THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 43, Issue of October 26, pp. 39653–39660, 2001

Printed in U.S.A.

Functional Interaction of Calcium-/Calmodulin-dependent Protein Kinase II and Cytosolic Phospholipase A_2*

Received for publication, April 9, 2001, and in revised form, July 29, 2001
Published, JBC Papers in Press, July 30, 2001, DOI 10.1074/jbc.M103136200

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Calcium-/calmodulin-dependent protein kinase II (CaM kinase II), a decoder of Ca^{2+} signals, and cytosolic phospholipase A_2 ($cPLA_2$), an enzyme involved in arachidonate release, are involved in many physiological and pathophysiological processes. Activation of CaM kinase II in norepinephrine-stimulated vascular smooth muscle cells leads to activation of $cPLA_2$ and arachidonic acid release. Surface plasmon resonance, mass spectrometry, and kinetic studies show that CaM kinase II binds to $cPLA_2$ resulting in $cPLA_2$ phosphorylation on Ser-515 and an increase in its enzymatic activity. Phosphopeptide mapping studies with $cPLA_2$ from norepinephrine-stimulated smooth muscle cells indicates that phosphorylation of $cPLA_2$ on Ser-515, but not on Ser-505 or Ser-727, occurs *in vivo*. This novel signaling pathway for arachidonate release is shown to be $cPLA_2$-dependent by use of a recently described and highly selective inhibitor of this enzyme.

Many cellular stimuli produce oscillations in the intracellular concentration of Ca^{2+}. Ca^{2+}-/calmodulin (CaM)[1]-dependent kinase II (CaM kinase II), a multifunctional protein kinase, decodes the frequency of Ca^{2+} spikes and regulates the activity of a range of cellular targets involved in many physiological processes including control of cell cycle, apoptosis, gene expression, neurotransmission, synaptic plasticity, learning and memory, and early after-depolarization (1, 2).

We have shown previously that the adrenergic transmitter norepinephrine (NE) and angiotensin II increase CaM kinase II activity in vascular smooth muscle cells (VSMC), leading to activation of cytosolic phospholipase A_2 ($cPLA_2$) (3–5). $cPLA_2$ liberates arachidonic acid by hydrolyzing arachidonyl phospholipids (6, 7). Arachidonic acid is the precursor of a variety of lipid mediators including leukotrienes and prostaglandins that modulate a number of cellular processes (8). $cPLA_2$ is an attractive target for the development of novel therapies because of its profound role in inflammatory processes, allergic responses, reproductive physiology, post-ischemic brain injury, cell proliferation, and cancer (9–13).

$cPLA_2$ is activated in a variety of cell types by growth factors, neurotransmitters, angiotensin II, vasopressin, lipopolysaccharides, colony-stimulating factor-1, thrombin, and other agonists (6, 14, 15). $cPLA_2$, like protein kinase C, GTPase-activating proteins, phospholipase C, and p65, contains a Ca^{2+}-dependent phospholipid binding domain that mediates translocation of $cPLA_2$ to the nuclear envelope and perinuclear region of cells (16–18). $cPLA_2$ is regulated post-translationally by submicromolar Ca^{2+} and phosphorylation (6, 19). p42/p44 mitogen-activated protein (MAP) kinase phosphorylate $cPLA_2$ on Ser-505 in many cell types (6, 20), whereas other members of the MAP kinase family, namely p38 stress-activated protein kinases, phosphorylate $cPLA_2$ in platelets (21). Rigorous studies to map the phosphorylation sites on $cPLA_2$ have been carried out only with platelets, HeLa cells, and CHO cells, and these studies reveal that $cPLA_2$ is phosphorylated on Ser-505 and Ser-727 (22). More recent work has shown that Ser-727 phosphorylation is catalyzed by MNK1 or a closely related isoform, which is a protein kinase that is activated by members of the MAP kinase family, and that both Ser-505 and Ser-727 phosphorylation are required for full activation of $cPLA_2$ (23). In most agonist/cell systems, $cPLA_2$ phosphorylation is not sufficient for its activation; a rise in intracellular Ca^{2+} is also required, and Ca^{2+}-independent $cPLA_2$ activation by an unknown mechanism has also been reported (6).

We reported recently that in VSMC, $cPLA_2$-catalyzed arachidonic acid release induced by NE or angiotensin II is significantly reduced by the CaM kinase II inhibitor KN-93 and by an antisense oligonucleotide that decreases CaM kinase II expression (3, 5). Moreover, we showed that activation of MAP kinase in VSMC also occurs in response to NE and angiotensin II. Antisense oligonucleotide directed against CaM kinase II and the CaM kinase II inhibitor KN-93 reduced MAP kinase activation by ~50%, whereas agents that decrease MAP kinase activity (antisense oligonucleotides directed against MAP kinase and a mitogen-activated protein kinase/extracellular signal-regulated kinase kinase inhibitor) have no effect on CaM kinase II activation (3). These results suggest that CaM kinase II acts upstream of MAP kinase in NE-stimulated VSMC (3). Although CaM kinase II seems to be the major activator of $cPLA_2$ in these cells (as measured by arachidonic acid release

* This work was supported in part by the American Heart Association Beginners Grant-in-aid (to M. M. M.) and by National Institutes of Health Grants 19134-26 (to K. U. M.), HL50040 (to M. H. G.), and R33CA 84698 and AI41109-01 (to R. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: CaM, calmodulin; CaM kinase II, calcium- and calmodulin-dependent protein kinase II; NE, norepinephrine; VSMC, vascular smooth muscle cells; $cPLA_2$, cytosolic phospholipase A_2; MAP, mitogen-activated protein; CHO, Chinese hamster ovary; ERK, extracellular signal-regulated kinase; DMEM, Dulbecco's modified Eagle's medium; RU, response units; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; P, peptide.

as noted above), MAP kinase may partially contribute to the activation of cPLA$_2$, because antisense oligonucleotides against this kinase, as well as a mitogen-activated protein kinase/extracellular signal-regulated kinase kinase inhibitor, reduce arachidonic acid release by ~30% (3). This signaling between CaM kinase II and cPLA$_2$ activation and MAP kinase activation may involve oxygenated metabolites of cPLA$_2$-released arachidonic acid, because pharmacological inhibition of cytochrome P450 and 12- and 15-lipoxygenase reduce NE- and angiotensin II-stimulated MAP kinase activity to near basal level (cyclooxygenase inhibitors were without effect) (4). Based on all of this evidence, it has been proposed that CaM kinase II activation in VSMC in response to NE and angiotensin II leads to cPLA$_2$ activation. cPLA$_2$-catalyzed arachidonic acid release may fuel an amplification of this response by providing fatty acid precursor to oxygenating enzymes for the production of mediators, including 20-hydroxyeicosatetraenoic acid, that activate MAP kinase (4). The latter could cause additional activation of cPLA$_2$.

In the current study, we set out to explore the possibility that CaM kinase II phosphorylates cPLA$_2$ directly, leading to its activation and arachidonic acid release in rabbit and human VSMC stimulated with the physiological agonist NE. By using rigorous protein chemical techniques, we provide strong evidence to suggest that CaM kinase II is the major kinase that activates cPLA$_2$ in these cells and that cPLA$_2$ phosphorylation occurs on a novel site (Ser-515).

EXPERIMENTAL PROCEDURES

Materials—Wild-type human cPLA$_2$ and the 4A mutant were purified from baculovirus-infected Sf9 cells as described (24). In some experiments, as noted below, wild-type cPLA$_2$ was dephosphorylated by treatment with phosphatase, and its concentration was determined by A_{280} using the extinction coefficient calculated from its amino acid sequence (25). Some samples of human recombinant cPLA$_2$ were obtained by expression in CHO cells (used as noted below). This enzyme and monoclonal anti-cPLA$_2$ antibody were kind gifts from Genetics Institute (Cambridge, MA). Rat brain CaM kinase II, recombinant CaM kinase IIα, CaM, and ERK1 MAP kinase were purchased from Calbiochem. ERK2 MAP kinase is from Upstate Biotechnology (Lake Placid, NY). NE, ATP, aprotinin, leupeptin, penicillin, streptomycin, and amphotericin are from Sigma. KN-92 and KN-93 ((2-[*N*-(2-hydroxyethyl)-*N*-(4-methoxybenzenesulfonyl)]amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine)) are from Seikagaku (Falmouth, MA). Monoclonal CaM kinase II antibody is from Transduction Laboratories. Polyclonal phospho-CaM kinase II antibody is from Promega (Madison, WI). M-199 medium is from Celgro. DMEM and phosphate-free DMEM are from Life Technologies, Inc. [^{32}P]Orthophosphate (7,000–8,000 Ci/mmol) and [γ-^{32}P]ATP (6,000 Ci/mmol) are from Amersham Pharmacia Biotech. 1-palmitoyl-2-[1-^{14}C]arachidonyl-phosphatidylcholine (57 mCi/mmol) is from American Radiolabeled Chemicals Inc. (St. Louis, MO). Sequencing-grade chymotrypsin is from Roche Diagnostics. The pS727-P phosphopeptide standard was obtained as described (22).

CaM Kinase II-cPLA$_2$ Binding Analysis—Studies were performed on a BIAcore 2000 biosensor system (BIAcore, Piscataway, NJ). CaM kinase II was cross-linked to the carboxylated dextran surface of a CM5 sensor chip to a density of ~2000 response units (RU). A 1:1 mixture of 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride and *N*-hydrosuccinimide was used to activate the sensor chip surface, followed by immobilization of the enzyme and blocking of unreacted sites with 1 M ethanolamine (pH 8.5). Immobilization was conducted at 25 °C using HBS (0.01 M HEPES, pH 7.4, 0.15 M NaCl) as the running buffer, and 10 mM sodium acetate, pH 5.0, was used for electrostatic pre-concentration of the protein. cPLA$_2$ (1 mg/ml, CHO cell expressed) was diluted 1000-fold in Ca^{2+}/CaM buffer (20 mM morpholinopropane sulfonic acid, pH 7.2, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM $CaCl_2$, 75 mM $MgCl_2$, 500 μM ATP). Flow cell 1 was activated and blocked in the absence of CaM kinase II and used as a control surface. For association, cPLA$_2$ solution was applied using the kinject program at 20–60 μl/min for 1 min. Dissociation was effected with HBS applied over the surface at 60 ml/min for 10 min. The surface was regenerated with a short pulse of 10 mM glycine, pH 3.0, which removes bound cPLA$_2$, as well as CaM. Sensorgrams were subjected to global analysis using BIAcore evaluation software 3.0.

In Vitro Phosphorylation of cPLA$_2$—For experiments shown in Fig. 2 and Fig. 3*A*, 3 μg of cPLA$_2$ (CHO cell or Sf9 cells expressed for the 4A mutant) expressed were incubated with 60 ng of rat brain CaM kinase II or CaM kinase IIα (200 units) and 0.4 μg of CaM at 30 °C in Ca^{2+}/CaM buffer containing 10 μCi of [γ-^{32}P]ATP and 100 μM ATP in a total volume of 30 μl. The reaction was terminated at the indicated times by the addition of 2× Laemmli sample buffer. Samples were boiled and subjected to SDS-PAGE on an 8% gel. Gels were dried and visualized by autoradiography. Phosphorylation of cPLA$_2$ (CHO cell expressed) by ERK2 MAP kinase was performed essentially as described above with the following modifications. In some experiments (see Fig. 2 and Fig. 3*A*), cPLA$_2$ (3 μg) was incubated with 4 units of active ERK2 MAP kinase at 30 °C for the indicated times in buffer containing 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EGTA, 2 mM dithiothreitol, 0.01% Brij 35, 100 μM ATP, and 10 μCi of [γ-^{32}P]ATP. The data obtained in Fig. 4*C* were obtained by phosphorylating 100 μg of cPLA$_2$ (wild-type, Sf9 cell expressed) with 40 units of ERK1 MAP kinase is 25 mM HEPES, pH 7.4, 1 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM EGTA, 100 μM ATP, 100 μM Na_3VO_4, and 200 μCi/ml of [γ-^{32}P]ATP in a total volume of 25 μl. After 30 min at 30 °C, the sample was submitted to SDS-PAGE, and cPLA$_2$ was submitted to proteolysis and HPLC analysis as described below.

Phosphoamino Acid Analysis—Following *in vitro* phosphorylation and separation by SDS-PAGE as described above, cPLA$_2$ was electrotransferred to a polyvinylidene difluoride membrane. The sections containing phosphorylated cPLA$_2$ were visualized by autoradiography, excised, and subjected to phosphoamino acid analysis. The polyvinylidene difluoride membrane was hydrolyzed for 60 min at 110 °C in 6 N HCl. The solution was concentrated to dryness in a SpeedVac. The sample was dissolved in 10 μl of pH 1.9 buffer (88% formic acid:acetic acid:water, 50:156:1794) containing 5 μg each of phosphoamino acid standards (phosphothreonine, phosphoserine, and phosphotyrosine). The sample was subjected to ascending chromatography on a 20 × 20-cm cellulose thin-layer chromatography plate with butanol:acetic acid:ethanol:water (1:1:1:1). Phosphoamino acid standards were visualized with 0.2% ninhydrin in acetone, and ^{32}P-labeled amino acids were identified by autoradiography.

Isolation, Culture, and Maintenance of VSMC—Human aortic smooth muscle cells obtained from human aorta were purchased from ATCC. The cells were cultured as described by ATCC. Rabbit aortic smooth muscle cells were obtained from thoracic aorta of male New Zealand White rabbits and cultured in M-199 medium (Sigma) with penicillin, streptomycin, and 10% fetal bovine serum as described previously (37). Cells between 4 and 6 passages were plated in 24-well plates or in 100-mm plates. Cells were maintained under 5% CO_2 at 37 °C.

Phosphorylation of cPLA$_2$ in Intact VSMC—VSMC, grown in 100-mm plates to 70% confluency, were arrested for 24 h in medium containing 0.05% fetal bovine serum. The cells were preincubated with phosphate-free DMEM medium for 30 min. Cells were labeled for 4 h in phosphate-free DMEM medium containing [^{32}P]orthophosphate (300 μCi/ml) along with CaM kinase II inhibitor KN-93 (20 μM) or vehicle and treated with NE (10 μM) for 15 min. Cells were washed with ice-cold phosphate-buffered saline and lysed in 1 ml of buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 5 mM EGTA, 10% glycerol, 1% Triton X-100, 2 mM $NaVO_4$, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 5 μg/ml leupeptin. The protein concentration was adjusted to 1 mg/ml, and the lysates were centrifuged at 10,000 × *g* for 10 min. cPLA$_2$ was immunoprecipitated from the supernatants by incubation with 10 μg of anti-cPLA$_2$ antibody for 4 h at 4 °C and then with protein A-agarose beads for 1 h. The immunoprecipitate was centrifuged at 10,000 × *g* for 5 min, and the pellets were washed four times with ice-cold phosphate-buffered saline containing phosphatase inhibitors. The samples were boiled for 5 min in 50 μl of 2× Laemmli sample buffer, and the supernatants were subjected to SDS-PAGE and autoradiography.

Assay of cPLA$_2$ Enzymatic Activity—To generate the data in Fig. 3*B*, wild-type cPLA$_2$, the 4A cPLA$_2$ mutant, or enzymatically dephosphorylated wild-type cPLA$_2$ (500 ng each) was incubated with 20 ng of rat brain CaM kinase II and 300 ng of CaM in 50 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 0.3 mM $CaCl_2$, 50 μM ATP, and 10 μCi of [γ-^{32}P]ATP in a total volume of 10 μl for 6 min at 25 °C. The reaction was quenched by boiling in Laemmli sample buffer, and proteins were resolved on a 7.5% Laemmli gel. The gel was dried, and radiophosphorylated cPLA$_2$ was visualized by autoradiography. The cPLA$_2$ band was excised from the gel and submitted to Cerenkov counting to determine the cpm of [^{32}P]phos-

phate incorporated into cPLA$_2$ and thus the stoichiometry of phosphorylation. For measuring the enzymatic activity of phosphorylated cPLA$_2$, CaM kinase II reactions were set up in parallel as above but in the absence of [γ-^{32}P]ATP. An aliquot containing 50 ng of cPLA$_2$ was submitted to the previously reported enzymatic assay (26). Reactions were terminated after 30 min at 37 °C, fatty acid was extracted with Dole's reagent, and the organic extract was passed through a small column of silica gel to remove traces of phospholipid. Using this assay, the amount of product was found to increase linearly with incubation time during the 30-min period and to be proportional to the amount of cPLA$_2$ in the range from 0 to 100 ng of cPLA$_2$.

cPLA$_2$ Phosphorylation Mapping Studies—cPLA$_2$ (20 μg, wild-type Sf9 cell expressed) was phosphorylated *in vitro* with CaM kinase II as described above. Radiophosphorylated cPLA$_2$ was purified by SDS-PAGE, eluted from the gel, precipitated, and washed as described previously (27). cPLA$_2$ was digested with trypsin as described (27) or with chymotrypsin (20% by weight) in 100 mM Tris-HCl, pH 7.8, 10 mM $CaCl_2$ for 20 h at 25 °C. To the digest was added 30 μg of pS727-P, and the entire digest was injected onto the C18 reverse-phase HPLC column (Vydac 218TP52) equilibrated previously with 100% Solvent A (0.06% trifluoroacetic acid in water). After loading, the column was developed with 100% Solvent A for 15 min, then to 30% Solvent B (0.06% trifluoroacetic acid in CH_3CN) in 60 min, then to 60% Solvent B in 30 min, then to 100% Solvent B in 10 min, and finally holding at 100% Solvent B for 45 min, all at a flow rate of 0.3 ml/min. Peptide standards were detected by monitoring the absorbance at 210 nm, and 1-min fractions were submitted to Cerenkov counting. During the manipulation of sample at all steps, the sample was submitted to Cerenkov counting to monitor yield of cpm.

HPLC fractions containing radiophosphorylated peptide were pooled and concentrated to dryness in a SpeedVac. The residue was dissolved in 10 μl of glacial acetic acid and submitted to two-dimensional chromatography on cellulose as described previously (27). Phosphopeptides were located on the plate by autoradiography or with a PhosphorImager.

Mass Spectrometry—An LCQ ion trap mass spectrometer (Finnigan MAT) was used with an HP1100 solvent delivery system (Agilent). A 45-min binary gradient with 5–40% Solvent B (0.005% heptafluorobutyric acid in CH_3CN; Solvent A is 0.005% heptafluorobutyric acid in water) was used at a flow rate of 0.4 μl/min for HPLC on a 75-μm × 10-cm fused silica capillary column packed in-house with Monitor C18, 5 μm spherical silica beads (Column Engineering). Samples were pressure-loaded onto the column and then eluted and analyzed by combined HPLC tandem mass spectrometry as described (28). SEQUEST (29) was used for searching the cPLA$_2$ sequence to identify the site of phosphorylation.

Arachidonic Acid Release—VSMC isolated from human and rabbit thoracic aortae were cultured in 24-well plates to 80% confluency. The cells were labeled with [^{3}H]arachidonic acid (0.25 μCi/ml, 100 Ci/mmol; American Radiolabeled Chemicals) for 16 h, washed three times with Hanks' balanced salt solution to remove unincorporated fatty acid, and stimulated with norepinephrine (10 μM) for 15 min. The release of [^{3}H]arachidonic acid in to the culture medium was measured as described previously (3).

RESULTS

CaM Kinase II Interacts with cPLA$_2$—To further understand the mechanism of activation of cPLA$_2$ by CaM kinase II, we examined the ability of CaM kinase II to interact with cPLA$_2$. Association and dissociation of CaM kinase II with cPLA$_2$ were studied by surface plasmon resonance performed on a BIAcore 2000 system. CaM kinase II (purified from rat brain as a hetero-oligomer of α- and β-subunits) was covalently cross-linked to the CM5 sensor chip. Approximately 2000 RU of CaM kinase II were immobilized on flow cell 2, whereas flow cell 1 was activated and blocked and used as a control surface. After control subtraction, ~170 RU of cPLA$_2$ binding was observed (Fig. 1*A*). cPLA$_2$ (1 μg/ml) was diluted 100-, 250-, and 500-fold in Ca^{2+}/calmodulin buffer and injected over the CaM kinase II surface. The overlaid sensorgrams shown in Fig. 1*B* demonstrate concentration-dependent binding of cPLA$_2$ to CaM kinase II. In the absence of CaM or Ca^{2+}, cPLA$_2$ did not bind to CaM kinase II (Fig. 1, *C* and *D*). However, removal of ATP did not affect binding of CaM kinase II to cPLA$_2$ (Fig. 1*E*). The addition of the specific CaM kinase II inhibitor KN-93 (10 μM)

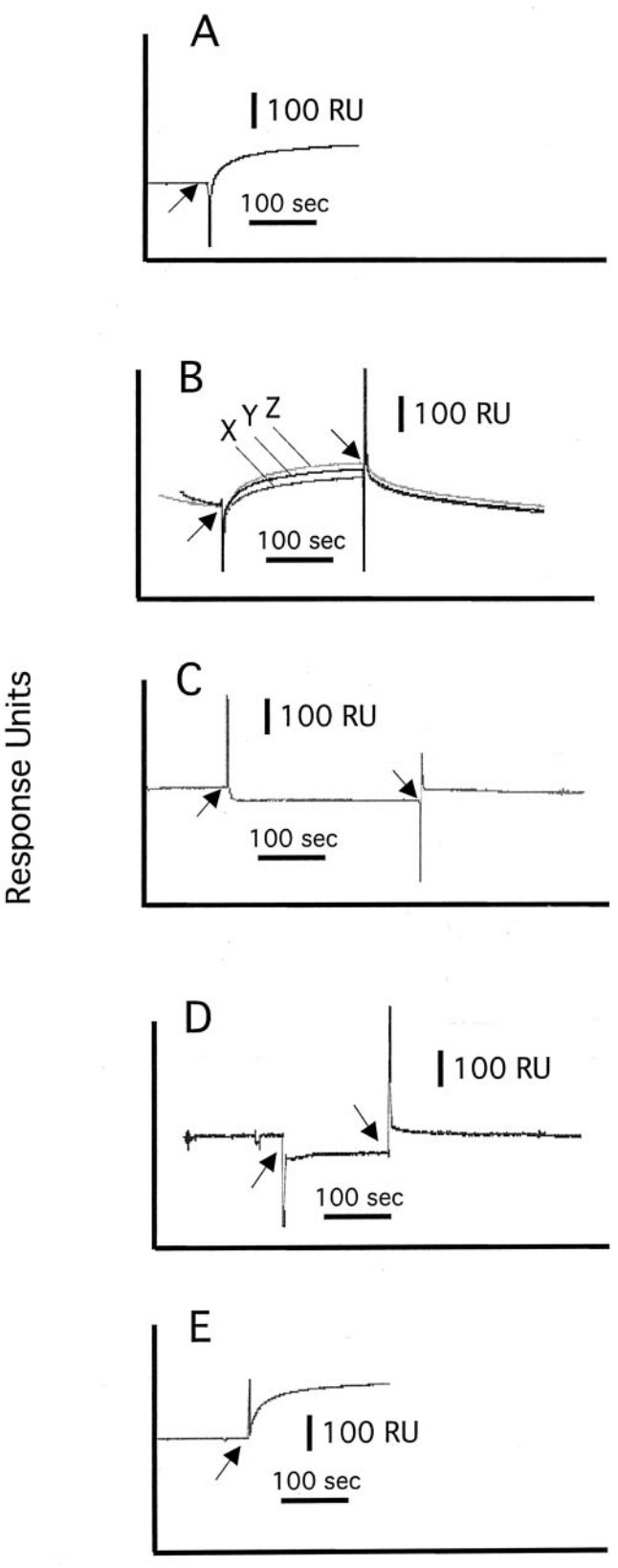

FIG. 1. **Sensorgrams depicting cPLA$_2$ interaction with CaM kinase II.** *A*, cPLA$_2$ binding to CaM kinase II in the presence of Ca^{2+}/CaM. RU are plotted as a function of time. cPLA$_2$ was injected at time 0 (indicated by an *upward arrow*) to achieve association with cross-linked CaM kinase II. Dissociation commences at the *downward arrow*. The data has been control-subtracted. *B*, overlaid sensorgrams of cPLA$_2$ dilutions (*X*, *Y*, and *Z* designate 500-, 250-, and 100-fold dilution of cPLA$_2$, respectively) binding to CaM kinase II in the presence of Ca^{2+}/CaM. *C*, sensogram in the absence of Ca^{2+}. *D*, sensogram in the absence of CaM. *E*, sensogram in the absence of ATP.

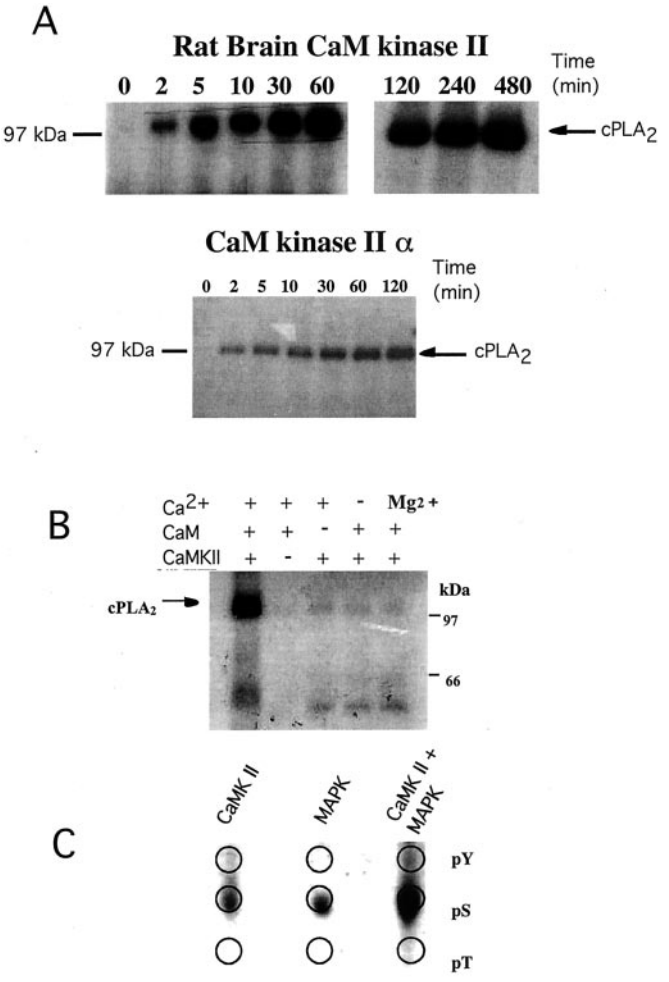

FIG. 2. **CaM kinase II and ERK2 MAP kinase phosphorylates cPLA$_2$ *in vitro*.** *A*, human cPLA$_2$ protein (3 μg) was phosphorylated with rat brain CaM kinase II (60 ng) or recombinant CaM kinase IIα (125 units) in the presence of 1 mM Ca^{2+}, 0.4 μg of CaM, [γ-^{32}P]ATP (10 μCi), and 100 μM ATP for the indicated duration. Control reactions without the kinase were also performed under the same conditions. The samples were submitted to SDS-PAGE (12% gel), and phosphorylated cPLA$_2$ was detected by autoradiography. A representative blot of three experiments is shown. *B*, same as *A* except reaction components were omitted as shown in the *legend above* the *figure*. $CaCl_2$ was replaced with 1 mM $MgCl_2$ in the *last lane*. *CaMKII*, CaM kinase II. *C*, phosphoamino acid analysis of CaM kinase II- and ERK2 MAP kinase (*MAPK*)-phosphorylated cPLA$_2$. cPLA$_2$ phosphorylated by CaM kinase II and ERK2 MAP kinase was hydrolyzed with HCl and separated along with unlabeled phosphotyrosine (*pY*), phosphothreonine (*pT*), and phosphoserine (*pS*) standards on cellulose thin-layer chromatography plates as described under "Experimental Procedures." Unlabeled standards were detected with ninhydrin, and ^{32}P-labeled amino acids were visualized by autoradiography.

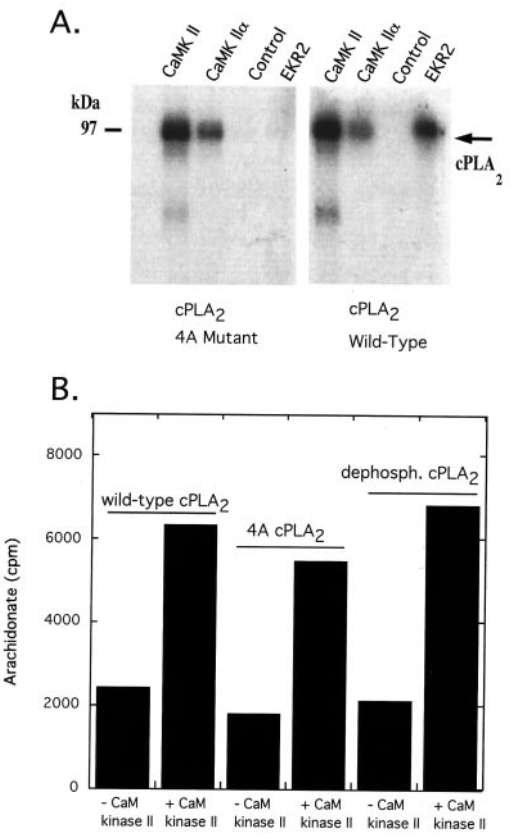

FIG. 3. ***In vitro* phosphorylation (*A*) of cPLA$_2$ by CaM kinase IIα (*CaMK IIα*), CaM kinase II (*CaMK II*), and ERK2 MAP kinase (*ERK2*) and cPLA$_2$ activation (*B*) by CaM kinase II.** *A*, *in vitro* kinase reactions were performed for 15 min at 30 °C. Reaction mixtures were submitted to SDS-PAGE, and phosphorylated cPLA$_2$ was visualized by autoradiography. *B*, purified wild-type, 4A mutant, or fully dephosphorylated cPLA$_2$ (*dephosph. cPLA$_2$*) (50 ng) was stoichiometrically phosphorylated *in vitro* by CaM kinase II as described under "Experimental Procedures." Control experiments were performed without the kinase. The cpm of released radiolabeled fatty acid from the cPLA$_2$-catalyzed hydrolysis of phospholipid vesicles is plotted.

reduced binding of cPLA$_2$ to CaM kinase II by 50–60% (data not shown). These observations indicated that CaM kinase II can bind directly to cPLA$_2$.

CaM Kinase II Phosphorylates cPLA$_2$ in Vitro—Rat brain CaM kinase II and the α-subunit of CaM kinase II (CaM kinase IIα) phosphorylated recombinant cPLA$_2$ *in vitro* in a time-dependent manner (Fig. 2*A*). No cPLA$_2$ phosphorylation was observed in the absence of CaM kinase II (Fig. 2*B*).

CaM kinase II is maintained in an inactive state under basal conditions because of its autoinhibitory domain, which blocks binding of both peptide and ATP substrates. CaM kinase II requires only Ca^{2+} and CaM for activation (30). Once activated, the kinase not only phosphorylates its target substrates but also autophosphorylates at Thr-286 (31). As shown in Fig. 2*B*, cPLA$_2$ was not phosphorylated by CaM kinase II if Ca^{2+} or CaM was omitted from the reaction mixture. Moreover, when Mg^{2+} was used in place of Ca^{2+}, CaM kinase II failed to phosphorylate cPLA$_2$ (Fig. 2*B*). These results establish the absolute requirement of Ca^{2+} and CaM in the phosphorylation of cPLA$_2$ by CaM kinase II.

cPLA$_2$ is phosphorylated on four serine residues (Ser-437, -454, -505, and -727) when expressed in a baculovirus/Sf9 cell system (27). In agonist-stimulated platelets, HeLa cells, and CHO cells, cPLA$_2$ is phosphorylated on Ser-505 and Ser-727 (22, 23). MAP kinase family members phosphorylate cPLA$_2$ on Ser-505, and Ser-727 phosphorylation is catalyzed by the MAP kinase-activated kinase MNK1, or a closely related isoform (23). To test whether CaM kinase II phosphorylates cPLA$_2$ on the other residues, we performed phosphorylation experiments using the mutant of cPLA$_2$ in which Ser-437, -454, -505, and -727 were mutated to alanine (4A mutant). CaM kinase II and CaM kinase IIα phosphorylated both wild-type and 4A mutant cPLA$_2$ to similar extents (Fig. 3*A*). On the other hand, as expected, MAP kinase failed to phosphorylate the 4A mutant (Fig. 3*A*). These results indicate that CaM kinase II and MAP kinase phosphorylate cPLA$_2$ on different sites and that CaM kinase II phosphorylates cPLA$_2$ on one or more sites other than Ser-437, -454, -505, and -727.

CaM Kinase II Phosphorylates cPLA$_2$ on Ser-515—Phosphoamino acid analysis of cPLA$_2$ that was phosphorylated *in vitro* with CaM kinase II shows exclusive phosphorylation on

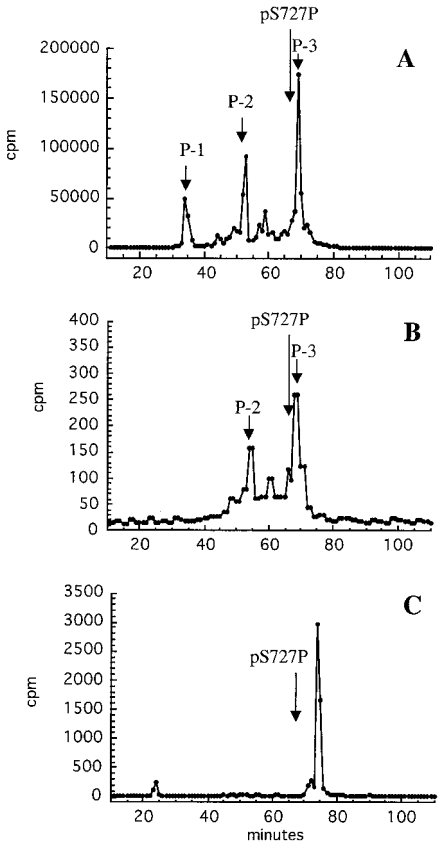

FIG. 4. **HPLC analysis of chymotrypsin digests of radiophosphorylated cPLA$_2$.** *A*, cPLA$_2$ was radiophosphorylated by CaM kinase II *in vitro*, gel purified, digested with chymotrypsin, and analyzed by HPLC. Eluted fractions were submitted to Cerenkov counting. The three major radioactive cPLA$_2$-derived phosphopeptides are labeled P-1, P-2, and P-3, and the elution position of added pS727P phosphopeptide standard is also shown. *B*, same analysis as for *A* except cPLA$_2$ is from human VSMC labeled with [^{32}P]orthophosphate and stimulated with NE. *C*, same analysis as for *A* using cPLA$_2$ that was *in vitro*-phosphorylated on Ser-505 with MAP kinase.

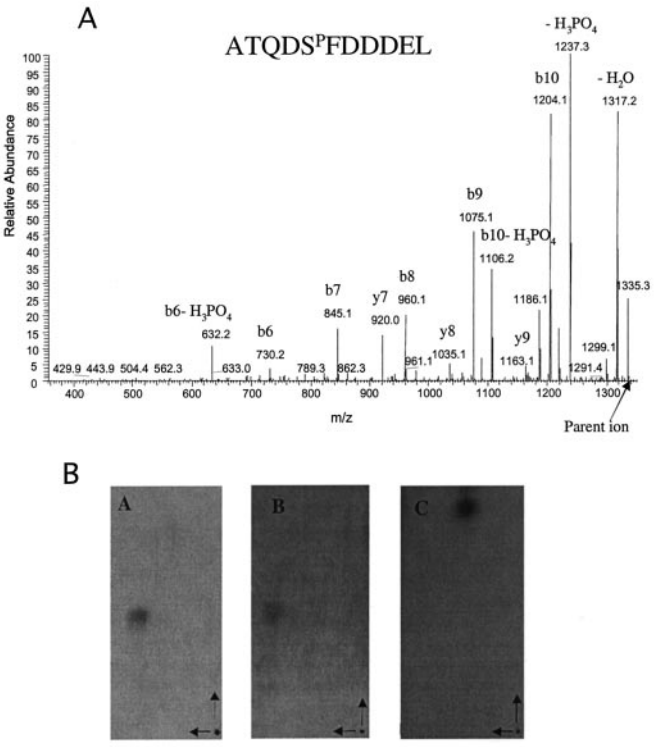

FIG. 5. **Ser-515 phosphorylation of cPLA2 by CaM kinase II.** *A*, combined HPLC-electrospray ionization tandem mass spectrum of the P-2 phosphopeptide obtained by chymotrypsin digestion of cPLA$_2$ that was *in vitro*-phosphorylated with CaM kinase II. The b-type and y-type ions are indicated and are derived from fragments due to peptide bond cleavage that contain the N- and C termini, respectively. *B*, two-dimensional chromatographic analysis of radiophosphorylated, cPLA$_2$-derived chymotryptic peptides. cPLA$_2$ is derived from [^{32}P]orthophosphate-labeled, NE-stimulated human VSMC (*A*), cPLA$_2$ radiophosphorylated with CaM kinase II *in vitro* (*B*), and cPLA$_2$ radiophosphorylated with MAP kinase *in vitro* (*C*). Electrophoresis was carried out in the horizontal direction followed by ascending chromatography in the vertical direction (sample applied at origin marked by *dot*).

serine (Fig. 2*C*). MAP kinase also phosphorylates cPLA$_2$ on serine as expected, and treatment of cPLA$_2$ with CaM kinase II and MAP kinase in the same reaction mixture leads to more phosphoserine than that obtained from cPLA$_2$ that was treated with each kinase alone (Fig. 2*C*).

cPLA$_2$ was radiophosphorylated *in vitro* with CaM kinase II, purified by SDS-PAGE, and digested with trypsin. When the digest was submitted to HPLC analysis and two-dimensional thin-layer chromatography on a cellulose plate (electrophoresis followed by ascending chromatography) as described (27), a single radiolabeled peptide was detected that co-migrated with the Ser-505-phosphorylated tryptic peptide (not shown). This result, together with the fact that CaM kinase II is able to phosphorylate the A4 mutant of cPLA$_2$, suggests that CaM kinase II phosphorylates cPLA$_2$ on a serine other than Ser-505 in the same tryptic peptide that contains Ser-505. Because the Ser-505 tryptic peptide is too long for structural analysis of the site of phosphorylation using tandem mass spectrometry, we explored the use of proteases other than trypsin to degrade CaM kinase II-phosphorylated cPLA$_2$.

As shown in Fig. 4*A*, digestion of CaM kinase II-phosphorylated, gel-purified cPLA$_2$ with chymotrypsin resulted in three radiophosphorylated peptides (P-1, P-2, and P-3) when analyzed by HPLC. Because trypsin degradation of the same sample gives only a single radiophosphorylated peptide, we suspected that the peptides P-2 and P-3 produced after chymotrypsin digestion represent partial degradation products. Indeed, increasing the amounts of chymotrypsin or lengthening the proteolysis reaction time lead to an increase in the amount of the P-1 and P-2 and a compensating decrease in the amount of the latest eluting peptide P-3 (not shown). The total cpm eluting from the HPLC column accounted for 70% of the cpm applied, indicating that additional major radiophosphorylated peptides were not lost in the analysis. The tandem mass spectrum of P-2 is shown in Fig. 5*A*. The fragmentation pattern is uniquely consistent with the phosphopeptide shown in the figure, thus identifying the site of CaM kinase II phosphorylation of cPLA$_2$ as Ser-515. As expected, this serine is part of the same tryptic peptide that includes Ser-505.

The classical consensus sequence for CaM kinase II phosphorylation is R*XX*(S/T); however, more recent studies have shown that this protein kinase also phosphorylates serines that are part of a consensus sequence S*X*D (31). The cPLA$_2$ Ser-515 phosphorylation site SFD fits this consensus pattern. Ser-515 is conserved in cPLA$_2$s found in GenBank™ (human, rat, mouse, horse, chicken, and zebrafish) as are the hydrophobic and acidic residues on the C-terminal side of Ser-515 (SFD, SVE and SLE). The Ser-515 CaM kinase II phosphorylation site is close in linear sequence to the Ser-505 MAP kinase phosphorylation site. Both occur on a loop of the catalytic domain of cPLA$_2$, which is presumed to be highly flexible, because it was not seen in the x-ray crystal structure of full-length enzyme (32).

Phosphorylation of cPLA$_2$ by CaM Kinase II Causes an In-

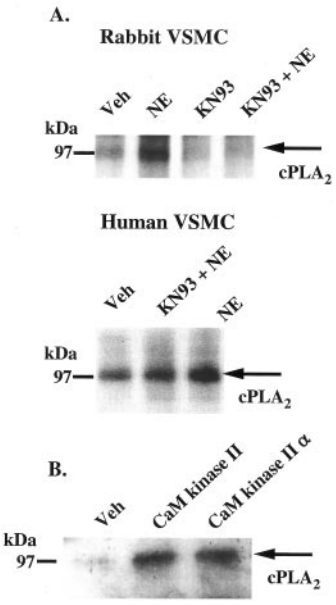

FIG. 6. **CaM kinase II mediates NE-stimulated phosphorylation of cPLA$_2$ in VSMC.** *A*, effect of KN-93 on NE-stimulated phosphorylation of cPLA$_2$. [^{32}P]Orthophosphate-radiolabeled VSMC were pretreated with KN-93 (20 μM) or vehicle (*Veh*) and then stimulated with NE for 15 min, lysed, and then immunoprecipitated with cPLA$_2$ antibody. The immunoprecipitates were subjected to SDS-PAGE, and the incorporation of ^{32}P into cPLA$_2$ protein was detected by autoradiography. *B*, *in vitro* phosphorylation of cPLA$_2$ from VSMC by CaM kinase II. cPLA$_2$ was immunoprecipitated from VSMC lysates (2 mg of total protein) with 7 μg of cPLA$_2$-specific monoclonal antibody for 4 h at 4 °C. The immune complex was then divided in half and incubated with or without CaM kinase II (50 ng) or CaM kinase IIα (125 units) in the presence of [γ-^{32}P]ATP (10 μCi) at 30 °C for 2 h. The samples were analyzed by SDS-PAGE (12% gel), and phosphorylated cPLA$_2$ was detected by autoradiography.

crease in Its Enzymatic Activity—We also determined whether phosphorylation of cPLA$_2$ by CaM kinase II results in increased enzymatic activity of cPLA$_2$. As shown in Fig. 3*B*, the activity of wild-type cPLA$_2$ was increased 2.6-fold following phosphorylation by rat brain CaM kinase II (stoichiometry of phosphorylation = 1.1 phosphates per cPLA$_2$). The cPLA$_2$ used in this experiment was produced by expression in the baculovirus/Sf9 cell system and is partially phosphorylated on Ser-437, -454, -505, and -727 (27). As shown in Fig. 3*B*, the same degree of activation was seen when the mutant of cPLA$_2$ in which all four of these serines were mutated to alanine (4A mutant) was stoichiometrically phosphorylated with CaM kinase II *in vitro*. Finally, CaM kinase II-catalyzed stoichiometric phosphorylation of cPLA$_2$ that had been previously fully dephosphorylated by treatment with phosphatase shows a similar degree of activation (Fig. 3*B*). We also confirmed the result of Kramer *et al.* (15) that phosphorylation of enzymatically dephosphorylated cPLA$_2$ by ERK2 *in vitro* leads to a 2- to 3-fold activation, although the stoichiometry of this phosphorylation was not measured. As expected, treatment of the 4A cPLA$_2$ mutant with ERK2 *in vitro* did not lead to an increase in its enzymatic activity (not shown).

CaM Kinase II Phosphorylates cPLA$_2$ at Ser-515 in Intact VSMC—We investigated the possibility of cPLA$_2$ phosphorylation by CaM kinase II in intact cells. As shown in Fig. 6*A*, stimulation of [^{32}P]orthophosphate-labeled VSMC obtained from rabbit and human thoracic aorta with NE led to the phosphorylation of cPLA$_2$. To investigate the possible role of CaM kinase II in the regulation of cPLA$_2$ phosphorylation, we used KN-93, a specific, water-soluble inhibitor of CaM kinase II (33). Treatment with the CaM kinase II inhibitor KN-93 blocked this phosphorylation (Fig. 6*A*) suggesting that CaM kinase II is part of the pathway in VSMC leading to cPLA$_2$ phosphorylation.

We investigated whether CaM kinase II is directly phosphorylating cPLA$_2$ in NE-stimulated VSMC. As shown in Fig. 6*B*, cPLA$_2$ immunoprecipitated from VSMC was phosphorylated by CaM kinase II and by CaM kinase IIα *in vitro*, which is consistent with the results obtained with purified cPLA$_2$ (Fig. 2). cPLA$_2$ was immunoprecipitated from [^{32}P]orthophosphate-labeled, NE-stimulated human VSMC. After purification by SDS-PAGE, gel-eluted and radiophosphorylated cPLA$_2$ was digested with chymotrypsin and submitted to HPLC. As shown in Fig. 4*B*, two radiophosphorylated peptides were seen (total cpm eluted from the column was 68% of that applied). The retention time of the earlier peak matched exactly that of the P-2 phosphopeptide seen in the chymotryptic digest of cPLA$_2$ phosphorylated *in vitro* with CaM kinase II (Fig. 4*A*). The later eluting peak (Fig. 4*B*) matched exactly the retention time of P-3 (Fig. 4*A*). P-1 phosphopeptide, which is derived from P-2 by additional chymotrypsin cleavage, was not seen with VSMC-derived cPLA$_2$ (Fig. 4*B*).

Because sufficient cPLA$_2$ from VSMC for mass spectrometry analysis of phosphopeptides was not available, we submitted the chymotrypsin digest to two-dimensional chromatography on cellulose. As shown in Fig. 5*B* (*panel A*), analysis of P-2 (obtained after HPLC) on the phosphopeptide map revealed a single radioactive spot. Furthermore, this spot co-migrated with P-2 obtained after chymotrypsin digestion of cPLA$_2$ phosphorylated *in vitro* with CaM kinase II (Fig. 5*B*, *panel B*). Thus, the single phosphopeptide obtained from cPLA$_2$ present in NE-stimulated human VSMC co-migrates with the Ser-515-phosphorylated chymotryptic peptide after chromatographic analysis in three dimensions. Because Ser-505 (the MAP kinase phosphorylation site) and Ser-515 are close to each other in the linear sequence of cPLA$_2$, it was important to explore whether chymotrypsin digestion of Ser-505-phosphorylated cPLA$_2$ produces a phosphopeptide that chromatographically resolves from the Ser-515-phosphorylated peptide. As shown in Fig. 4*C*, chymotrypsin digestion of Ser-505-phosphorylated cPLA$_2$ (prepared by treating cPLA$_2$ with MAP kinase *in vitro*) gave a single phosphopeptide that eluted later than P-3 seen in the chymotrypsin digest of CaM kinase II-phosphorylated cPLA$_2$. When this material was analyzed by two-dimensional cellulose chromatography, a single spot was seen at a different map position (Fig. 5*B*, *panel C*) than that seen with cPLA$_2$ from VSMC (Fig. 5*B*, *panel A*). All together, the results strongly suggests that cPLA$_2$ is phosphorylated in these cells on Ser-515 by CaM kinase II.

cPLA$_2$ Is Responsible for Arachidonic Acid Release in NE-stimulated Human and Rabbit VSMC—Previous studies with antisense oligonucleotide technology to reduce the level of cPLA$_2$ in rabbit VSMC suggest that cPLA$_2$ is required for NE-stimulated arachidonic acid release in these cells (3). To assess the role of cPLA$_2$ in NE-stimulated arachidonate release by an independent method and to examine cPLA$_2$ involvement in this process in human VSMC for the first time, we carried out studies with Pyrrolidine-1, a recently described cPLA$_2$ inhibitor (compound 4c from Ref. 33). Pyrrolidine-1 inhibits cPLA$_2$ *in vitro* in the 50–100 nM range, does not inhibit other phospholipases A$_2$, and blocks arachidonic acid release in a number of mammalian cells known to require cPLA$_2$ (for example CHO cells transfected with cPLA$_2$) (34, 35). Pyrrolidine-1 inhibits the release of [^{3}H]arachidonic acid from both human and rabbit VSMC stimulated with NE (Table I). The ED$_{50}$ for Pyrrolidine-1 is about 50 nM (Table I). At higher concentrations (0.5–5 μM), Pyrrolidine-1 also reduced the basal release of [^{3}H]arachidonic acid in the rabbit VSMC (Table I).

TABLE I

Effect of the cPLA$_2$ inhibitor Pyrrolidine-1 on NE-stimulated arachidonic acid release in VSMC

VSMC were labeled with [^{3}H]arachidonic acid (0.1 μCi/well) for 18 h and pretreated with the cPLA$_2$ inhibitor Pyrrolidine-1 for 30 min prior to stimulation with 10 μM NE for 10 min. Arachidonate release was measured as described under "Experimental Procedures." Data are expressed as the percent of total cpm (released + cell bound) that is released into the medium.

[PLA2 inhibitor]	Human VSMC		Rabbit VSMC	
	Vehicle	10 μM NE	Vehicle	10 μM NE
μM				
0	2.11 ± 0.08	3.40 ± 0.10	5.11 ± 1.52	8.51 ± 1.19
0.05	2.18 ± 0.10	2.78 ± 0.20	4.46 ± 0.26	5.09 ± 0.29
0.1	2.20 ± 0.11	2.60 ± 0.09	3.79 ± 0.36	4.69 ± 0.41
0.5	2.12 ± 0.15	2.29 ± 0.21	2.93 ± 0.79	3.31 ± 0.25
5	2.19 ± 0.18	2.19 ± 0.16	3.17 ± 0.52	3.05 ± 0.29

DISCUSSION

Collectively, the data in this study indicate that CaM kinase II phosphorylates and activates cPLA$_2$ both *in vitro* and in NE-stimulated rabbit and human VSMC. CaM kinase II interacts directly with cPLA$_2$ and phosphorylates it at a single site, Ser-515. This phosphorylation leads to a 2- to 3-fold increase in cPLA$_2$ enzymatic activity measured with an *in vitro* assay. Several laboratories have reported that phosphorylation of cPLA$_2$ on Ser-505 by MAP kinases also leads to a 2- to 3-fold increase in cPLA$_2$ enzymatic activity (15).

In platelets, HeLa cells, and CHO cells, cPLA$_2$ is phosphorylated by members of the MAP kinase family on Ser-505. Phosphorylation of cPLA$_2$ also occurs on Ser-727 in these cells and is dependent on a protein kinase that is activated by the MAP kinase family member (23). This explains why suppression of both Ser-505 and Ser-727 phosphorylation is seen when platelets, HeLa cells, and CHO cells are treated with inhibitors of the MAP kinases. In NE-stimulated VSMC, Ser-505 phosphorylation was not detected. This is based on the fact that cPLA$_2$ radiophosphorylated on Ser-505 by MAP kinase treatment *in vitro* gives rise to a chymotryptic peptide that elutes from the HPLC column at 75 min, and no corresponding peak could be detected in the HPLC trace of the chymotrypsin digest of cPLA$_2$ isolated from NE-stimulated, radiolabeled human VSMC (Fig. 4). Ser-727 cPLA$_2$ phosphorylation was also not detected following HPLC analysis of the trypsin digest of cPLA$_2$ isolated from NE-stimulated, radiolabeled human VSMC (not shown). This was expected based on the lack of observation of Ser-505 phosphorylation, because Ser-505 and Ser-727 phosphorylation are linked as noted above.

In our previous studies we have shown that antisense suppression of CaM kinase II or addition of the CaM kinase II inhibitor KN-93 completely suppresses NE-induced arachidonic acid release in rabbit VSMC (3). MAP kinase is activated in these cells downstream of CaM kinase II activation suggesting that MAP kinase could contribute to cPLA$_2$ activation in these cells. Both an antisense oligonucleotide and the mitogen-activated protein kinase/extracellular signal-regulated kinase kinase inhibitor PD-098509 suppress NE-stimulated arachidonic acid release in rabbit VSMC, but the effect is small (30% reduction in arachidonic acid release compared with complete reduction in the presence of CaM kinase II inhibitors) despite the fact that MAP kinase activation was inhibited completely (3). We cannot rule out the possibility that a small amount of Ser-505 phosphorylation occurs in NE-stimulated human VSMC but that the cpm incorporated is below the detection limit of our radiometric analysis of chymotryptic peptides. Alternatively the modest, MAP kinase-dependent activation of cPLA$_2$ may occur by a mechanism other than phosphorylation of cPLA$_2$ by MAP kinase. It is not known whether MAP kinase can phosphorylate cPLA$_2$ once it has translocated from the cytosol to the membrane. In NE-stimulated VSMC, MAP kinase activation occurs subsequent to CaM kinase II activation and a rise in intracellular calcium. This variation in the temporal activation of CaM kinase II and MAP kinase may be the reason why cPLA$_2$ is mainly phosphorylated on Ser-515 by CaM kinase II in VSMC. In any case, it is clear that Ser-515 phosphorylation of cPLA$_2$ by CaM kinase II is the major mechanism of cPLA$_2$ activation in these cells.

After the completion of our studies, Handlogten *et al.* (36) reported that cPLA$_2$ is activated in human embryonic kidney 293 cells that have been transfected with the calcium-sensing receptor in a MAP kinase-independent way involving Gq, phospholipase C, Ca^{2+}-CaM, and CaM kinase II (as shown by transfection studies and use of a CaM kinase II inhibitor) (36). It would be interesting to determine the site(s) of cPLA$_2$ phosphorylation in these cells.

Acknowledgments—We thank Dr. Sheree Long from Biacore Inc., Piscataway, NJ for assistance with the Biacore studies and Genetics Institute Inc., Cambridge, MA for providing recombinant cPLA$_2$ protein and its monoclonal antibody. We also thank Dr. Cagen for editorial comments and Anne Estes for excellent technical assistance.

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