffer. Both tubes were incubated for 45 min at 30 °C. The mixtures were submitted to SDS-PAGE on a 7.5% gel. The gel was stained with Coomassie blue, and bands corresponding to cPLA$_2$α were excised and dissolved in 0.5 ml of 30% H_2O_2 overnight prior to scintillation counting. To generate cPLA$_2$α-515P for kinetic studies, the above phosphorylation was repeated in the presence of non-labeled ATP.

Preparation of buffers with various concentrations of free Ca^{2+}. To 200 ml of buffer A was added a concentrated $CaCl_2$ solution to give ~0.5 mM $CaCl_2$. A 1 ml aliquot was placed in a fluorescence cuvette, the fluorimeter reading was set to zero, 1 μL of 40 μM Fluo-3 (Molecular Probes) in water was added, the fluorescence was read (ex 505 nm, em 526 nm), and then read again after adding an aliquot of concentrated $CaCl_2$ to give 1.5 mM (maximum signal with Fluo-3 saturated with calcium). The fraction of maximal fluorescence response was used with the dissociation constant for the fluo-3-calcium complex (0.325 μM in 10 mM MOPS, pH 7.2, 100 mM KCl, Molecular Probes handbook) to calculate the free calcium concentration. This was repeated after addition of more $CaCl_2$ or buffer A to the original buffer solution as needed until the desired free calcium concentration was obtained. The same process was used for free calcium > 5 μM except 20 nM Calcium-Green-5N (Molecular Probes, ex 506 nm, em 532 nm, dissociation constant 14 μM) was used.

Radiometric cPLA$_2$α assay. The standard radiometric cPLA$_2$α assay was carried out as follows. A polypropylene microfuge tube was charged with 1 μl of 2 mM $CaCl_2$

followed by 100 μL of supernatant (see above), and finally 4 μL of ^{14}C-PAPC in DMSO (100,000 dpm per assay, 50 Ci/mol). The mixture was mixed on a vortex mixture briefly and incubated for 30 min at 37 °C. The reaction was quenched by addition of 370 μL of $CHCl_3$:MeOH:concentrated HCL (200:100:1), followed by 460 μL of MeOH:$CHCl_3$:glacial acetic acid (2/1/0.8), followed by 250 μL of $CHCl_3$ and finally 250 μL of water. After mixing on a vortex mixture, the tube was centrifuged briefly to separate the solvent layers, and the lower layer was transferred to a glass tube. The other layer was extracted with 400 μL of $CHCl_3$. After mixing and centrifugation, the lower layer was combined with the first extract and solvent was removed with a stream of N_2. The residue was dissolved in petroleum ether:ether:glacial acetic acid (70:30:1), and the sample was processed by silica gel chromatography (1.5-2 cm bed of silica in a glass wool-plugged Pasteur pipet) and scintillation counting as described (3).

Isolation of human blood platelets. Human blood was drawn with a 21.5 Gauge needle and butterfly tubing (discarding the first 1 ml of blood) into a syringe that was pre-filled with ACD (85 mM trisodium citrate, 64 mM citric acid, 111 mM glucose, filter sterilized) using 1 volume of ACD per 6 volumes of blood. The blood/ACD mixture is mixed gently by inverting the 50 ml sterile plastic tube. Tubes are centrifuged at 200x$g_{average}$ for 15 min at room temperature without braking in a swinging bucket rotor, and ~80% of the upper layer (platelet rich plasma) is transferred to a new tube with a plastic transfer pipet. Prostacyclin stock solution (1 mg in 2 mL of 50 mM Tris, 0.1 M NaCl, pH 12, stored -80°C) is diluted fresh before use with the same buffer to give 0.05 mg/ml. This stock is added to platelet rich plasma to give 0.1 μg/ml. The sample is centrifuged at 1000x$g_{average}$ in a fixed angle rotor for 15 min at room temperature, and the supernatant is removed by aspiration. The platelets in the pellet are resuspended by gentle up and down motion with a pasture pipet in room temperature platelet buffer (25 mM Hepes, pH 7.3, 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, filter sterilized), and indomethacin is added to give a concentration of 10 μM. Platelets are counted with a hemacytometer (after dilution 1000-10,000 fold with platelet buffer). Prostacyclin is added to give 0.1 μg/ml, and the suspension is centrifuged at 1000x$g_{average}$ for 15 min at room temperature. After aspiration of the supernatant, the platelets in the pellet are resuspended in platelet buffer to give 1-1.5x10^9 platelets per ml.

Thrombin stimulation of platelets. The above platelet suspension is placed in a water bath 30 °C for 30 min, and EGTA is added from a 100 mM stock solution in water to give 1 mM (mix by gentle inversion of the tube). The cell suspension is transferred to a 37 °C water bath, and thrombin is added to give 4-5 Units/ml (Enzyme Research Laboratories, 3318 Units/mg). The tube is mixed by gentle inversion and left in the 37 °C bath for 5 min with period mixing by gentle inversion.

Partial purification of cPLA$_2$α-505P/727P from thrombin-stimulated platelets. The following procedure is adapted from Kramer et al. (4) starting from 600 ml of blood. To the suspension of thrombin stimulated platelets (see above) is added staurosporin and genistein (Sigma) to give 10 μM each. Platelets are pelleted in a Sorvall GSA rotor spun at 7000 rpm for 1 min at 4 °C. The supernatant is removed by aspiration, and the platelets are resuspended in 8 ml of ice-cold lysis buffer (10 mM Hepes, pH 7.4, 140 mM

NaCl, 27 mM KCl, 2 mM EGTA, 1 mM DTT containing freshly added protease inhibitors (1 mM PMSF, 100 μM leupeptin, 100 μg/ml aprotinin, 10 μM pepstatin A) and freshly added kinase inhibitors (1 μM genistein, 1 μM staurosporin). The suspension was sonicated on ice using a microtip (4x10 sec bursts, Heat Systems Ultrasonics Model W225 at 30% duty cycle and power control at 3) using eight 1.5 ml polypropylene microfuge tubes. Phosphatase inhibitors are added to give 50 mM NaF, 0.2 mM Na_3VO_4, 1 μM microcystin-LR (Calbiochem). The tubes are centrifuged at 104,000xg for 40 min at 4 °C, and the supernatant is transferred to a new tube on ice. Total protein is measured using the Bradford dye binding assay (BioRad) using BSA as a standard. About 23 mg of platelet protein is obtained at this point and can be stored at -80 °C. The lysate is thawed on ice and 2 volumes of ice-cold 25 mM Tris, pH 8.0, 150 mM NaCl, 4 mM DTT is added, and the sample is centrifuged at 10,000 rpm in an SS34 rotor for 10 min at 4 °C to pellet any insoluble material. The supernatant is loaded onto a Mono-Q column (5/50 GL, GE Biosciences 17-5166-01) pre-equilibrated with 25 mM Tris, pH 8.0, 150 mM NaCl, 2 mM 2-mercaptoethanol at 4 °C. The column is washed with equilibration buffer at 1 ml/min at 4 °C until the OD280 reaches a stable minimum. Then a gradient of 150-575 mM NaCl in equilibration buffer (30 ml total volume) is run at 1 ml/min with collection of 0.4 min fractions. The peak of cPLA2a is found by using the radiometric enzyme assay (see main text). Fractions containing at least 40% of maximal activity are pooled, and the sample is concentrated in a YM-30 Centricon (Amicon) to a small volume. The buffer is exchanged into 25 mM Tris, pH 7.5, 150 mM KCl, 2 mM DTT by 4-5 cycles of dilution and re-concentration. Glycerol is added to give 30% by volume, and the $cPLA_2\alpha$ stock is stored at -20 °C. Based on the standard radiometric assay with calibration using recombinant $cPLA_2\alpha$ from baculovirus/Sf9 cell expression (see main text) about 3 μg of $cPLA_2\alpha$ is obtained. Analysis of the $cPLA_2\alpha$ by SDS-PAGE shows that the enzyme is fully phosphorylated (gel shifted, see main text).

As shown in Supplemental Information Fig. 5, western blot analysis of partially purified platelet $cPLA_2\alpha$ shows a complete gel shift relative to $cPLA_2\alpha$-PAP indicating that the enzyme is fully phosphorylated in thrombin-stimulated platelets. We have shown previously that this enzyme is phosphorylated stoichiometrically at both Ser-505 and Ser-727 (5)

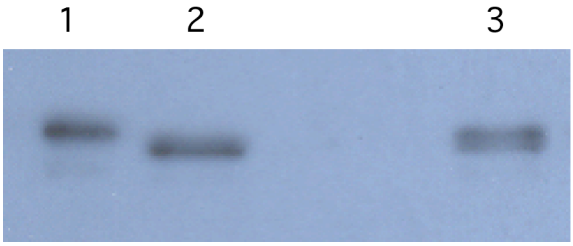
Studies with PIP_2 with low phospholipid concentration. We found that when an aliquot of the supernatant above the pelleted PAPC/PIP_2 vesicles was transferred to the standard radiometric $cPLA_2\alpha$ assay that the amount of dpm in the assay was significantly higher ((typically 3-fold) than the signal obtained when 5 ng of enzyme from the original stock solution was used (if enzyme did not bind to vesicles, 5 ng would have been transferred to the radiometric assay solution). This indicates that some of the PIP_2 was leaching out of the vesicles, i.e. it did not all pellet with the PAPC. To correct the interfacial binding curves for this effect, we took an aliquot of supernatant above pelleted PAPC/PIP_2 vesicles from a sample that did not contain enzyme and mixed it with 5 ng of enzyme from the stock solution and added this mixture to the standard radiometric assay to get the 100% value used to generate the interfacial binding plots. By use of radiolabeled ^{14}C-PAPC were are sure that all of this phospholipid pellets after ultracentrifugation.

The ~8-fold increase in specific activity of $cPLA_2\alpha$ when 10 mole % PIP_2 was added to 200 mM PAPC vesicles (Table Gelb 2) was not observed when the total

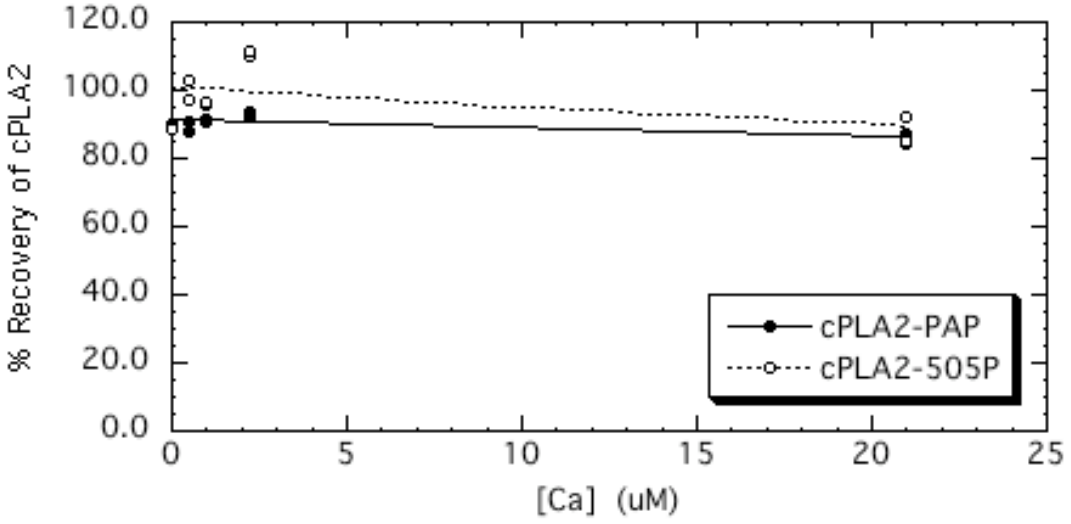
phospholipid concentration was reduced to 10 μM (data not shown). The only reasonable explanation for this is that most of the PIP_2 is not partitioned into 10 μM PAPC vesicles, which is consistent with the data presesnted in the above paragraph showing the PIP_2 is found in the supernatant above pelleted vesicles. The results suggest that PIP_2 is present as solitary monomers in the aqueous phase above 10 μM PAPC vesicles. This in turn suggests that the critical micelle concentration of the PIP_2 used in this study is not far from 10 μM. This is not unreasonable given that dioctanoylphosphatidylinositol, which contains shorter fatty acyl chains but lacks two head group phosphates, has a critical micelle concentration of 60 μM. Since the critical micelle concentration of the PIP_2 that we used is likely to be ~10 μM, we can estimate that most of the PIP_2 is present in the PAPC vesicles when 200 μM phospholipid is used.

References

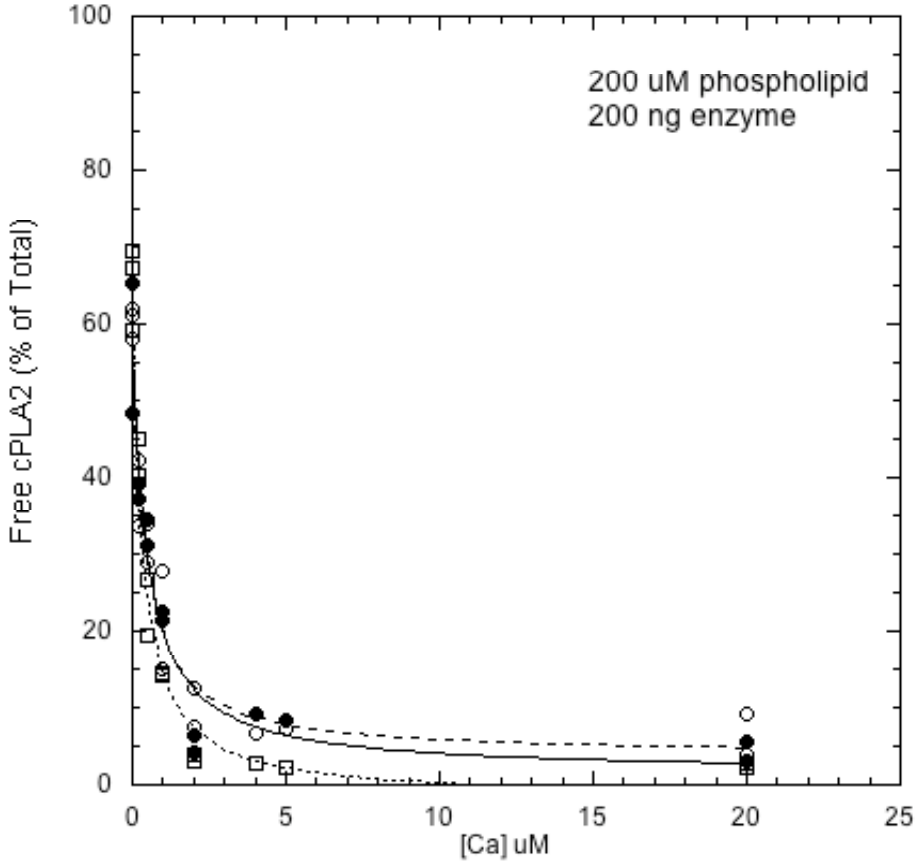
1. Hixon, M. S., Ball, A., Gelb, M . H. (1998) Calcium-dependent and independent interfacial binding and catalysis by cytosolic group IV phospholipase A_2. *Biochemistry* **37**, 8516-8526.
2. de Carvalho M. G., McCormack, A. L., Olson, E. Ghomashchi, F., Gelb, M. H., Yates, J. R., 3rd, Leslie, C. C. (1996) Identification of phosphorylation sites of human 85-kDa cytosolic phospholipase A_2 expressed in insect cells and present in human monocytes. *J. Biol. Chem*. **271**, 6987-6997.
3. Ghomashchi, F., Schuttel, S., Jain, M. K., Gelb, M. H. (1992) Kinetic analysis of a high molecular weight phospholipase A_2 from rat kidney: divalent metal-dependent trapping of enzyme on product-containing vesicles. Biochemistry, 31, 3814-3824.
4. Kramer, R. M., Roberts, E. F., Manetta, J. V., Hyslop, P. A., and Jakubowski, J. A. (1993) Thrombin-induced phosphorylation and activation of Ca(2+)-sensitive cytosolic phospholipase A_2 in human platelets. *J. Biol. Chem.* **268**, 26796-26804.
5. Borsch-Haubold, A. G., Bartoli, F., Asselin, J., Dudler, T., Kramer, R. M., Apitz-Castro, R., Watson, S. P., Gelb, M. H. (1998) Identification of the phosphorylation sites of cytosolic phospholipase A_2 in agonist-stimulated human platelets and HeLa cells. *J. Biol. Chem*. **273**, 4449-4458.



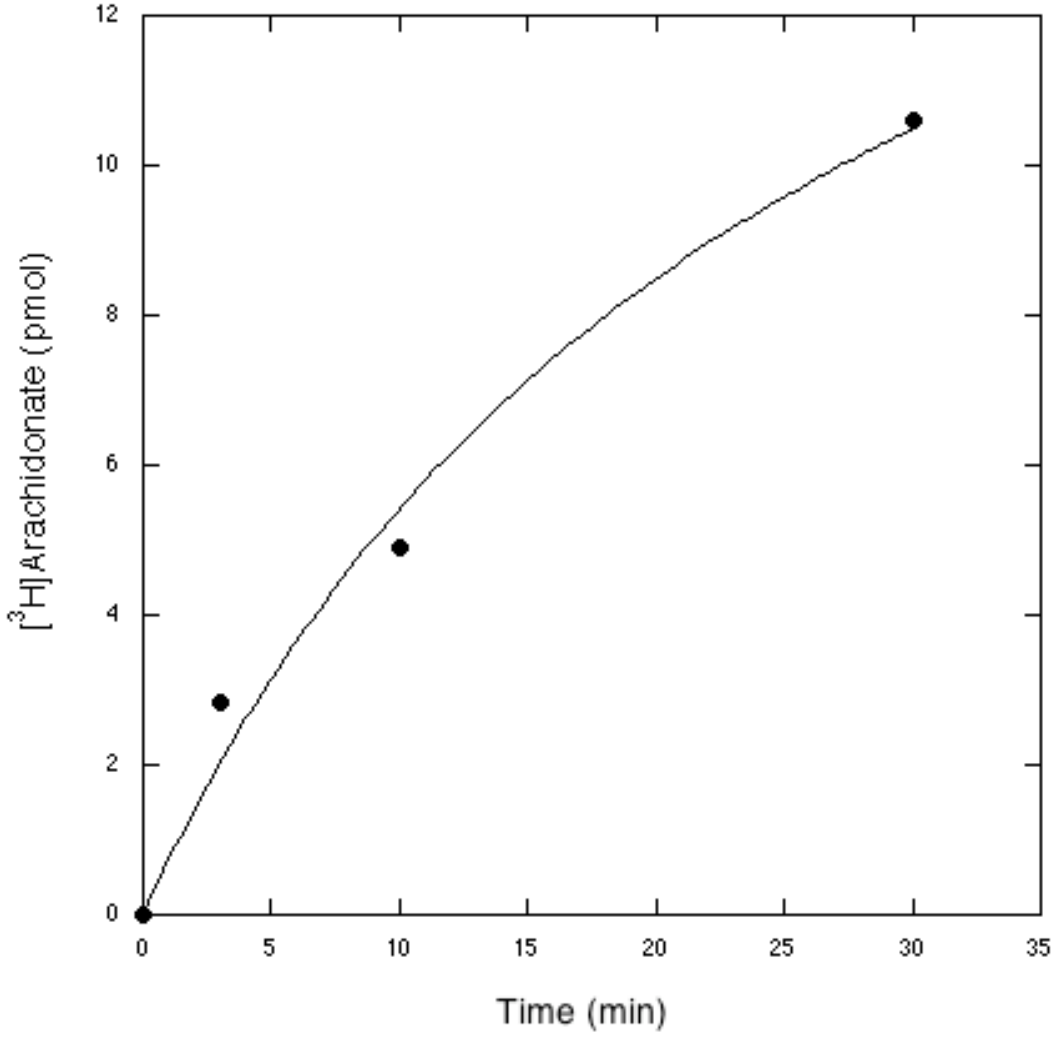
Supplemental Fig. 1. Western blot analysis of cPLA$_2\alpha$ in thrombin-stimulated platelets. Lane 1, partially purified cPLA$_2\alpha$ from thrombin-stimulated platelets; lane 2, cPLA$_2\alpha$-PAP; lane 3 platelet and cPLA$_2\alpha$-PAP co-loaded.



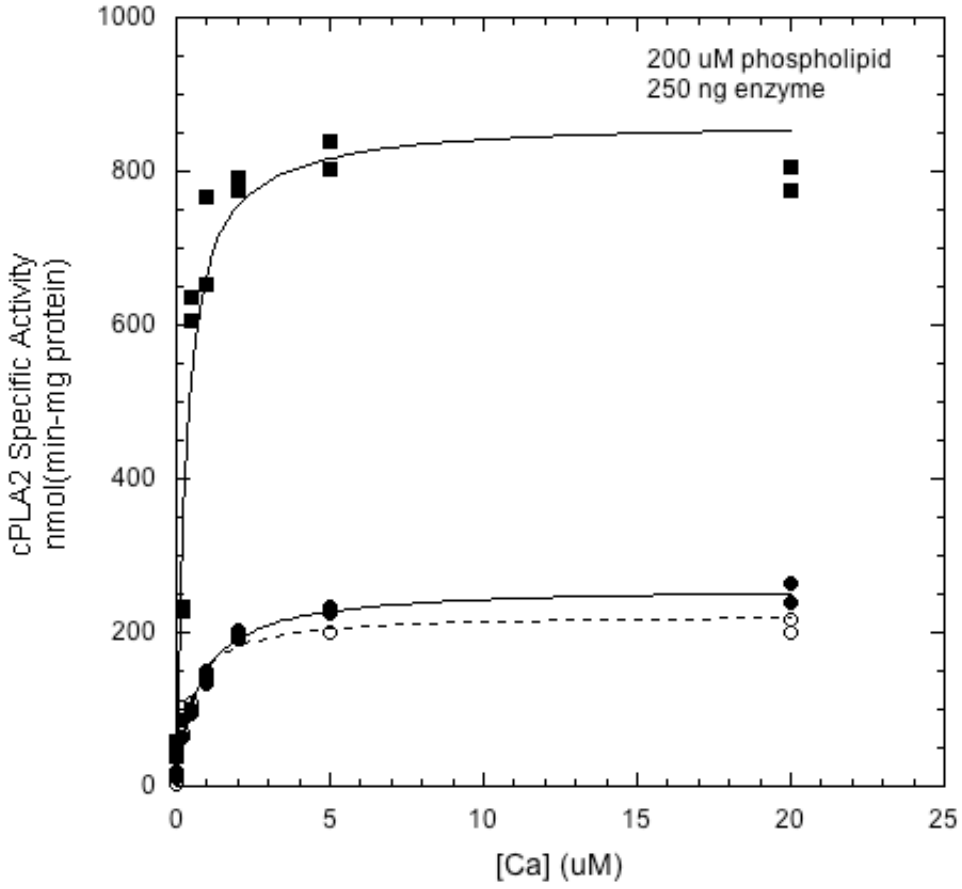
Supplemental Fig. 2. Lack of non-specific binding of cPLA$_2\alpha$ to the ultracentrifuge tube. Each sample contained 75 ng of cPLA$_2\alpha$-PAP or cPLA$_2\alpha$-505P in 1 ml of buffer A with 0.5 mg/ml BSA and with the indicated concentration of free calcium (no phospholipid present). Samples were submitted to ultracentrifugation followed by standard radiometric enzyme assay of a 100 μl aliquot of the supernatant (see Methods) to determine the percent recovery of enzyme, where 100% enzyme recovery is the standard radiometric assay dpm obtained with 7.5 ng of enzyme taken directly from the enzyme stock solution.



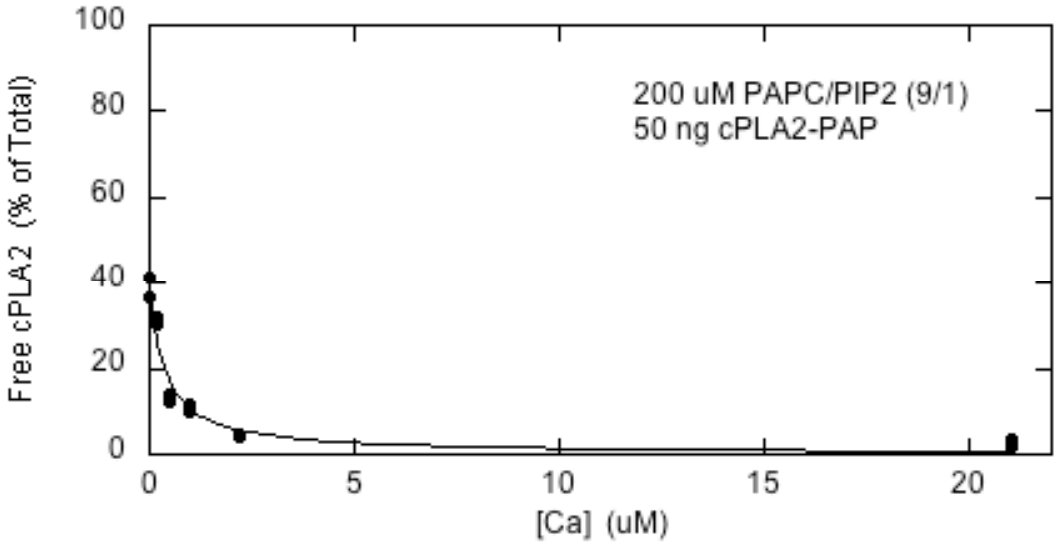
Supplemental Fig. 3. Interfacial binding of cPLA$_2\alpha$-PAP and cPLA$_2\alpha$-515P to PAPC vesicles. Conditions are the same as in Fig. 9 (main text): cPLA$_2\alpha$-PAP binding to PAPC vesicles (filled circles, solid line); cPLA$_2\alpha$-515P binding to PAPC vesicles (open circles, dashed line); cPLA$_2\alpha$-515P binding to PAPC/PIP2 (9/1) vesicles (open squares, dotted line). Each experiment was carried out in duplicate, and both are shown.



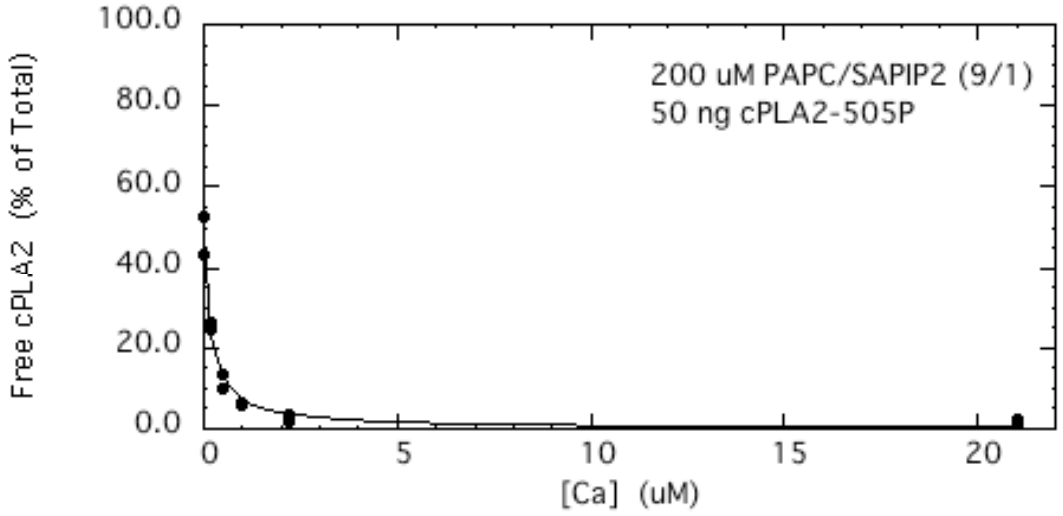
Supplemental Fig. 4. Time course for hydrolysis of ^{14}C-PAPC vesicles by cPLA$_2\alpha$-PAP. Reactions contained 200 μM phospholipid, 2 μM calcium and 200 ng of enzyme.



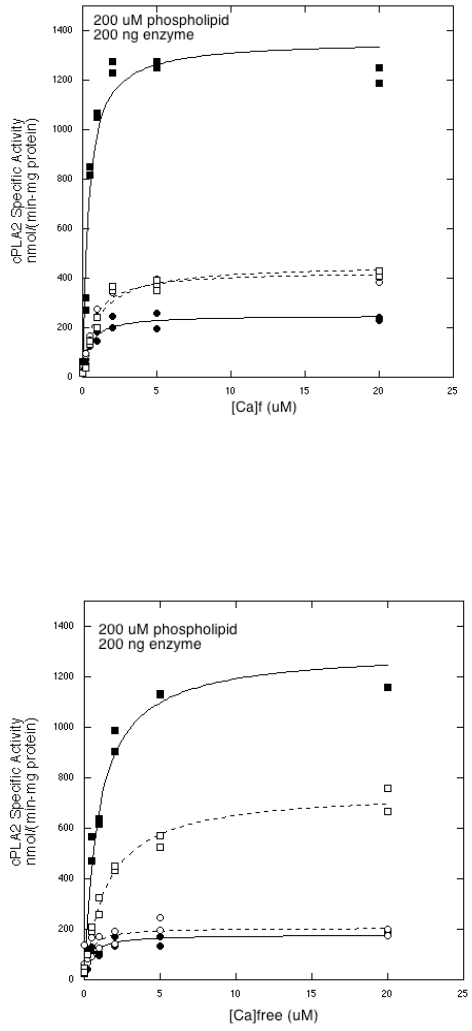
Supplemental Fig. 5. Interfacial kinetics of cPLA$_2$α-515P versus cPLA$_2$α-PAP. Reactions contained ^{14}C-PAPC with or without 10 mole % PIP$_2$ (200 μM total phospholipid), 250 ng of enzyme in buffer A containing 0.5 mg/ml BSA and the indicated calcium concentration. filled circle/solid line, ^{14}C-PAPC vesicles with cPLA$_2$α-PAP; open circles/dashed line, ^{14}C-PAPC vesicles with cPLA$_2$α-515P; filled squares/solid line, ^{14}C-PAPC/PIP$_2$ (9/1) vesicles with cPLA$_2$α-515P. Each experiment was carried out in duplicate, and both are shown.



Supplemental Fig. 6. Interfacial kinetics of cPLA$_2\alpha$-PAP to PAPC vesicles containing 10 mole% PIP$_2$. Conditions are the same as in Fig. 9 of the main text.



Supplemental Fig. 7. Interfacial kinetics of cPLA$_2\alpha$-505P to PAPC vesicles containing 10 mole% PIP$_2$. Conditions are the same as in Fig. 9 of the main text.



Supplemental Fig. 8. Interfacial kinetics of wild type versus mutant cPLA$_2\alpha$-PAP versus the calcium concentration. (Top) closed circles/solid line, cPLA$_2\alpha$-PAP on ^{14}C-PAPC; closed squares/solid line, cPLA$_2\alpha$-PAP on ^{14}C-PAPC/PIP; open circles/dashed line, cPLA$_2\alpha$-PAP-K488N/K543N/K544N on ^{14}C-PAPC; open squares/dashed line, cPLA$_2\alpha$-PAP-K488N/K543N/K544N on ^{14}C-PAPC/PIP$_2$. (B) closed circles/solid line, cPLA$_2\alpha$-PAP on ^{14}C-PAPC; closed squares/solid line, cPLA$_2\alpha$-PAP on ^{14}C-PAPC/PIP$_2$; open circles/dashed line, cPLA$_2\alpha$-PAP-K271N/K273N/R274N on ^{14}C-PAPC, $^{app}K_{Ca}$ = 0.3 ± 0.2; open squares/dashed line, cPLA$_2\alpha$-PAP-K271N/K273N/R274N on ^{14}C-PAPC/PIP. For both panels A and B, the anionic lipid is present at 10 mole % in ^{14}C-PAPC vesicles, and the total lipid concentration is 200 μM using 250 ng of enzyme. Analyses were carried out in duplicate and both data points are plotted.