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Identification of the Phosphorylation Sites of Cytosolic Phospholipase A_2 in Agonist-stimulated Human Platelets and HeLa Cells*

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Angelika G. Börsch-Haubold‡§, Fulvia Bartoli‡¶, Judith Asselin§, Thomas Dudler‡, Ruth M. Kramer¶, Rafael Apitz-Castro‖, Steve P. Watson§, and Michael H. Gelb‡ ‡‡**

From the ‡Departments of Chemistry and Biochemistry, University of Washington, Seattle, Washington 98195, the §Department of Pharmacology, University of Oxford, Mansfield Road, Oxford OX1 3QT, United Kingdom, the ‖Laboratory of Thrombosis, Centro de Biofisica y Bioquimica, IVIC, Caracas, Venezuela, and ¶Lilly Research Laboratories, Indianapolis, Indiana 46285

The present study identifies the phosphorylation sites of the 85-kDa cytosolic phospholipase A_2 ($cPLA_2$) in human platelets and HeLa cells. Tryptic digests of ^{32}P-phosphorylated and -immunoprecipitated $cPLA_2$ were analyzed by microbore high performance liquid chromatography and two-dimensional phosphopeptide mapping against synthetic phosphopeptide standards. Thrombin stimulated significant phosphorylation of platelet $cPLA_2$ at two sites, Ser-505 and Ser-727. Exclusive phosphorylation on these two sites was also seen in collagen-stimulated platelets and HeLa cells stimulated with interferon-α or arsenite; no tyrosine phosphorylation was detected. The inhibitor of the 38-kDa stress-activated protein kinase ($p38^{mapk}$), SB 203580, reduced phosphorylation of both Ser-505 and Ser-727 by 50 and 60%, respectively, in thrombin-stimulated platelets. An additional $p38^{mapk}$ inhibitor SB 202190 also partially (60%) inhibited the phosphorylation of $cPLA_2$ in arsenite-stimulated HeLa cells. These studies extend the previous work on the identification of multiple phosphorylation sites on $cPLA_2$ expressed in a baculovirus/insect cell system to $cPLA_2$ in mammalian cells stimulated with physiological agonists. They also underscore the necessity of high resolution phosphopeptide mapping combined with microbore high performance liquid chromatography for quantification of phosphorylation levels, which has lead to the conclusion that Ser-505 and Ser-727 are common phosphorylation sites on $cPLA_2$ in different mammalian cells stimulated with multiple agonists.

Cytosolic phospholipase A_2 ($cPLA_2$)[1] catalyzes the cleavage of arachidonic acid from the *sn*-2 position of phospholipids (1, 2). The 85-kDa enzyme is present in many mammalian cells (3), and strong evidence is accumulating for the role of $cPLA_2$ in the generation of tissue mediators that are metabolites of arachidonic acid, such as prostaglandins, leukotrienes, and thromboxanes. In contrast to the small molecular weight phospholipase A_2s that are secreted and are active on the outside of cells, $cPLA_2$ is regulated by intracellular signals that are propagated from surface receptors. One important regulatory mechanism appears to be a rise in the intracellular Ca^{2+} concentration which causes translocation of $cPLA_2$ from the cytosol to internal membranes (4–7) where it binds through a Ca^{2+}-dependent lipid-binding domain (8). A second, well established mechanism of the regulation of $cPLA_2$ activity is by phosphorylation on Ser-505 through a mitogen-activated protein kinase (MAPK) (9) which modestly increases the intrinsic activity of the lipase measured *in vitro* (3, 10, 11). Phosphorylation of $cPLA_2$ together with release of arachidonic acid has been observed in a variety of cells (11–16).

A thorough characterization of the phosphorylation sites of human $cPLA_2$ heterologously expressed in *Spodoptera frugiperda* (Sf9) cells by high performance liquid chromatography (HPLC), mass spectrometry, and protein sequencing has revealed four sites of phosphorylation: Ser-437, Ser-454, Ser-505, and Ser-727 (17). The kinases phosphorylating the three novel serine residues in Sf9 cells are not known. Interestingly, the protein phosphatase inhibitor okadaic acid causes a marked increase in arachidonic acid release from these Sf9 cells and a selective increase in the phosphorylation of $cPLA_2$ at Ser-727 (17). Moreover, phosphorylation of Ser-727 decreased the electrophoretic mobility of $cPLA_2$ on an SDS-polyacrylamide gel, a characteristic that has been frequently used in numerous previous studies of $cPLA_2$ phosphorylation in mammalian cells as a "selective" method to investigate Ser-505 phosphorylation. Thus, detailed phosphopeptide mapping and phosphorylation quantification studies are needed to obtain reliable information about the pattern of $cPLA_2$ phosphorylation in agonist-stimulated cells.

We have previously studied the regulation of $cPLA_2$ in human platelets stimulated by collagen and thrombin, two key physiological platelet agonists. Studies using the $p42/p44^{mapk}$ kinase inhibitor PD 98059 (18, 19) clearly showed that these kinases do not mediate the phosphorylation of $cPLA_2$ stimu-

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** Royal Society Research Fellow.

‡‡ To whom correspondence should be addressed: Depts. of Chemistry and Biochemistry, Box 351700, University of Washington, Seattle, WA 98195. Fax: 206-685-8665; E-mail: gelb@chem.washington.edu (or A. G. Börsch-Haubold, Dept. of Pharmacology, Mansfield Road, Oxford OX1 3QT, UK. Fax: 44-1865-271853; E-mail: angelika.borsch@pharmacology.oxford.ac.uk).

[1] The abbreviations used are: $cPLA_2$, cytosolic phospholipase A_2; MAPK, mitogen-activated protein kinase; Sf9 cell, *Spodoptera frugiperda* cell; HPLC, high performance liquid chromatography; $p38^{mapk}$, 38-kDa stress-activated protein kinase; SB 203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole; PAGE, polyacrylamide gel electrophoresis; mbHPLC, microbore HPLC; MAPKAP-K2, MAPK-activated protein kinase 2; SB 202190, 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl) imidazole; S437-PP, serine 437-containing phosphopeptide; S454-PP, serine 454-containing phosphopeptide; S727-PP, serine 727-containing phosphopeptide; S505A $cPLA_2$, $cPLA_2$ with serine 505 mutated to alanine; Me_2SO, dimethyl sulfoxide.

lated by thrombin or collagen, and that they are not involved in the process of arachidonic acid release from platelets (20). In addition, protein kinase C does not seem to directly regulate platelet $cPLA_2$ (21). In contrast, inhibition of the 38-kDa stress-activated protein kinase ($p38^{mapk}$) by the anti-inflammatory drug 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole (SB 203580) (22, 23) reduced both thrombin-stimulated incorporation of ^{32}P into $cPLA_2$ and thrombin-stimulated increase in $cPLA_2$ activity (measured in platelet lysates) by 50% (24). Under these conditions of partial inhibition by SB 203580 in the presence of thrombin, the $cPLA_2$ SDS-polyacrylamide gel electrophoresis (PAGE) gel shift, which is the commonly used assay of $cPLA_2$ phosphorylation, was completely blocked by treatment with the kinase inhibitor (24). In contrast, both ^{32}P incorporation into $cPLA_2$ and *in vitro* activity were nearly completely inhibited by SB 203580 when platelets were stimulated with collagen (25). Similar results were also obtained by the use of agonists that only partially stimulate platelet responses (26, 27). These results and the observation of multiple phosphorylation sites on $cPLA_2$ expressed in Sf9 cells raise the possibility that platelet $cPLA_2$ is phosphorylated on one or more residues other than Ser-505, and they also leave doubts about the usefulness of the indirect gel shift assay for assessing the level and site(s) of $cPLA_2$ phosphorylation. Thus, high resolution phosphopeptide mapping studies are needed, and these are described in the present study.

Recent studies suggest that $cPLA_2$ is phosphorylated on tyrosine in HeLa cells stimulated with interferon-α (28). $cPLA_2$ could be co-immunoprecipitated with the protein tyrosine kinase Jak1, and transfection studies suggest that Jak1 is necessary for $cPLA_2$ activation in this system. In light of studies in other cell types showing phosphorylation of $cPLA_2$ only on serine, the possibility of $cPLA_2$ tyrosine phosphorylation was investigated in the present study using phosphopeptide mapping.

Analysis of $cPLA_2$ phosphorylation described here was carried out in part with microbore HPLC (mbHPLC) for the accurate quantification of the levels of site-specific $cPLA_2$ phosphorylation when only small amounts of protein obtained from non-transfected mammalian cells are available. It is shown that mbHPLC gives reliable quantification data; two-dimensional phosphopeptide mapping on cellulose plates does not because of sample losses.

MATERIALS AND METHODS

Reagents and Antibodies—Polyclonal anti-$cPLA_2$ antiserum was obtained as described previously (11, 17). Antibody against MAPK-activated protein kinase 2 (MAPKAP-K2) was purchased from Upstate Biotechnology Inc. (TCS Biologicals Ltd., Bucks, UK), and substrate peptide was synthesized by Alta Bioscience Ltd. (Birmingham, UK). SB 203580 was kindly provided by Dr. J. C. Lee (SmithKline Beecham, King of Prussia, PA), and SB 202190 (4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)imidazole) was purchased from Calbiochem. $^{32}P_i$ (dissolved in H_2O, 8,500–9,120 Ci/mmol) and [γ-^{32}P]ATP (3,000 Ci/mmol) were obtained from NEN Life Science Products Inc. Sequencing grade, modified trypsin came from Promega and activated recombinant $p42^{mapk}$ from Calbiochem. mbHPLC standard peptides S437-PP ($cPLA_2$ amino acids 428–445, Ser-437 phosphorylated), S454-PP (446–467, Ser-454 phosphorylated), and S727-PP (721–736, Ser-727 phosphorylated) were synthesized by Multiple Peptide Systems (San Diego, CA). Peptides were purified by reverse phase HPLC, and their structures were confirmed by electrospray mass spectrometry. Prostacyclin was kindly donated by Wellcome Laboratories (Beckenham, Kent, UK). Bovine thrombin, staurosporine, genistein, okadaic acid, aprotinin, leupeptin, and pepstatin A were purchased from Sigma, and microcystin-LR came from Calbiochem. Collagen was obtained from Nycomed Arzneimittel (Munich, Germany). Grace's supplemented medium was obtained from Life Technologies, Inc. Phosphate-free Grace's medium with glutamine was obtained from Speciality Media Inc. (catalog GR-005-B) and supplemented with yeastolate (catalog IM-123-D) and lactalbumin hydrolysate (catalog IM-113-D) from the same company.

Phosphorylation Studies on $cPLA_2$ Derived from Sf9 Cells—$cPLA_2$ expressed in Sf9 cells was radiolabeled with $^{32}P_i$ essentially as described (17) except that the following amounts of cells and $^{32}P_i$ were used. To each of two T-75 flasks was added 10 ml of Sf9 cells (0.9×10^6/ml) in Grace's supplemented medium containing 10% fetal bovine serum and penicillin G/streptomycin/fungizone (100-fold dilution of catalog number 15240-062 from Life Technologies, Inc.) at 27 °C for 1 h. Adherent cells were infected with the recombinant baculovirus expressing human $cPLA_2$ (29) at a multiplicity of infection of 18. Twenty-four hours after infection, cells in each flask were rinsed twice in Grace's supplemented phosphate-free medium containing 10% fetal bovine serum and penicillin G/streptomycin/fungizone and were labeled with 10 mCi of $^{32}P_i$ at 27 °C. After 48 h, cells were rinsed twice in phosphate-free medium, and then stimulated with 1 μM okadaic acid for 3 h. After two rinses, cells from each flask were lysed on ice in 1 ml of lysis buffer (50 mM HEPES, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 100 μM leupeptin, 200 μM Na_3VO_4, 10 mM $Na_4P_2O_7$, 3 μM *p*-nitrophenyl phosphate) and insoluble material was removed by microcentrifugation. $cPLA_2$ was immunoprecipitated using polyclonal anti-$cPLA_2$ antiserum (R11783 antiserum, 1:7.5 dilution) as described (17). The combined supernatants from each immunoprecipitate were loaded into a 55-mm wide preparative well (or two 28-mm wide wells) of a 1 mm-thick 10% SDS-PAGE mini-gel (Bio-Rad Mini-PROTEAN II). Gels were run at 130 V until the bromphenol blue dye front just reached the bottom, and the bottom 2 cm of the gels were discarded for safety reasons. Gels were dried and phosphorylation was visualized by autoradiography.

Preparation of $^{32}P_i$ Radiolabeled Platelets—Blood (100 ml) was drawn on the day of experiment from healthy volunteers by venepuncture using acidic citrate dextrose solution as anticoagulant (21). Platelet-rich plasma was separated from red blood cells by centrifugation at $200 \times g$ for 20 min, and platelets were isolated by centrifugation at $1{,}000 \times g$ for 10 min in the presence of 0.1 μg/ml prostacyclin at 30 °C. The platelet pellet was gently resuspended in 1 ml of phosphate-free platelet buffer (25 mM HEPES, pH 7.3, 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose), and indomethacin (10 μM) was added to block cyclooxygenase. Platelets were radiolabeled with 25 mCi of $^{32}P_i$ at 30 °C for 1.5–2 h. The platelet suspension was washed with platelet buffer containing acidic citrate dextrose solution and prostacyclin and centrifuged at $1{,}000 \times g$ for 10 min. The pellet was resuspended in platelet buffer at an approximate concentration of 1×10^9 platelets/ml. Indomethacin (10 μM) was added, and platelets were left at 30 °C for 30 min prior to experimentation. EGTA (1 mM) was added just before stimulation.

Stimulation of Platelets and Immunoprecipitation of $cPLA_2$—The platelet suspension was distributed into 500-μl aliquots and prewarmed to 37 °C. When required, platelets were incubated in the presence of Me_2SO (1%) or SB 203580 (20 μM) for 15 min. Platelets were gently shaken on a moving platform during stimulation with thrombin or stirred in an aggregometer at 1,200 rpm during stimulation with collagen. After addition of 500 μl of ice-cold lysis buffer (final concentrations: 1% Triton X-100, 0.5% SDS, 0.75% deoxycholate, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 100 μg/ml aprotinin, 10 μM pepstatin A, 1 μM microcystin-LR, 5 μM staurosporine, 5 μM genistein, 10 mM $Na_4P_2O_7$, 50 mM sodium fluoride, 200 μM Na_3VO_4), insoluble material was removed by microcentrifugation, and lysates were pre-cleared using 20 μl of protein A-Sepharose CL-4B (1:2 in H_2O, v/v). $cPLA_2$ was immunoprecipitated at 4 °C overnight using 2 μl of polyclonal anti-$cPLA_2$ antiserum (11) and 20 μl of protein A-Sepharose CL-4B slurry. Immunoprecipitates were washed as described previously (21), extracted with Laemmli sample buffer, and proteins were resolved by 10% SDS-PAGE. For the preparation of $cPLA_2$ for mbHPLC analysis, the extracts of 8 immunoprecipitates, derived from 8 aliquots of platelet lysate, were pooled in a 55-mm wide preparative well. To control for the degree of phosphorylation in each experiment, single immunoprecipitates from stimulated and non-stimulated platelets were put onto a separate gel.

Phosphorylation of $cPLA_2$ by $p42^{mapk}$ in Vitro—Wild-type human $cPLA_2$ was expressed in Sf9 cells and purified (29). His-tagged S505A $cPLA_2$ mutant was expressed in Sf9 cells and purified using standard Ni(II) immobilized-ion affinity chromatography procedures (30). Both proteins were dialyzed against 25 mM HEPES, pH 7.4. Ten micrograms of dialyzed $cPLA_2$ was incubated with 4 units of activated $p42^{mapk}$ (1 unit = 1 pmol of phosphorylation of myelin basic protein/min) at 30 °C in kinase buffer (25 mM HEPES, pH 7.4, 1 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM EGTA, 100 μM ATP, 100 μM Na_3VO_4, 200 μCi/ml [γ-^{32}P]ATP) in a total volume of 25 μl. The kinase reaction was stopped

after 30 min by the addition of 4 × Laemmli sample buffer. Samples were boiled, subjected to 10% SDS-PAGE, and gels were dried.

Preparation of Tryptic Digests of cPLA$_2$—The exact position of cPLA$_2$ on dried gels was located by autoradiography. ^{32}P of cPLA$_2$ in dried SDS-PAGE gels was quantified either with a PhosphorImager or by densitometry analysis of autoradiographs obtained with different exposure times. cPLA$_2$ was eluted from the gel, precipiated with trichloroacetic acid, washed with acetone, and digested with trypsin as described (17). Yields were constantly monitored by Cerenkov counting. Fifty-two to 60% of the counts/min of platelet cPLA$_2$ (67–75% of cPLA$_2$ from *in vitro* kinase experiments) was eluted from the gel slices (two elutions), and >95% of the eluted cPLA$_2$ was recovered after precipitation with trichloroacetic acid and washing with acetone.

mbHPLC—Each 200-μl frozen tryptic digest was thawed and analyzed by mbHPLC. To the digest was added a mixture of synthetic peptide standards (S437-PP, S454-PP, and S727-PPox), and the entire mixture was injected onto the mbHPLC system. Sufficient amounts of synthetic phosphopeptides were added to give an approximate full-scale detector response using the mbHPLC system described below (predetermined by analyzing the standards alone). mbHPLC was carried out with a Titanium Dynamax SD-200 HPLC system (Rainin) with PEEK tubing. The eluant from the pumps was connected to a low dead volume PEEK splitter (VICI Instruments MY1CPK). One outlet was connected to the inlet of a titanium Rheodyne injector (1-ml PEEK sample loop), and the other outlet to a standard analytical HPLC column (218TP52, Vydac Inc., virtually any analytical column can be used since its purpose is to provide back pressure to the splitter). The outlet of the injector was connected to the mbHPLC column (Vydac 901-3151-00, 0.5 × 150 mm, Michrom BioResources, Inc.). The outlet of the mbHPLC column was connected to the UV detector (Dionex detector equipped with a 2-μl PEEK flow cell). The outlet of the flow cell was connected to a 12-inch length of narrow-bore PEEK tubing (0.005 inch inner diameter) to provide sufficient back pressure to prevent outgassing in the flow cell. The flow rate through the mbHPLC column was adjusted to give 30 μl/min (total system flow rate was about 0.2 ml/min). The column was developed with 100% solvent A (0.06% CF_3COOH in Milli-Q H_2O (Millipore)) for 15 min, to 30% solvent B (0.06% CF_3COOH in CH_3CN (HPLC grade, Baker)) over 60 min, to 60% solvent B over 30 min, to 100% solvent B over 10 min, and then for 45 min at 100% solvent B. Eluting peptides were detected by monitoring the absorbance at 210 nm with the detector range set at 0.1–0.2 absorbance units full scale. Fractions were collected every 15 min during the first hour and then every 2 min into silicanized polypropylene tubes (VWR number 20172-935). Radioactivity was monitored by Cerenkov counting. Appropriate radioactive fractions were pooled for oxidation and/or two-dimensional phosphopeptide mapping.

The yield of eluted phosphopeptides from the mbHPLC column was determined by summing the counts/min from all mbHPLC fractions from 0 to 160 min and dividing this number by the counts/min injected onto the column. This latter number was obtained by Cerenkov counting of the tryptic digest tube before and after its contents were injected onto the mbHPLC. Typically, >90% of the counts/min in the digest was injected onto mbHPLC. The yield of eluted phosphopeptides in platelet experiments where less than 3,000 cpm were applied onto the column was 82–98%.

Phosphopeptide Maps—Pooled mbHPLC fractions or tryptic digests were dried in a Speed-Vac. Phosphopeptides were dissolved in 10 μl of glacial CH_3COOH and spotted onto 20 × 20-cm cellulose thin-layer chromatography (TLC) plates (Kodak number 13255), which were developed by electrophoresis (1,400 V for 20 min) and ascending chromatography as described (17). ^{32}P-Phosphorylated peptides were located with a PhosphorImager or by autoradiography.

Oxidation of Peptides with Performic Acid—mbHPLC fractions were dried in a Speed-Vac at low temperature. Oxidation with performic acid was as described (31). Peptides were dissolved in 10 μl of glacial CH_3COOH for two-dimensional maps or in 50 μl of H_2O for mbHPLC, and solubility was checked by Cerenkov counting.

Measurement of MAPKAP-K2 Activity—MAPKAP-K2 was immunoprecipitated from platelet lysates (5×10^8 platelets/lysate) for 2.5 h at 4 °C using 1 μl of antiserum and 20 μl of protein A-Sepharose CL-4B slurry. Immunoprecipitates were collected by microcentrifugation and washed three times in 20 mM Tris, pH 7.6, containing 137 mM NaCl and 0.1% Tween 20. For the *in vitro* kinase assay, immunoprecipitates were incubated with 40 μl of 50 mM Tris, pH 7.0, 100 μM EGTA, 10 mM $MgCl_2$, 100 μM ATP, 50 μCi/ml [γ-^{32}P]ATP, and 30 μM substrate peptide KKLNRTLSVA (32) at 37 °C. After 10 min, samples were briefly microcentrifuged. The kinase reaction was stopped by pipetting 30 μl of supernatant onto P-81 phosphocellulose paper (Whatman) and washing these in 75 mM phosphoric acid. Radioactivity of the dried filter papers was determined by liquid scintillation counting.

Studies with HeLa Cells—HeLa S3 cells were obtained from ATCC and cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.; 11965-092) containing 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 at 37 °C. For radiolabeling, cells were grown in 25-cm^2 flasks (T25) to 70% confluence. Cells were washed three times with phosphate-free Dulbecco's modified Eagle's medium (Life Technologies, Inc.; 11971-025) containing 0.5% bovine serum albumin and no fetal bovine serum and then labeled in 1 ml of the same medium containing 1 or 5 mCi of carrier-free $^{32}P_i$. After a 4-h labeling period, cells were either left untreated or stimulated for 20 min with 1,000 units/ml interferon-α (Accurate Chemical & Scientific Inc.) or for 30 min with 0.5 mM sodium arsenite. Stimulations were stopped by placing the flasks on ice, and cells were washed twice with ice-cold medium. Cells were lysed with 0.5 ml of cold lysis buffer, and the cleared supernatant was treated with 1 μl of R11683 anti-cPLA$_2$ antiserum to immunoprecipitate cPLA$_2$ as described (17). Immunoprecipitates were submitted to SDS-PAGE, and gel bands were processed for trypsin as described above for platelet studies. Half of each digest was submitted to mbHPLC, one-third to two-dimensional phosphopeptide mapping, both as described above for platelets, and one-sixth to phosphoamino acid analysis as described (17). In these mbHPLC studies a conventional analytical flow cell was used in the detector (Rainin Dynamax with a 7200–075 flow cell). The delay between UV detection and emergence of counts/min from the tubing outlet was measured by co-injecting bovine serum albumin and $^{32}P_i$ and monitoring UV in real time and counts/min in 2-min fractions.

RESULTS

Analysis of the Tryptic Digest of cPLA$_2$ Produced in Sf9 Cells—The previous characterization of phosphorylation sites of cPLA$_2$ expressed in Sf9 cells has been performed on a C18 reverse phase HPLC column with samples that contained approximately 100,000 cpm (17). To be able to analyze the ^{32}P-phosphorylation of cPLA$_2$ derived from native cells, where the amount of cPLA$_2$ comprises only a fraction of the cPLA$_2$ expressed in Sf9 cells, we set up a C18 mbHPLC column. As internal standards, the three phosphopeptides S437-PP, S454-PP, and S727-PP corresponding to possible phosphorylated tryptic digest fragments of cPLA$_2$ (17) were synthesized. The peptide bearing the MAPK phosphorylation site Ser-505 (residues 489–533) was too long to be readily chemically synthesized, and thus it was generated by trypsin digestion of cPLA$_2$ that had been phosphorylated *in vitro* with p42^{mapk} (see below). Typical retention times of the standard peptides from the mbHPLC column were 70 min for S437-PP, 84 min for S727-PP, and 84 and 85 min for S454-PP, which was resolved as a doublet (due to the presence of an impurity). Since S727-PP was previously found to be oxidized on a cysteine residue during the process of its isolation (17), it was oxidized (S727-PPox) with performic acid which resulted in a retention time of 79–80 min and thus well separated from S454-PP. The retention times of these standard peptides were highly reproducible between runs (variation < 1 min), and they were co-injected with trypsin digests in every run. The standard peptides eluted from the mbHPLC column in the same order as seen using a Vydac 218TP52 C18 column (S437-PP 63 min, S727-PPox 73 min, S454-PP 77 min; not shown) but the retention times were delayed by 7 min. We therefore expected the Ser-505 containing peptide to elute well after 105 min, the retention time on the 218TP52 column (17).

To evaluate the mbHPLC method and to generate authentic cPLA$_2$ tryptic peptides, studies were first carried out with cPLA$_2$ from Sf9 cells, and the results compared with the earlier study (17). Tryptic digests of ^{32}P-phosphorylated cPLA$_2$ from okadaic acid-stimulated Sf9 cells were prepared and subjected to mbHPLC together with a mixture of the three standard peptides. Major radioactive fractions eluted with retention times of 72, 90, and 134 min (peaks), and the standard peptides were detected at 70.7 (S437-PP), 80.4 (S727-PPox), and 85.1

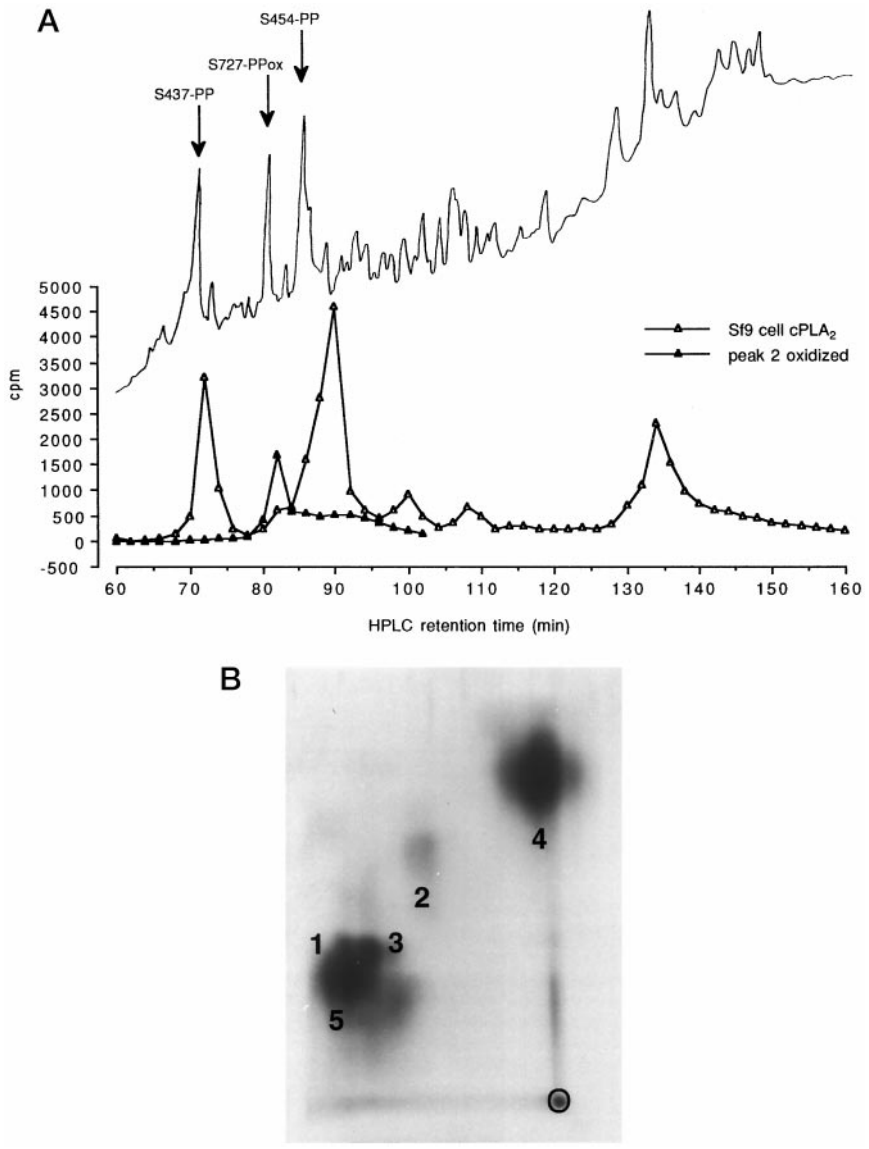

FIG. 1. **mbHPLC and two-dimensional phosphopeptide map of tryptic peptides derived from cPLA$_2$ from okadaic acid-stimulated Sf9 cells.** *A,* part of the digest (50,000 cpm) was applied onto the mbHPLC column (Δ). Fractions constituting peak 2 were pooled, and the material was oxidized with performic acid and subjected to mbHPLC (▲). The trace at the *top* is the absorbance at 210 nm. *B,* a portion of the tryptic digest (15,000 cpm) was spotted onto a cellulose plate for a two-dimensional phosphopeptide map (origin marked by ○). Proteins were separated by electrophoresis (horizontal dimension, anode at *left*) and ascending chromatography (vertical dimension). Phosphorylated peptides were detected by autoradiography. R_F values (second dimension) are: *spot 1*, 0.38; *spot 2*, 0.61; *spot 3,* 0.37; *spot 4*, 0.81.

min (S454-PP) (Fig. 1*A*). The first radioactive peak is the Ser-437-phosphorylated peptide. The third radioactive peak observed after 130–140 min co-eluted with a large peptide peak detected by UV absorbance (132.2 min) on the HPLC chart (Fig. 1*A*). This is similar to the co-elution of the Ser-505-containing tryptic fragment around 105 min with a major peptide peak from the 218TP52 column (17). This 130–140-min peak co-eluted with the radiolabeled tryptic phosphopeptide prepared by *in vitro* phosphorylation of cPLA$_2$ with p42^{mapk} (see below).

The identification of the 90-min peak was less obvious, as this peak was relatively broad and eluted well after the S727-PPox and the S454-PP standards. We explored the possibility that this 90-min peak is the unoxidized S727-PP. The radioactive fractions of this peak were therefore pooled, oxidized with performic acid, and again subjected to mbHPLC. This treatment resulted in a shift of the peak to a shorter retention time (82 min) that corresponded to the retention time of the standard S727-PPox (81.2 min) (Fig. 1*A*). S454-PP does not contain any oxidizable amino acids and may constitute a minor part of the 90-min peak (before oxidation) and it may possibly be responsible for the small amount of radioactivity of the fractions eluting around 90 min in the analysis of the oxidized sample. This is in agreement with the previous finding that Ser-454 is only a minor phosphorylation site of cPLA$_2$ in okadaic-treated Sf9 cells (17). These results suggest that in the present study, unlike previously (17), the putative Ser-727 phosphopeptide was isolated in its unoxidized form.

To further analyze the phosphopeptides derived from tryptic digests of cPLA$_2$, the [^{32}P]phosphopeptides recovered from the mbHPLC separation and those of the whole tryptic digests were resolved on cellulose TLC plates in two dimensions. The positions of the major spots were compared with previous results (17) and numbered in the same way. Spot 4 is the Ser-505-containing phosphopeptide, which virtually does not move in the first dimension, during electrophoresis, but travels close to the solvent front in the second dimension, on ascending TLC (R_F = 0.81; Fig. 1*B*). An equivalent spot was detected as the sole radioactive peptide when fractions of different mbHPLC experiments that were eluting after 134 min were applied (discussed below). Spots 1 (S437-PP) and 3 (S454-PP) moved furthest in direction of the anode and had R_F values of 0.38 and 0.37, respectively (Fig. 1*B*). Spot 5 (just below and to the left of spot 1) was previously identified as a degradation product of the S437-PP (17). Spot 2, putatively S727-PP, was detected near a diagonal connecting spot 4 and spots 1/3 and had an R_F value of 0.61. When peptides of the mbHPLC fractions eluting around 84 min (Fig. 1*A*) were oxidized, spot 2 was located at a position nearer to the anode with an R_F value of 0.53, the

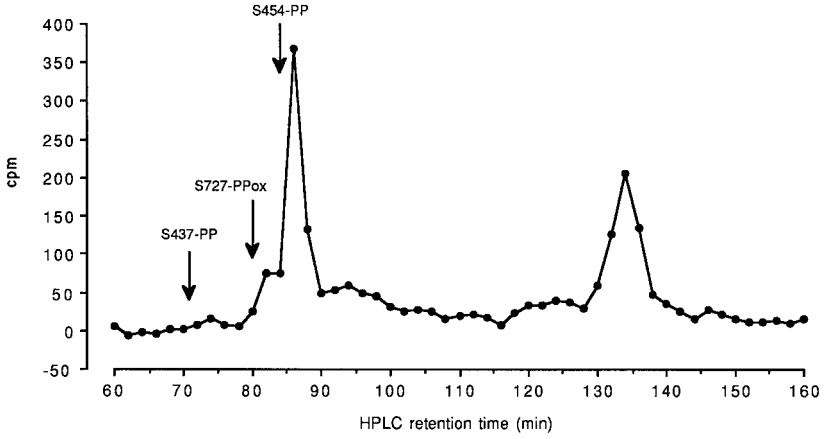

FIG. 2. **mbHPLC of tryptic peptides derived from cPLA$_2$ immunoprecipitated from thrombin-stimulated human platelets.** Platelets were stimulated with 2 units/ml thrombin for 5 min at 37 °C and a portion of the tryptic digest of cPLA$_2$ (3,800 cpm) was loaded onto the mbHPLC column. Retention times of the standard peptides were: S437-PP 71 min, S727-PPox 80 min, and S454-PP 85 min. A major OD_{210} peptide peak was detected after 133 min. Similar results were obtained in four independent experiments.

position of the oxidized S727-PP (17), as will be discussed later.

Two Major Phosphorylation Sites of cPLA$_2$ in Thrombin-stimulated Human Platelets—Having shown that the mbHPLC system is reliable for the analysis of cPLA$_2$-derived phosphopeptides, we turned to the identification of the phosphorylation sites of cPLA$_2$ from human platelets. After $^{32}P_i$ labeling of platelets and stimulation with thrombin, cPLA$_2$ was purified by immunoprecipitation and SDS-PAGE, digested with trypsin, and subjected to mbHPLC. Two radioactive fractions eluted at 82–88 min and at 130–138 min (Fig. 2). The column was eluted for up to 180 min (0.06% CF_3COOH in CH_3CN from 115 to 180 min), and there were no further phosphopeptides detected. The yield of counts/min in all mbHPLC fractions was typically 82–98% of those applied, and approximately 6% (6–11% in other runs) of the counts/min eluted in the void volume. Thus it can be concluded that the two phosphopeptides detected represent the major sites of phosphorylation on cPLA$_2$ from thrombin-stimulated platelets. The ratio of radioactivity of the two peaks was approximately 1:1, as determined by summing up the counts/min of the 4–5 radioactive fractions constituting each peak. This ratio did not change when platelets were stimulated with thrombin for 2 min instead of 5 min, or in the presence of 1 mM external Ca^{2+} instead of EGTA. Also, doubling the amount of trypsin in the digest did not alter this pattern (not shown), which suggests that the phosphopeptides are not partial tryptic fragments.

The radioactive fractions after 134 min co-eluted with a major OD_{210} peptide peak that was detected at 133 min of the run, and, according to the Sf9 cell standard described above, seems to be the Ser-505-containing peptide. The peak eluting at 82–88 min (Fig. 2) is likely to be unoxidized S727-PP since it co-migrated with authentic S727-PP. To obtain more information about the phosphopeptide(s) eluting from the mbHPLC column between 82 and 88 min (Fig. 2), these fractions were oxidized with performic acid and again subjected to mbHPLC. The radioactive peak shifted to a shorter retention time (78–80 min) and co-eluted with the S727-PPox standard (not shown) similar to the results described above for the Sf9 cell experiment (Fig. 1).

The entire tryptic digest of cPLA$_2$ from thrombin-stimulated platelets was subjected to two-dimensional TLC (Fig. 3*A*). Two closely migrating spots (4 and 4a) were seen which stayed close to the origin in the first dimension and which both ran in the second dimension with an R_F of 0.86. This is characteristic of the Ser-505 phosphopeptide (17). In this previous study it was shown that the Ser-505 phosphopeptide can be partly oxidized during its isolation and that the oxidized material migrates just to the left of the unoxidized phosphopeptide in the first dimension. Similarly, the phosphopeptide recovered from the second mbHPLC peak (Fig. 2) stayed near the origin during

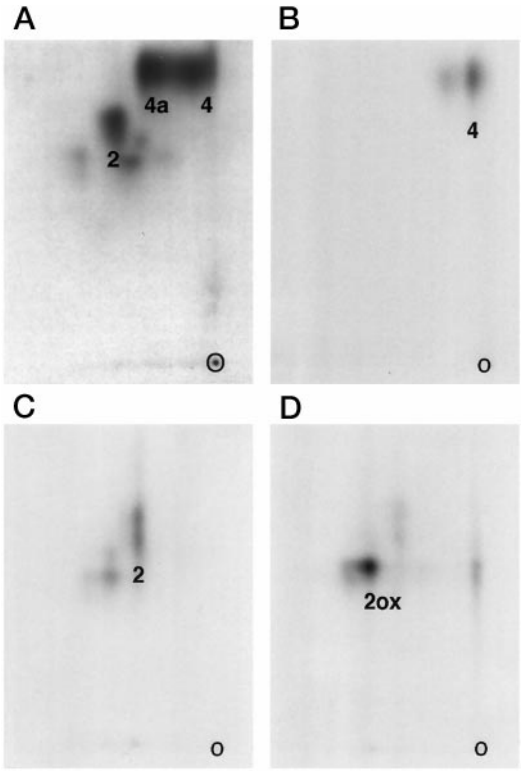

FIG. 3. **Two-dimensional tryptic phosphopeptide maps of ^{32}P-labeled cPLA$_2$ from thrombin-stimulated platelets.** *A*, the whole tryptic digest (800 cpm) was applied. *B*, the pool of mbHPLC fractions eluting between 128 and 138 min (mbHPLC peak 2, Fig. 2) was dried in a Speed-Vac, and 400 cpm was applied. *C*, a portion of the pool of mbHPLC fractions eluting between 78 and 90 min (mbHPLC peak 1, Fig. 2) was applied (400 cpm). *D*, a portion of the pool of mbHPLC fractions eluting between 78 and 90 min was oxidized with performic acid and 400 cpm was applied. Phosphopeptides were detected with a PhosphorImager and by autoradiography.

electrophoresis and moved with an R_F value of 0.88 during chromatography (Fig. 3*B*). Thus, the map location of the phosphopeptide constituting the second radioactive peak of the mbHPLC analysis identifies it as the Ser-505-phosphorylated tryptic fragment.

Spot 2 of the tryptic digest in Fig. 3*A* had an R_F value of 0.69 and moved similarly to the S727-PP derived from Sf9 cells (spot 2, Fig. 1*B*). Since this peptide was previously characterized in its oxidized form (17), the radioactive mbHPLC fractions of the first peak (Fig. 2) were pooled, and half of the amount of the peptide was oxidized with performic acid. The unoxidized sample resolved on the TLC plate into two phosphopeptides (Fig. 3*C*), moving in the second dimension with R_F values of 0.63

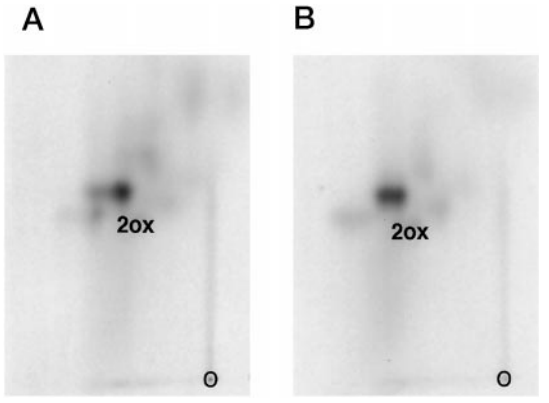

FIG. 4. **Two-dimensional maps of the tryptic phosphopeptides derived from platelet and Sf9 cell cPLA$_2$ co-eluting on mbHPLC with the S727-PPox standard.** $^{32}P_i$-labeled cPLA$_2$ was purified from thrombin-stimulated platelets and Sf9 cells and digested with trypsin. The pool of mbHPLC fractions eluting between 78 and 90 min was oxidized with performic acid. The Sf9 cell sample was further purified by mbHPLC, and fractions eluting between 78 and 84 min were pooled and dried. *A*, the platelet sample (900 cpm) was co-spotted with the Sf9 cell sample (900 cpm). *B*, the Sf9 cell sample was applied (1,800 cpm). Radioactive phosphopeptides were visualized with a PhosphorImager. The R_F values of the spots 2ox are 0.54 (*A*) and 0.53 (*B*).

(spot 2) and 0.50, the location of spot 2ox. After oxidation, the sample consisted mainly of one phosphopeptide (spot 2ox; R_F = 0.51; Fig. 3*D*).

It is necessary for identification of phosphopeptides resolved in two dimensions on cellulose plates to co-spot radiolabeled phosphopeptide with an authentic standard. To make sure that the phosphopeptide from the thrombin experiment that eluted at 82–88 min from the mbHPLC column is S727-PP, we oxidized the respective mbHPLC fractions derived from platelet and from Sf9 cell cPLA$_2$ with performic acid, and co-spotted equal amounts of counts/min onto the cellulose plate. To separate any oxidized S727-PP from S454-PP in the Sf9 cell sample, the oxidized sample was first subjected to mbHPLC, and only the radioactive fractions co-eluting with the S727-PPox standard were used for TLC analysis. Approximately 75% of the radioactivity, as quantified with a PhosphorImager, were detected in one location (R_F = 0.54; Fig. 4*A*). Most of the minor spots surrounding this peptide were also detected in a control experiment where only the Sf9 cell phosphopeptide was applied onto the cellulose plate (spot 2ox: R_F = 0.53; Fig. 4*B*). Each of the minor spots represented 5–10% of the total radioactivity. The only difference between the pattern of Fig. 4, *A* and *B*, was a minor spot directly to the left of spot 2ox in Fig. 4*A* which was not present in the pure Sf9 sample and must therefore be derived from platelet cPLA$_2$. We have observed an equivalent minor spot in all experiments where the first mbHPLC peak of digested platelet cPLA$_2$ was oxidized with performic acid before TLC analysis (for example, see Fig. 3*D*). This phosphopeptide may represent a degradation product of the oxidized S727-PP, a partial tryptic fragment, or a minor phosphopeptide other than those containing Ser-505 and Ser-727 phosphorylation. Since equal counts/min of the two phosphopeptides were co-spotted, it may be concluded that the major phosphopeptide present in the platelet- and Sf9-derived cPLA$_2$ behave identically on two-dimensional TLC. Judging from both mbHPLC and two-dimensional phosphopeptide maps, thrombin stimulates the phosphorylation of two sites on platelet cPLA$_2$, namely Ser-727 and Ser-505.

mbHPLC Analysis of cPLA$_2$ Phosphorylated by p42^{mapk} in Vitro—To further confirm that the late eluting radioactive peak from the mbHPLC column in the platelet experiments is due to phosphorylation at Ser-505, we performed *in vitro* assays with activated p42^{mapk} using wild-type human cPLA$_2$ and the S505A cPLA$_2$ mutant as substrates. Following SDS-PAGE and autoradiography, prominent ^{32}P-phosphorylation was seen in cPLA$_2$/p42^{mapk} samples, but a small amount of cPLA$_2$ phosphorylation was also detected in kinase reactions that lacked exogenous p42^{mapk} (not shown). The tryptic digests of cPLA$_2$ eluted from the gel were then analyzed on mbHPLC (Fig. 5). The major radioactive fraction of the cPLA$_2$/p42^{mapk} sample (retention time 130–140 min) co-eluted with a prominent OD_{210} peptide peak (133.2 min) and represented 85% of the total counts/min eluting from the mbHPLC column. Its retention time was the same as that of the Ser-505-containing phosphopeptide seen in the platelet and Sf9 cell experiments. On two-dimensional maps, the phosphopeptides recovered from this mbHPLC peak moved in positions of spots 4 and 4a (not shown). In the absence of p42^{mapk} there was a trace of radioactivity detectable with a similar retention time (132–138 min). However, this peak was not seen in experiments where the mutated S505A cPLA$_2$ was used as substrate for p42^{mapk} (Fig. 5). This suggests that the minor peak is caused by a MAPK contamination in the purified cPLA$_2$ used in these *in vitro* experiments.

The samples containing cPLA$_2$ or S505A cPLA$_2$ and p42^{mapk} gave a novel radioactive peak at 122–124 min (Fig. 5). This peak was not present in control reactions where p42^{mapk} was omitted (Fig. 5). The radioactivity constituted less than 7% of the counts/min of the cPLA$_2$/p42^{mapk} sample that eluted from the mbHPLC column. It may represent a minor p42^{mapk} phosphorylation site on cPLA$_2$. One candidate is Ser-676 within the sequence PESP. *In vitro* phosphorylation of a cPLA$_2$ peptide consisting of the amino acids 545–749 by p42^{mapk} resulted in a weak phosphorylation of the peptide (10). However, it is clear that this possible second p42^{mapk} phosphorylation site is not detectably phosphorylated in platelet cPLA$_2$ (Fig. 2).

The cPLA$_2$ reaction mixtures with and without p42^{mapk} contained an equal and very small amount of radioactivity that eluted at 86 to 88 min, the retention time of unoxidized S727-PP (Fig. 5). This peak also appears to be present in S505A cPLA$_2$ reaction mixtures. When these fractions were analyzed on two-dimensional TLC, two phosphopeptides in the location of spot 2 (R_F = 0.68) and spot 2ox (R_F = 0.48) were detected (not shown). It is possible that the preparation of human wild-type cPLA$_2$ from insect cells contained a trace of kinase capable of phosphorylating Ser-727 during the kinase reaction.

Effect of the p38^{mapk} Inhibitor SB 203580 on the Phosphorylation of cPLA$_2$ in Thrombin-activated Platelets—Our previous studies on the phosphorylation of cPLA$_2$ in human platelets treated with thrombin showed that the p38^{mapk} inhibitor SB 203580 inhibits cPLA$_2$ phosphorylation by 50% (24). To verify the effect of SB 203580 on p38^{mapk} activity in platelets, the activity of MAPKAP-K2, an *in vivo* substrate of p38^{mapk}, was determined. SB 203580 inhibited activation of MAPKAP-K2 by thrombin in a concentration dependent manner with an IC_{50} of 0.2 μM, and inhibition was complete with 10 μM inhibitor (not shown). SB 203580 did not interfere with vital platelet responses such as activation of protein kinase C, 5-hydroxytryptamine release, and tyrosine phosphorylation of platelet proteins.[2]

When platelets were incubated with SB 203580 (20 μM) before stimulation with thrombin (1 unit/ml, 2 min), ^{32}P-phosphorylation of cPLA$_2$ was reduced by approximately 50% (Fig. 6*A*) which is consistent with our previous results (24). The separation of the tryptic digest of cPLA$_2$ on mbHPLC resulted in three radioactive fractions with retention times of 82, 86,

[2] A. G. Börsch-Haubold and S. P. Watson, unpublished data.

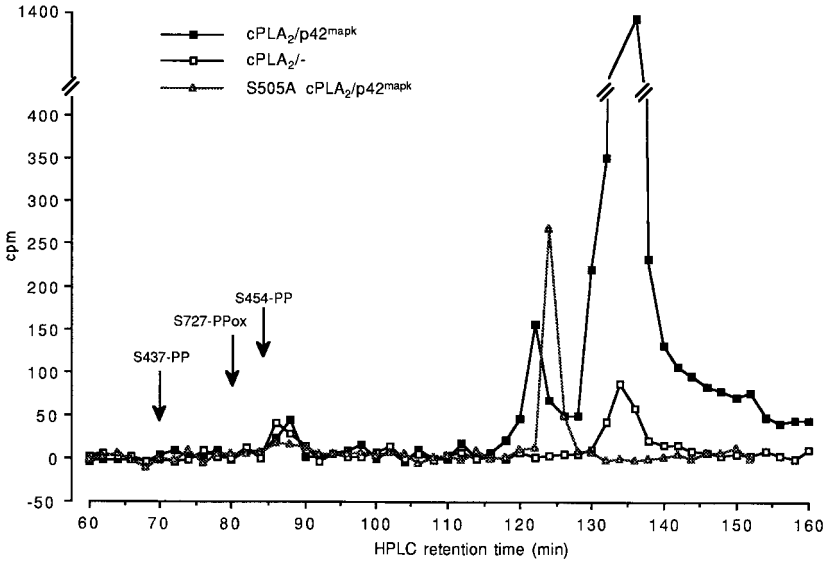

FIG. 5. ***In vitro* phosphorylation of human cPLA$_2$ and mutant S505A cPLA$_2$ by p42^{mapk}.** After the kinase reaction, cPLA$_2$ was eluted from SDS-PAGE and digested with trypsin. The entire digests applied onto mbHPLC contained 6,890 cpm (cPLA$_2$/p42^{mapk}, ■), 389 cpm (cPLA$_2$/-, □) and 480 cpm (S505A cPLA$_2$/p42^{mapk}, △). The retention times of the standard peptides were: S437-PP 70.4 min, S727-PPox 80.2 min, S454-PP 84.5/85.6 min (□, *arrows*); S437-PP 72.4 min, S727-PPox 80.9 min, S454-PP 85.0/86.1 min (■); and S437-PP 71.1 min, S727-PPox 79.6 min, S454-PP 84.0/85.2 min (Δ). Data shown are representative of two similar experiments.

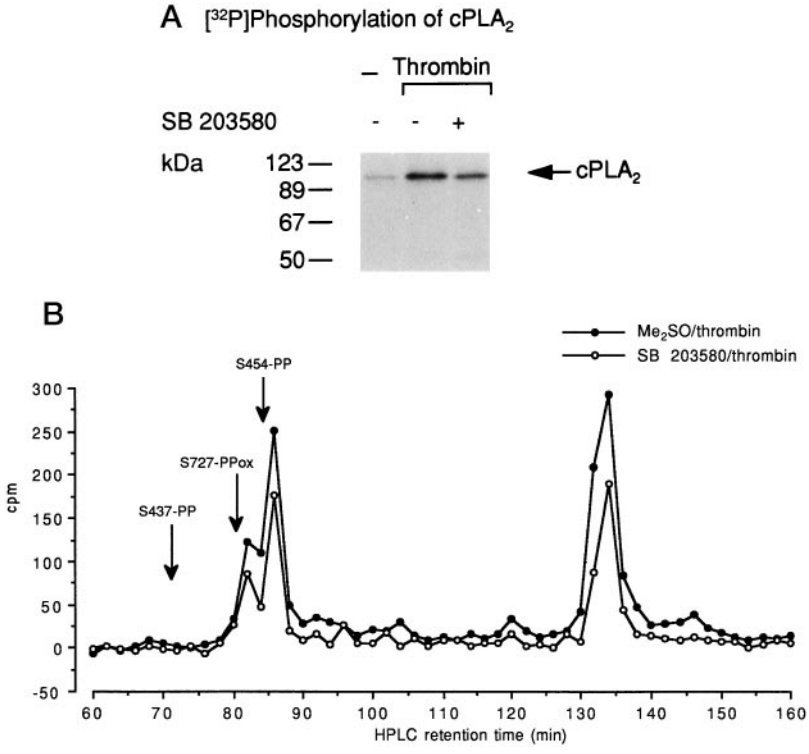

FIG. 6. **Effect of the p38^{mapk} inhibitor SB 203580 on the phosphorylation of cPLA$_2$ in thrombin-stimulated human platelets.** $^{32}P_i$-radiolabeled platelets were incubated with Me_2SO (1%) or SB 203580 (20 μM) for 15 min at 37 °C and stimulated with buffer (basal) or 1 unit/ml thrombin for 2 min. *A,* autoradiograph of one cPLA$_2$ immunoprecipitation per lane. *B,* eight immunoprecipitations were combined, and cPLA$_2$ was eluted and digested with trypsin. The Me_2SO/thrombin sample loaded onto mbHPLC contained 2,580 cpm (●), the SB 203580/thrombin sample 1,200 cpm (○). Elution over 160 min yielded 82% (●) and 99% (○) of the radioactivity applied to the column. The retention times of the peptide standards were: S437-PP 71.5 min, S727-PPox 80.3 min, S454-PP 84.4/85.5 min (●, *arrows*) and S437-PP 70.2 min, S727-PPox 79.9 min, S454-PP 84.2/85.2 min (○).

and 134 min (peaks) in both the thrombin and the SB 203580/thrombin sample (Fig. 6*B*). The 82-min peak co-eluted with the S727-PPox standard, the 86-min peak with unoxidized S727-PP (which co-elutes with S454-PP, see above), and the retention time of the 134-min peak corresponded to the authentic Ser-505 phosphopeptide (Fig. 5). Based on these results, TLC analysis, and the previous studies (17), it is clear that the S727-PP undergoes variable oxidation in different preparations. This behavior further confirms that the platelet-derived phosphopeptide is S727-PP.

SB 203580 reduced the radioactivity of the first fraction (80–88 min) by 60% and the second fraction (130–138 min) by 50%, as calculated from the counts/min eluting from the column (Fig. 6*B*). Thus, the treatment did not significantly alter the peak 1:peak 2 ratio which was 0.84:1 (thrombin) and 1.05:1 (SB 203580/thrombin).

Phosphorylation of cPLA$_2$ in Collagen-activated Platelets and Effect of the 38^{mapk} Inhibitor SB 203580—Collagen (100 μg/ml) stimulated ^{32}P-phosphorylation of cPLA$_2$ by 2.6-fold over basal levels (Fig. 7*A*). Radioactivity was incorporated into 2 tryptic fragments, as resolved by mbHPLC (Fig. 7*B*), and the retention times of these fractions (peaks: 86 and 134 min) were virtually identical to those of the two phosphopeptides obtained from thrombin-activated platelet cPLA$_2$ (Fig. 6*B*). The phosphopeptide recovered from the first peak was oxidized by performic acid and subjected to mbHPLC, which resulted in a shift of the peak to a shorter retention time (80 min), now co-eluting with the S727-PPox standard (retention time 80.1 min; Fig. 7*B*).

The phosphopeptides of the two radioactive mbHPLC fractions after oxidation (80 and 134 min) were subjected together to two-dimensional TLC. Two major radioactive spots were detected (Fig. 7*C*). Spot 4 was located in the region of the Ser-505-containing phosphopeptide (R_F = 0.85), and spot 2ox (R_F = 0.48) was in a similar position to the oxidized S727-PP derived from Sf9 cells and from thrombin-activated platelets. In longer exposures, there was also a minor fraction of unoxi-

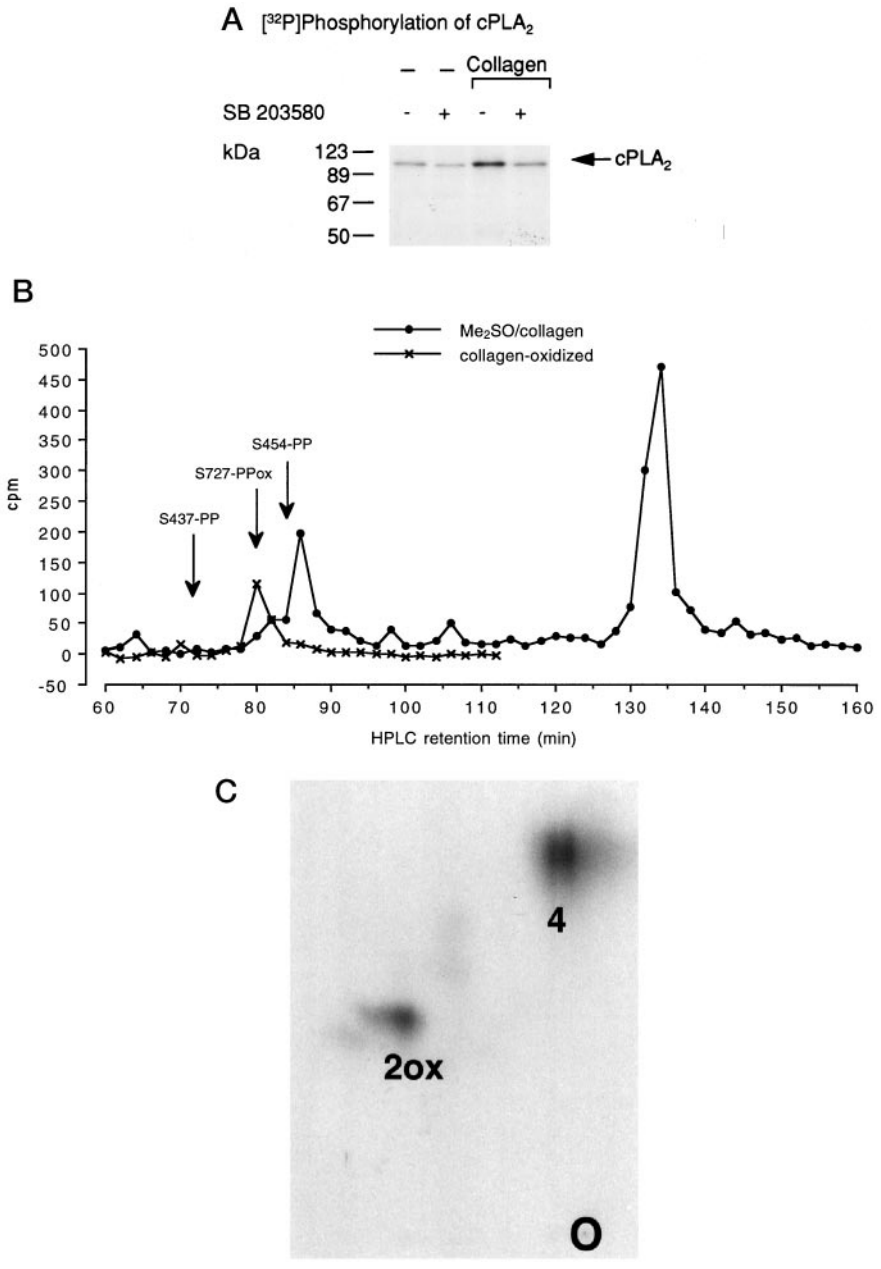

FIG. 7. **Phosphorylation of cPLA$_2$ in collagen-stimulated human platelets.** $^{32}P_i$-radiolabeled platelets were incubated with Me_2SO (1%) or SB 203580 (20 μM) for 15 min at 37 °C and stimulated with buffer (basal) or 100 μg/ml collagen for 5 min in an aggregometer. *A,* autoradiograph of one cPLA$_2$ immunoprecipitation per lane. *B,* eight immunoprecipitations were combined, cPLA$_2$ was eluted and digested with trypsin. The Me_2SO/collagen sample loaded onto mbHPLC contained 2,660 cpm (●), and elution over 160 min yielded 96% of the radioactivity applied to the column. The phosphopeptide eluting after 82–90 min from the collagen sample was oxidized with performic acid and analyzed on mbHPLC (×). The retention times of the standard peptides were: S437-PP 72.0 min, S727-PPox 80.6 min, S454-PP 84.6/85.7 min (●, *arrows*) and S437-PP 70.6 min, S727-PPox 80.1 min, S454-PP 84.8 min (×). *C,* radioactive fractions eluting after 78–84 min (×) and 128–140 min (●) were dried in a Speed-Vac, proteins were dissolved in CH_3COOH and applied onto a cellulose TLC plate. Phosphopeptides were separated in two dimensions and detected with a PhosphorImager. R_F values are: *spot 2ox*, 0.48; *spot 4*, 0.85.

dized S727-PP detectable (R_f = 0.60). Thus, both mbHPLC and phosphopeptide maps showed that cPLA$_2$ is phosphorylated on Ser-505 and Ser-727 in collagen-stimulated platelets.

The IC_{50} for the action of SB 203580 on p38^{mapk} activity in collagen-stimulated platelets, as determined by the activity of MAPKAP-K2, is 0.2 μM, and incubation of platelets with 20 μM SB 203580 fully blocks MAPKAP-K2 activation (25). SB 203580 inhibited the collagen-induced cPLA$_2$ phosphorylation by 85% (Fig. 7*A*).

Phosphorylation of cPLA$_2$ in HeLa S3 Cells—In light of the cPLA$_2$ phosphorylation results with Sf9 cells and platelets, we were intrigued by the recent report that cPLA$_2$ in interferon-α-stimulated HeLa S3 cells is phosphorylated on tyrosine (28). Thus, we carried out mbHPLC studies of cPLA$_2$ derived from HeLa cells. HeLa S3 cells were radiolabeled with $^{32}P_i$ in the presence and absence of 1,000 units/ml interferon-α. HeLa cells were also stimulated with sodium arsenite, which is a known activator of stress-activated protein kinases such as p38^{mapk}. As shown in Fig. 8*A*, two radioactive tryptic peptides were detected with both stimulants. The earlier peak eluted with the S727-PP standard, and the later peak eluted in the region expected for the Ser-505 phosphopeptide. These assignments were confirmed by submitting each mbHPLC peak to two-dimensional phosphopeptide mapping (not shown). Furthermore, two-dimensional phosphopeptide mapping of the entire tryptic digest (without mbHPLC) revealed only these two phosphopeptides (not shown). Treatment of HeLa cells with interferon-α resulted in an increase in cPLA$_2$ phosphorylation. Cerenkov counting of the SDS-PAGE bands from immunoprecipitates gave 5,000 cpm in the control sample, and 6,400 cpm in the stimulated sample. cPLA$_2$ from arsenite-treated cells contained 11,300 cpm. Interferon-α treatment led to a 2-fold increase in phosphorylation at Ser-727 and a 1.6-fold increase in phosphorylation at Ser-505, and the corresponding increases with arsenite treatment were 6.5-fold for Ser-727 and 1.9-fold for Ser-505 (Fig. 8*A*). To explore the possibility that a putative phosphotyrosine peptide was lost in the mbHPLC analysis or in the transfer of the tryptic digest to the two-dimensional TLC plate, the entire tryptic digest was submitted to phosphopeptide amino acid analysis (performed in the same tube as the

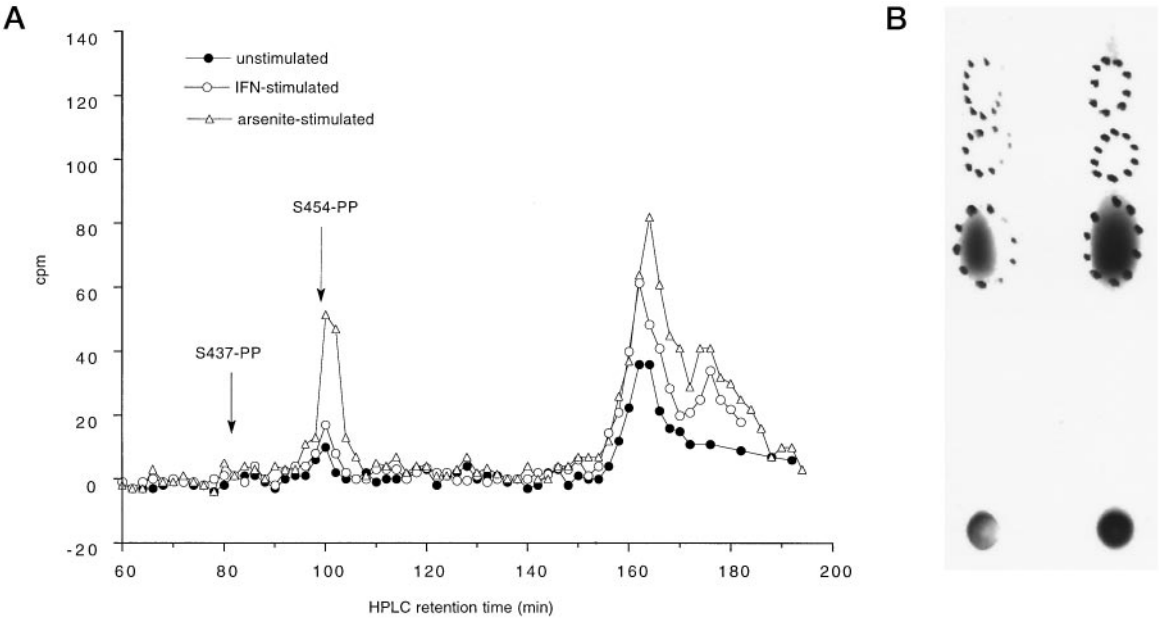

FIG. 8. **Analysis of cPLA$_2$ phosphorylation in HeLa S3 cells.** *A*, HeLa S3 cells were labeled with 1 mCi of $^{32}P_i$ and unstimulated (●), stimulated with 1,000 units/ml interferon-α (○), or stimulated with 0.5 mM sodium arsenite (Δ). Tryptic peptides were submitted to mbHPLC (the absolute values of the peptide retention times are somewhat larger than those seen in earlier mbHPLC runs (*i.e.* Fig. 1) presumably due to column aging). *B*, cPLA$_2$ immunoprecipitated from unstimulated or interferon-α-stimulated HeLa S3 cells were subjected to phosphoamino acid analysis. *Left lane*, unstimulated; *right lane*, interferon-α-stimulated. Phosphoamino acid standard spots visualized with ninhydrin are *circled* (from *top* to *bottom*: phosphoserine, phosphothreonine, and phosphotyrosine). The origin is the *lowest spot*.

trypsin digestion). As shown in Fig. 8*B*, only phosphoserine was detected, and the non-radiolabeled standards (phosphoserine, -threonine, and -tyrosine) were clearly visible as well defined spots by ninhydrin detection. When HeLa cells were labeled with 5 mCi of $^{32}P_i$, phosphorylation of cPLA$_2$ in control cells (28,500 cpm) was increased to 46,700 cpm in the presence of arsenite, and co-treatment with SB 202190 (20 μM) and arsenite resulted in incorporation of 32,000 cpm of phosphate into cPLA$_2$. Thus, as seen with platelets, SB 202190 causes partial inhibition of cPLA$_2$ phosphorylation.

DISCUSSION

All of the phosphorylation sites of cPLA$_2$ expressed in a baculovirus/Sf9 cell system have been previously determined by a combination of HPLC, electrospray mass spectrometry, and Edman sequencing (17). As pointed out in this previous study, two-dimensional phosphopeptide mapping analysis is problematic for accurately quantifying the amount of each phosphopeptide, and this point should be considered in any analysis of protein phosphorylation. This is because the multiple lyophilizations needed to remove buffer salt prior to analysis causes a significant fraction of the radiolabeled phosphopeptides to irreversibly adhere to the walls of the tube, and thus the counts/min of each phosphopeptide spot cannot be used for quantification. In this previous study, quantification was carried out by conventional HPLC analysis of trypsin digests since the yield of counts/min from the reverse phase column was >80%. In these studies, tens of micrograms of cPLA$_2$ were available (typically 100,000 cpm), and thus no extraordinary measures were needed to handle the phosphopeptides. This approach had to be modified for the present study by use of mbHPLC to analyze relatively tiny amounts of phosphopeptides derived from immunoprecipitates of platelet and HeLa cell cPLA$_2$. The small diameter of the mbHPLC column (0.5 mm) enabled us to elute samples containing 100–3,000 cpm with a yield of over 90%. Thus, mbHPLC is a valuable tool for phosphopeptide mapping and quantification of phosphorylation of proteins present at natural abundance in mammalian cells.

From mbHPLC and two-dimensional phosphopeptide maps we conclude that in thrombin- and collagen-stimulated platelets, cPLA$_2$ is phosphorylated on Ser-505 and Ser-727. Tryptic phosphopeptide fragments generated from the Sf9 baculovirus expression system (17) were compared with those from platelets and were an important component for identification of the phosphorylation sites of platelet cPLA$_2$. Our firm demonstration that cPLA$_2$ is not phosphorylated on tyrosine in interferon-α- and arsenite-stimulated HeLa S3 cells despite the fact that both agents increase cPLA$_2$ phosphorylation is at odds with the published conclusion that cPLA$_2$ is tyrosine phosphorylated in these cells (28). In the previous study, phosphotyrosine formation was assessed only by immunoblotting with a tyrosine phosphate-specific antibody, whereas in the present studies it was assessed by direct protein chemical approaches including phosphoamino acid analysis. It is inconceivable that a putative tyrosine phosphate-containing cPLA$_2$ peptide was lost due to surface absorption during sample handing because the entire tryptic digest was hydrolyzed with HCl in the same tube as the trypsin digest itself. By labeling HeLa S3 cells with 5 mCi of $^{32}P_i$, we were able to obtain immunoprecipitated cPLA$_2$ that contained tens of thousands of counts/min, and thus even if 1% of these counts/min represented tyrosine phosphate this amino acid would have been detected by phosphoamino acid analysis. Although we cannot say with absolute certainty that cPLA$_2$ is not phosphorylated on tyrosine (our HeLa S3 cells are probably not genetically identical to the ones used by Flati *et al.* (28), and handling of the cells could be somewhat different in the two laboratories), our direct structural results cast significant doubt that cPLA$_2$ is tyrosine phosphorylated in interferon-α-treated HeLa S3 cells. Phosphorylation of cPLA$_2$ on tyrosine has been claimed to occur in transforming growth factor-α-stimulated HEL-30 keratinocytes, but again this is based only on an anti-phosphotyrosine immunoblot (33).

In the present study we have found that SB 203580 inhibited

phosphorylation of cPLA$_2$ on both Ser-505 and Ser-727 by 60 and 50%, respectively, in thrombin-stimulated platelets. This partial inhibition occurs under conditions in which MAPKAP-K2 activation is completely inhibited by SB 203580. These results suggest either that a SB 203580-sensitive p38^{mapk} is only partially responsible for cPLA$_2$ phosphorylation, or that the K_m for cPLA$_2$ as a substrate for p38^{mapk} is relatively low compared with other substrates. We cannot address the last possibility at the present time because of the limited solubility of SB 203580 and SB 202190, which precludes testing at concentrations >20 μM. The recently described stress-activated protein kinases SAPK3 and SAPK4, neither of which is inhibited by SB 203580 and SB 202190 (34), or an unidentified kinase may be responsible for a portion of the cPLA$_2$ phosphorylation that occurs in thrombin-stimulated platelets. The recently described isoform of p38^{mapk}, p38β, which is inhibited by SB 203580 and SB 202190 (35), is also a candidate kinase for cPLA$_2$ phosphorylation.

The reduction of cPLA$_2$ phosphorylation on Ser-727 by SB 203580 was surprising since this site does not lie within a MAPK consensus sequence. Indeed, p42^{mapk} did not phosphorylate cPLA$_2$ at Ser-727 *in vitro* (Fig. 5). Moreover, S505A cPLA$_2$ was a poor substrate for the partially-purified platelet p38^{mapk} preparation that phosphorylated wild type cPLA$_2$ (26), which suggests that Ser-727 is not directly phosphorylated by p38^{mapk}. The following possibilities may account for the inhibition of Ser-727 phosphorylation by SB 203580: 1) Ser-505 and Ser-727 are phosphorylated by different kinases and both are inhibited by SB 203580; 2) the Ser-727 kinase is downstream of the SB 203580-sensitive Ser-505 kinase in the activation pathway; 3) phosphorylation of Ser-505 by a SB 203580-sensitive kinase (p38^{mapk} or an isoform) leads to a conformational change in cPLA$_2$ that exposes Ser-727 to the same kinase (this would explain why platelet-derived p38^{mapk} did not phosphorylate S505A cPLA$_2$ *in vitro* (27)) or to a distinct kinase. Clearly, additional work is needed to firmly identify the kinase(s) responsible for Ser-505 and Ser-727 phosphorylation.

We have previously shown that the kinase inhibitor staurosporine completely blocks phosphorylation of cPLA$_2$ in thrombin-activated platelets without inhibiting the release of arachidonic acid (21). However, staurosporine increases the thrombin-induced rise of intracellular Ca^{2+} which might counteract the effect of inhibition of cPLA$_2$ phosphorylation on arachidonic acid formation. In light of the fact that specific inhibitors which fully block cPLA$_2$ phosphorylation at Ser-505 and Ser-727 in platelets and HeLa cells are not available, the role of cPLA$_2$ phosphorylation in promoting agonist-induced arachidonic acid release cannot yet be conclusively resolved.

Our results underscore the importance of direct chemical approaches for analyzing the sites of cPLA$_2$ phosphorylation. Furthermore, mbHPLC provides the level of phosphorylation to be accurately quantified, and this is critical for the study of the effect of kinase inhibitors on cPLA$_2$ phosphorylation. The widely used SDS-PAGE gel shift analysis may not be reliable for determination of the sites or levels of cPLA$_2$ phosphorylation.

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