cPLA₂ phosphorylation at serine-515 and serine-505 is required for arachidonic acid release in vascular smooth muscle cells

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Abstract  Cytosolic phospholipase A₂ (cPLA₂) is activated by phosphorylation at serine-505 (S505) by extracellular regulated kinase 1/2 (ERK1/2). However, rat brain calcium/calmodulin-dependent kinase II (CaMKII) phosphorylates recombinant cPLA₂ at serine-515 (S515) and increases its activity in vitro. We have studied the sites of cPLA₂ phosphorylation and their significance in arachidonic acid (AA) release in response to norepinephrine (NE) in vivo in rabbit vascular smooth muscle cells (VSMCs) using specific anti-phospho-S515- and -S505 cPLA₂ antibodies and by mutagenesis of S515 and S505 to alanine. NE increased the phosphorylation of cPLA₂ at S515, followed by phosphorylation of ERK1/2 and consequently phosphorylation of cPLA₂ at S505. The CaMKII inhibitor 2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzene-sulfonyl)amin-N-(4-chlorocinnamyl)-methylbenzylamine attenuated cPLA₂ at S515 and S505, whereas the ERK1/2 inhibitor U0126 reduced phosphorylation at S505 but not at S515. NE in cells transduced with adenovirus carrying enhanced cyan fluorescent protein cPLA₂ wild type caused phosphorylation at S515 and S505 and increased AA release. Expression of the S515A mutant in VSMCs reduced the phosphorylation of S505, ERK1/2, and AA release in response to NE. Transduction with a double mutant (S515A/S505A) blocked the phosphorylation of cPLA₂ and AA release. These data suggest that the NE-stimulated phosphorylation of cPLA₂ at S515 is required for the phosphorylation of S505 by ERK1/2 and that both sites of phosphorylation are important for AA release in VSMCs.—Pavicevic, Z., C. C. Leslie, and K. U. Malik. cPLA₂ phosphorylation at serine-515 and serine-505 is required for arachidonic acid release in vascular smooth muscle cells. J. Lipid Res. 2008. 49: 724–737.

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Norepinephrine (NE) released from postganglionic sympathetic fibers stimulates arachidonic acid (AA) release for the synthesis of prostaglandins, which, in turn, act as a physiological modulator of neurotransmitter release in various tissues, including blood vessels (1). In vascular smooth muscle cells (VSMCs), NE stimulates the release of AA via the α1-adrenergic receptor by the activation of cytosolic phospholipase A₂ (cPLA₂) (2, 3), a group IV phospholipase A₂ member (4). NE-induced cPLA₂ activation and AA release require calcium (Ca²⁺), calmodulin (CaM), and calmodulin-dependent kinase II (CaMKII) in VSMCs (2, 3). cPLA₂ activity has been reported to be regulated by phosphorylation by extracellular regulated kinase 1/2 (ERK1/2), p42/p44 mitogen-activated protein kinase, and Ca²⁺-dependent translocation to the nuclear envelope, allowing its access to arachidonyl-containing phospholipid substrate at the sn-2 position (6). ERK1/2 phosphorylates cPLA₂ at serine-505 (S505) in several cell types (5–7). However, the phosphorylation and activation of cPLA₂ by a mechanism independent of ERK1/2 has also been reported (8) and may be mediated by other members of the mitogen-activated protein kinase family, such as p38 stress-activated protein kinase (9, 10). The existence of multiple phosphorylation sites on cPLA₂ (S431, S454, S505, and S727) suggested cPLA₂ as a substrate for other kinases (11). Studies in platelets, HeLa cells, and CHO cells have shown that cPLA₂ is phosphorylated on S505 and S727 (12). S727 is phosphorylated by MNK1 or a closely related isoform, a protein activated by MAP kinase family members and the small GTPase Rac1. However, the role of these sites of phosphorylation in AA release is not clear.

Abbreviations:  AA, arachidonic acid; AdECFPcPLA₂ wt, adenoviral enhanced cyan fluorescent protein cytosolic phospholipase A₂ wild type; Ca²⁺/CaMKII, calcium/calmodulin kinase II α; cPLA₂, cytosolic phospholipase A₂; ECFP, enhanced cyan fluorescent protein; ERK1/2, extracellular regulated kinase 1/2; KN-92, 2-[N-(4-methoxybenzene-sulfonyl)]amin-N-(4-chlorocinnamyl)-N-methylbenzylamine; KN-93, 2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzene-sulfonyl)]amin-N-(4-chlorocinnamyl)-methylbenzylamine; MOI, multiplicity of infection; NE, norepinephrine; TCID₅₀, tissue culture infective dose 50; VSMC, vascular smooth muscle cell.

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kinase that is activated by members of the mitogen-activated protein kinase family, and phosphorylation of both S505 and S727 is required for the full activation of cPLA2 (13). However, it has been shown that cPLA2 phosphorylation is insufficient for its activation; an increase in intracellular Ca\(^{2+}\) is also required, and Ca\(^{2+}\)-independent cPLA2 activation by an unknown mechanism has also been reported (14).

Studies conducted in rabbit and rat VSMCs have shown that the activity of ERK1/2 in response to agonists such as NE, angiotensin II, or ionomycin is regulated in part by Ca\(^{2+}\)/CaMKII and that both CaMKII and ERK1/2 are involved in cPLA2 activation (3, 15–17). Further studies revealed that NE-stimulated cPLA2 activates cPLA2 and releases AA and that oxygenated metabolites of AA generated via lipoxygenase and cytochrome P450 stimulate ERK1/2 and amplify cPLA2 activity and release additional AA (15). In vitro surface plasmon resonance, mass spectrometric, and kinetic studies showed that CaMKII binds directly to cPLA2 and phosphorylates it on S515 and increases its enzymatic activity (18). Whether NE promotes cPLA2 phosphorylation on S515 in VSMCs, and whether phosphorylation on S515 mediates ERK1/2 activation and phosphorylation on S505 for AA release in vivo, are not known. Therefore, to assess the significance of cPLA2 phosphorylation by CaMKII on S515 in its activation and its effect on S505 phosphorylation by ERK1/2 and AA release, we examined the expression, distribution, and activation of adenoviral constructs of wild-type cPLA2 and those mutated on S505, S515, and S505/515 to alanine, which were chosen for subcloning into adenoviral shuttle vector pacAd5CMV (obtained from the University of Iowa, Iowa Viral Vector Facility, Iowa City), for mutation of both S505 and S515 to alanine (S505A/S515A) inserted in pacAd5CMV were cotransfected with ECFPcPLA2 wt and its mutants S505A, S515A, and S505A/S515A fusion constructs in the adenoviral vector

**EXPERIMENTAL PROCEDURES**

**Materials**

Arterenol NE and ampicillin were from Sigma (St. Louis, MO); the CaMKII inhibitor 2-\([N-(4-hydroxyethyl)]N-(4-methoxybenzenesulfonyl)\) amino-\([N-(4-chlorocinnamyl))-N-methylbenzylamine (KN-93) and its inactive analog 2-\([N-(4-methoxybenzenesulfonyl)]\)-amino-\([N-(4-chlorocinnamyl))-N-methylbenzylamine (KN-92) were from Calbiochem (San Diego, CA); \(^{3}H\)AA (0.1 \(^{3}C\)/ml) was from ARC, Inc. (St. Louis, MO); agarose was from Invitrogen-Life Technologies (Gaithersburg, MD); FuGENE 6 transfection reagent was from Roche (Indianapolis, IN); and HEK293 cells were obtained from the American Type Culture Collection (Manassas, VA).

**Preparation of VSMCs**

Male New Zealand White rabbits (1–2 kg) were anesthetized with 30 mg/kg pentobarbital (Abbott Laboratories, North Chicago, IL), and the thorax and abdomen were opened by a midline incision. The aorta was rapidly removed, and VSMCs were isolated as described previously (2). Cells between the fourth and eighth passages were cultured in 6- or 24-well, or 60 or 100 mm, plates for experiments. Cells were maintained under 5% CO\(_2\) in M-199 medium (Sigma Aldrich, St. Louis, MO) containing 10% FBS, 1% penicillin/streptomycin, and 0.1% amphotericin B.

**HEK293 cell culture**

Cells were cultured in Dulbecco’s modified Eagle’s medium that was supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 \(\mu\)g/ml streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO\(_2\) in air. The medium was changed three times per week, and the cells were passaged two times per week. Cells were detached from the culture flask by adding 0.25% trypsin and 2:21 mM EDTA in HBSS.

**Preparation of ECFPcPLA2 wt and its mutants S505A, S515A, and S505A/S515A fusion constructs in the adenoviral vector**

Transient transfection by liposomal reagent usually leads to a low level of transfection and variable levels of expression from cell to cell as well as to nonphysiological levels of expression in some cells (19). Therefore, we used adenovirus carrying ECFPcPLA2 wt and its mutants. Plasmids carrying ECFPcPLA2 wt and its mutants S515A, S505A, and S505A/S515A were prepared as described (20, 21). These plasmids contained coding sequence (methionine-1 to alanine-749) of the human cPLA2 gene (GenBank accession number M72993). This gene was inserted into multiple cloning sites HindIII/Smal of pECFP-C3 plasmid (Clontech, Mountain View, CA) so that fluorescent tag ECFP was at the N terminus of cPLA2. Two restriction sites were chosen for subcloning into adenoviral shuttle vector pacAd5CMV (obtained from the University of Iowa, Iowa Viral Vector Facility, Iowa City); 5′ end EcoRV Iioschizomer A/J, and Smal at the 3′ end; this restriction digest contained the sequence of ECFPcPLA2 wt. Adenoviral pacAd5CMV vector was linearized using the EcoRV site in multiple cloning sites of the vector. Ligation reaction of the ECFPcPLA2 wt fragment and adenoviral pacAd5CMV vector was made using the Ready-To-Go T4 DNA Ligase kit (Amersham Pharmacia Biotech, Piscataway, NJ). The ligation product was transformed into chemically competent DH5α cells according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA) and grown on agar plates containing ampicillin. Single colonies were picked, and adenoviral pshuttle vector containing ECFPcPLA2 wt was isolated using the Maxiprep kit (Qiagen, Valencia, CA).

The new construct was confirmed by digestion with a unique PstI restriction site that gave a band of 10 kb on 1% agarose gels and by sequencing. Mutants of ECFPcPLA2 (505, 515, and 505/515) were made using ECFPcPLA2 wt inserted in pacAd5CMV vector as the template and appropriate primers [for mutation of S505 to alanine (S505A), forward primer 5′-CATCTTATCCAC-TGGCTCCTTTGAGTGAC-3′ and reverse primer 5′ GTCCTTTGTAGTGAC-3′ reverse primer 5′-GTACTC-CAAAAGGACCAGTGATAAGATG-3′; for mutation of S515 to alanine (S515A), forward primer 5′-GACTTTGCCACACAG-GACGCCCTTGTAGATGAAGATG-3′; and reverse primer 5′-CAGTTTCACATCATCATAAAGGGCCTGGTGTTGGAAAAGCTG-3′ (IDT, Inc., Caralville, IA)] for each mutant and the Quick-Change XL Site-Directed Mutagenesis Kit according to the manufacturer’s instructions (Stratagene, La Jolla, CA). To make the double S505A/S515A mutant, single mutants were used as the templates in reactions of Quick-Change XL site-directed mutagenesis. ECFPcPLA2 wt and its mutants S505A, S515A, and S505A/S515A inserted in pacAd5CMV were cotransfected with
adenoviral ΔE1 backbone vector into low-passage HEK293 cells (American Type Culture Collection) using the FuGENE 6 transfection reagent (Roche, Palo Alto, CA). A cytopathic effect signaled virus formation, and fluorescence measurements were used to check the efficiency of cotransfection. Viruses were amplified in HEK293 cells and purified using ultracentrifugation in a CsCl2 gradient and dialysis in PBS. Viral particle concentration was determined by measuring optical density at 260 nm by tissue culture infection dose 50 (20).

Transduction of rabbit VSMCs with adenoviral ECFPcPLA2 wt and its mutants

Adenoviruses carrying cDNA ECFPcPLA2 wt and its mutants S515A, S505A, and S515A/S505A were transduced in rabbit VSMCs at a concentration of 60 or 120 multiplicity of infection (MOI) per cell. The cells were then incubated with viral particles for 48 h, and expression of ECFPcPLA2 protein was confirmed by fluorescence microscopy (Olympus IX 50) using filter set exciter wavelength 440 nm, dichroic wavelength 455 nm, and emitter wavelength 480 nm at 40X magnification. Viral transduction efficiency was assessed by Western blot analysis of cell lysates using anti-green fluorescent protein antibody (Santa Cruz Biotechnology, Santa Cruz, CA) that cross-reacts with ECFP and anti-cPLA2 antibody (Santa Cruz Biotechnology). Transduction efficiency of VSMCs with adenoviral enhanced cyan fluorescent protein cytosolic phospholipase A2 wild type (AdECFPcPLA2 wt) and its mutants, as determined by fluorescence microscopy, was >95% and was similar for both AdECFPcPLA2 wt and its mutants.

Preparation of anti-phospho-S515 cPLA2 antibody

Anti-phospho-S515 antibody was produced against human cPLA2 sequence amino acids (510–520) by Quality Controlled...
Biochemicals (Hopkinton, MA). This antibody was raised in rabbits immunized with Ac-CFATQD(pS)FDDDE-amide peptide and purified from serum of immunized animals by affinity purification. The specificity of this antibody was examined by transfecting HEK293 cells with plasmid pacAd5CMV containing ECFPcPLA2 wt and mutants S515A, S505A, and S505A/S515A ECFPcPLA2. The cell lysates were subjected to SDS-PAGE, and Western blot analysis was performed. The blots were probed with anti-phospho-S515 and anti-phospho-S505 antibodies. To further determine the specificity of the phospho-S515 antibody, competition experiments were performed by adding phospho-S515 peptide Ac-CFATQD(pS)FDDDE-amide to the same blocking solution used for Western blot analysis. Phospho-S515 cPLA2 antibody prepared against human cPLA2 peptide sequence (FATQDsFDDDE) was able to recognize the rabbit cPLA2 peptide sequence (DFTQEpSFDDDE).

**Microscopic imaging of fluorescent protein localization in VSMCs transduced with AdECFPcPLA2**

To determine the localization of ECFPcPLA2 wt and its mutants S505A, S515A, and S505A/S515A and ECFP (from empty virus) expressing VSMCs, we used fluorescence microscopy (Olympus IX 50) at 40× magnification with filter set exciter wavelength 440 nm, dichroic wavelength 455 nm, and emitter wavelength 480 nm. AdECFPcPLA2 wt and mutants were transduced into VSMCs, grown on tissue culture plates at 60 MOI in M199 medium containing 0.1% FBS, and incubated for 48 h.

**Fig. 2.** Time course of the phosphorylation of endogenous cPLA2 at S515 and S505 and of ERK1/2 in rabbit vascular smooth muscle cells (VSMCs). Cells arrested in serum-free M199 medium for 48 h were exposed to norepinephrine (NE; 10 μM) for 1, 2, 5, 10, 15, and 20 min. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with primary anti-p-S515 (A, upper panel), anti-p-ERK1/2 (B, upper panel), and anti-p-S505 (C, upper panel) cPLA2 and secondary anti-rabbit HRP conjugate, and chemiluminescence reagent was applied as described in Experimental Procedures. The same membranes were stripped and reprobed with anti-cPLA2 and anti-ERK1/2 antibodies to determine total cPLA2 and ERK1/2 proteins, respectively. Membranes were also probed with anti-α-smooth muscle cell actin antibody to determine equivalent loading (data not shown). The density of bands on Western blots was quantified using ImageJ software and calculated as the ratio of the density of the phosphorylated band over the density of their respective total proteins; it was expressed as percentage increase from that obtained with vehicle (Veh) of NE (A, lower panel, phospho-S515 cPLA2; B, lower panel, phospho-ERK1/2; C, lower panel, phospho-S505 cPLA2). Values are means ± SEM from at least three different experiments with the same treatment in triplicate performed in different batches of cells. Asterisks indicate values significantly different from the corresponding values obtained in the presence of vehicle of NE (P < 0.05).
Cells were imaged using an Olympus IX 50 inverted fluorescence microscope equipped with a 40× objective.

**Distribution of ECFP and ECFPcPLA2 wt and its mutants (S505A, S515A, and S505A/S515A) in VSMCs in response to NE**

To determine the distribution of ECFPcPLA2 wt and its mutants in response to NE, VSMCs were transduced with AdECFPcPLA2 wt and its mutants at 60 MOI as described above and then washed with serum-free medium. After this period, the cells were exposed to NE (10 μM) or its vehicle and viewed with a fluorescence microscope (Nikon Eclipse TE300) using Metamorph software version 6.1. Time-lapse images were taken every 5 s before and after adding NE for 15 min. Two time points were selected, 0 and 20 s after adding NE for data analysis. Experiments were performed in triplicate for each ECFP alone, ECFPcPLA2 wt, and mutant ECFP (S505A, S515A, and S505A/S515A) expressing VSMCs. Images were analyzed using ImageJ software.

**Western blot analysis**

Lysates of VSMCs with and without transduction with AdECFPcPLA2 wt and its mutants were prepared in RIPA-modified lysis buffer (Upstate Biotechnology, Charlottesville, VA). Samples containing 30 μg of proteins were resolved by SDS-PAGE before transferring to nitrocellulose membranes. The membranes were blocked with 3% milk in TBST at room temperature for 1 h and then in a cold room overnight with primary monoclonal antibodies (1:1,000 dilution) against phospho-S505 cPLA2, phospho-ERK1/2, ERK1/2 (Cell Signaling Technology, Danvers, MA), and α-actin (Sigma). Anti-phospho-S515 cPLA2 antibody was used at a dilution of 1:100. The blots were developed using biotinylated secondary antibodies and horseradish peroxidase (Amersham Pharmacia Biotech), and signals were detected using ECL Western blot de-

![Fig. 3.](image-url)
tection reagent SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). The density of bands was measured using ImageJ software. Western blot experiments were performed at least three times on the cells transfected with mutants.

cPLA₂ phosphorylation studies

The phosphorylation of endogenously and exogenously expressed ECFPcPLA₂ wt and its mutants S505A, S515A, and S505A/S515A in VSMCs in response to NE was studied using specific anti-phospho-S515 and anti-phospho-S505 antibodies. The cells with and without transduction with AdECFPcPLA₂ wt and its mutants as described above were treated with NE (10 μM) for 10 min and lysed; total cell proteins were determined using the Bradford assay (22) and separated by SDS-PAGE, and blots were probed with anti-phospho-S515 or anti-phospho-S505 cPLA₂ antibody.

[³H]AA release

VSMCs were plated on 24-well plates at a density of 10⁵ cells per well and infected with viral particles carrying AdECFPcPLA₂. The cells were transfected with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). The density of bands was measured using ImageJ software. Western blot experiments were performed at least three times on the cells transfected with mutants.

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**Fig. 4.** Expression of exogenous ECFPcPLA₂ wt and its mutants S515A, S505A, and S505A/S515A in VSMCs. The cells were transduced with adenovirus (Ad) carrying ECFPcPLA₂ wt and its mutants S515A, S505A, and S505A/S515A [60 multiplicity of infection (MOI) viral particles per cell] or empty vector (EV) for 48 h in serum-free M199 medium. After this period, fluorescence images were taken at 60× magnification or expression of wild-type cPLA₂ and its mutants was determined by Western blot analysis using cPLA₂ antibody. A: Fluorescence micrographs showing the distribution of fluorescence in live cells transduced with AdECFPcPLA₂ wt and its mutants. The fluorescence is uniformly distributed throughout the cytoplasm. B: Western blot of lysates of VSMCs transduced with AdECFPcPLA₂ wt and its mutants S515A, S505A, and S505A/S515A and probed with anti-cPLA₂ antibody. The upper bands show the ectopically expressed ECFPcPLA₂ wt and its mutants (~130 kDa), and the lower bands show the endogenous cPLA₂ (~100 kDa). Fluorescence micrographs and Western blots are representative of three experiments performed with different batches of VSMCs.
wt or its mutants (S515A, S505A, and S505A/S515A) at 60 or 120 MOI. Cells were incubated with viral particles for 48 h in serum-free M199 medium, and then they were labeled with 0.1 μCi/ml [3H]AA in serum-free M199 medium for 18 h at 37°C. After that, the medium was removed and the cells were washed twice with HBSS, incubated with M199 containing 0.1% BSA, and treated with NE (10 μM) or its vehicle for 15 min. The reaction medium was then removed, and radioactivity was measured by liquid scintillation spectroscopy (Beckman, Irvine, CA). The cells were digested in 1 N NaOH overnight for measurement of total cell radioactivity. [3H]AA release into the medium was expressed as a percentage of the total radioactivity of cells plus supernatant.

Trypan blue viability assay

To determine any possible cytotoxic effect on the viability of VSMCs transduced with AdECFPcPLA2 wt and its mutants, we counted the number of viable cells at 72 h after transduction with AdECFPcPLA2 wt and its mutants using trypan blue 0.4% solution (Sigma). More than 99% of cells were trypan blue-negative. We did not find any difference in the number of trypan blue-positive cells between transduced and nontransduced cells.

Statistical analysis

The results of [3H]AA release are presented as mean relative increases elicited by NE as a percentage of that obtained in samples treated with NE vehicle (±SEM). The n value in figure legends refers to the number of experiments that were performed in triplicate for each treatment. The data were analyzed by one-way ANOVA; the unpaired Student’s t-test was used to determine the difference between two groups, and the Newman-Keuls a posteriori test was used to determine the difference between multiple groups. P < 0.05 was considered significant. The phospho-S515/total cPLA2, phospho-S505/total cPLA2, and phospho-ERK1/2/total ERK1/2 ratios were calculated from the

Fig. 5. Distribution of ectopically expressed ECFPcPLA2 wt and its mutants S515A, S505A, and S505A/S515A in response to NE as measured by time-lapse fluorescence imaging. Rabbit VSMCs were transduced with AdECFPcPLA2 wt and its mutants S505A, S515A, S505A/S515A (60 MOI of viral particles) or AdECFP (empty vector). Cells were incubated with viral particles for 72 h before fluorescence imaging experiments. Time-lapse images were acquired using Metamorph 6.1 software so that images were taken before adding NE (10 μM) and after adding NE every 5 s for 15 min. Images presented are at 0 and 20 s after adding NE. Images were taken from cells expressing ECFPcPLA2 wt and its mutants S515A, S505A, and S505A/S515A and AdECFP alone. A–D: ECFPcPLA2 wt and its mutants showed a mostly cytoplasmic distribution before treatment with NE. Fluorescence is shown as a ground-glass appearance in the cytoplasm but not in the nucleus, and there was increased intensity of fluorescence in the perinuclear region because the cells are thicker in this region and have more membranous organelles. E: Cells expressing AdECFP alone showed high intensity of fluorescence in the nucleus and showed a ground-glass appearance of the fluorescence in the cytoplasm without accumulation of the fluorescence perinuclearly. In VSMCs expressing ECFPcPLA2 wt, exposure to NE (10 μM) caused a fluorescence shift from the cytoplasm to the membranes, especially in the perinuclear region at 20 s after adding NE, and it remained there during exposure of cells to NE for 15 min. This pattern of translocation of fluorescence in response to NE was similar in cells expressing mutants S515A, S505A, and S505A/S515A ECFPcPLA2 (A–D). NE did not alter the nuclear or the cytoplasmic localization of fluorescence in cells expressing AdECFP (E). The micrographs are representative of cells from five experiments performed with different batches of cells at 60× magnification.
densitometry analysis of the bands; the value at time point 0 min was arbitrarily chosen as 100%. Values are expressed as x-fold change from the value obtained at time 0 for the subsequent time course during treatment with NE. Means ± SEM of each ratio were calculated from three different Western blot analyses.

RESULTS

Specificity of anti-phospho-S515 cPLA2 antibody

To determine the specificity of anti-phospho-S515 cPLA2 antibody, HEK293 cells were transfected with plasmid pacAd5CMV carrying ECFPcPLA2 wt (pECFPcPLA2 wt) and its mutants S515A and S505A, as described in Experimental Procedures. The expression of pECFPcPLA2 wt and its mutants relative to the levels of endogenous cPLA2 in HEK293 cells as detected by anti-cPLA2 antibody is shown in Fig. 1A. Exogenous ECFPcPLA2 and its mutants expressed in HEK293 cells were detected as a single band (~130 kDa), but the level of endogenous cPLA2 was very low and was not detected consistently. Analysis of cell lysates by Western blots that were probed with anti-phospho-S515 antibody showed a single phosphorylated band from lysates of HEK293 cells transfected with pECFPcPLA2 wt or its mutants S515A and S505A. At 48 h after transduction with AdECFPcPLA2 wt, AdECFPcPLA2S515A, or AdECFPcPLA2S505A (60 MOI of viral particles), VSMCs were treated with NE (10 μM, 10 min). Cells were lysed and protein was separated by SDS-PAGE and probed with anti-p-S515 and anti-p-S505 antibodies (upper panel of blots), and after that membranes were stripped and reprobed with anti-cPLA2 antibody (lower panel of blots). The density of bands on the blots was quantified using ImageJ software and calculated as the ratio of the density of the phosphorylated band over the density of their respective total proteins; it was expressed as percentage increase from that obtained with vehicle (Veh) in the absence of NE. NE increased phosphorylation at S515 and S505 in cells expressing ECFPcPLA2 wt (A, B, I, J) and of endogenous cPLA2 (E, F, M, N). On the other hand, NE-induced phosphorylation at S505 was reduced in cells expressing ECFPcPLA2S515A mutant (C, D) and endogenous cPLA2 (G, H), whereas in samples of cells expressing ECFPcPLA2S505A mutant, phosphorylation on S15 was not reduced but phosphorylation at S505 of ectopically expressed protein ECFPcPLA2 was absent (K, L). The phosphorylation of endogenous cPLA2 at S15 was also not reduced, but phosphorylation at S505 was reduced in cells expressing ECFPcPLA2 mutant (O, P). Values are means ± SEM from three to five experiments performed with different batches of cells. Asterisks indicate values significantly different from the corresponding values obtained in the presence of vehicle of NE (P < 0.05).
with pECFPcPLA2 wt and pECFPcPLA2S505A mutant but not pECFPcPLA2S515A (Fig. 1B, upper panel). When the blots were probed with anti-phospho-S505 antibody, a single phosphorylated band was observed on Western blots from the samples of HEK293 cells transfected with pECFPcPLA2 wt and pECFPcPLA2S515A mutant but not pECFPcPLA2S505A (Fig. 1B, middle panel). The level of total cPLA2 in HEK293 cells transfected with these mutants was detected by probing the blots with anti-cPLA2 (Fig. 1B, lower panel). These results show that anti-phospho-S515 antibody and anti-phospho-S505 antibody are specific in recognizing phospho-S515 cPLA2 and phospho-S505 cPLA2, respectively. Moreover, in a competition experiment, when anti-phospho-S515 antibody and phospho-S515 peptide were added together in the same blocking solution, no phospho-S515 band was observed on the Western blots prepared from lysates of cells transfected with pECFPcPLA2 wt (Fig. 1C). This further confirms the specificity of antiphospho-S515 cPLA2 antibody. In HEK293 cells expressing pECFPcPLA2 wt, stimulation with ionomycin (5 μM) for 10 min increased its phosphorylation by 5-fold at S515, by 2-fold at S505, and by 7-fold at ERK1/2 above basal (Fig. 1D). These results indicate that pECFPcPLA2 wt overexpressed in HEK293 cells is constitutively phosphorylated on both S515 and S505 and that its phosphorylation is enhanced by ionomycin, an agent known to increase the influx of extracellular Ca2+.

Phosphorylation of endogenous cPLA2 at S515 and S505 in response to NE is mediated by CaMKII and ERK1/2, respectively, in VSMCs.

To determine whether NE-activated CaMKII phosphorylates cPLA2 at S515 and leads to the phosphorylation and activation of ERK1/2 and, in turn, the phosphorylation of cPLA2 at S505 in vivo in VSMCs, we examined the time course of phosphorylation of endogenous cPLA2 at S515 and S505 using specific anti-phospho-S515 and -S505 antibodies. Stimulation of rabbit VSMCs with NE (10 μM) at different time points increased the phosphorylation of cPLA2 at S515, as detected by anti-phospho-S515 antibody. CaMKII was rapidly activated (<1 min) by NE (unpub-
lished data) and phosphorylated cPLA2 at S515 that was sustained for up to 20 min (Fig. 2A).

NE (10 μM) increased ERK1/2 phosphorylation at 5 min, which reached its maximum at 10 min (Fig. 2B). The phosphorylation of ERK1/2 was associated with the phosphorylation of S505 cPLA2, as determined by specific anti-phospho-S505 cPLA2 antibody (Fig. 2C). The phosphorylation of both ERK1/2 and cPLA2 at S505 by NE followed the phosphorylation of cPLA2 at S515. KN-93, which inhibits CaMKII activity in VSMCs, but not its inactive analog KN-92 (3), attenuated the phosphorylation of cPLA2 at S515 and S505 as well as ERK1/2 phosphorylation (Fig. 3A–C), whereas the inhibitor of ERK1/2, U0126, attenuated the phosphorylation at S505, but not at S515, of cPLA2 elicited in response to NE (Fig. 3D), indicating that the phosphorylation of cPLA2 at S505 depends upon its phosphorylation at S515 by CaMKII in VSMCs.

**Expression and distribution of exogenous AdECFPcPLA2 wt and AdECFPcPLA2 S505A, S515, and S505A/S515A mutants in VSMCs**

To further establish that NE phosphorylates cPLA2 at S515, which in turn leads to the phosphorylation of cPLA2 at S505, we made fusion constructs of ECFPcPLA2 wt and its mutants in which S515 and S505 were mutated to alanine (S515A, S505A, and S515A/S505A), inserted them into adenovirus, and transduced them in VSMCs, as described in Experimental Procedures. The expression of ECFPcPLA2 wt and its mutants was determined by fluorescence microscopy and by Western blot analysis. Fluorescence microscopic examination showed the expression of ECFPcPLA2 wt and its mutants S505A, S515A, and S505A/S515A in VSMCs (Fig. 4A). Most of the fluorescence was seen in the cytoplasm. Western blot analysis using anti-cPLA2 antibody also showed higher expression of ECFPcPLA2 wt and its mutants (Fig. 4B, upper bands; ~130 kDa) compared with endogenous cPLA2 (Fig. 4B, lower bands; ~100 kDa) in VSMCs transduced with AdECFPcPLA2 wt and its mutants (S505A, S515A, and S505A/S515A).

**Translocation of ECFP, ECFPcPLA2 wt, and its mutants S515A, S505A, and S505A/S515A in response to NE in VSMCs**

To determine whether ECFPcPLA2 wt and its mutants (S505A, S515A, and S505A/S515A) translocate from the cytosol to the membranes, VSMCs were transduced with AdECFPcPLA2 wt and its mutants (S505A, S515A, and S505A/S515A). Fluorescence images were obtained at 72 h after transduction. AdECFPcPLA2 wt and its mutants (S505A, S515A, and S505A/S515A) showed a similar pattern of distribution in the cytoplasm, a ground-glass appearance, and there was no fluorescence detected in the nucleus (Fig. 5A–D). The cells transduced with AdECFP alone showed the presence of fluorescence inside the nucleus that was more intense than in the cytoplasm because of the smaller size of AdECFP. Images were taken before the addition of NE (10 μM) and then sequentially every 5 s for up to 15 min after adding NE. Translocation of ECFPcPLA2 wt was increased in the perinuclear region within 20 s after stimulation with NE and remained there for up to 15 min during NE treatment (Fig. 5A). This pattern of translocation was also detected in cells transduced with mutants (Fig. 5B–D). In cells that were transduced with AdECFP alone, NE did not alter the distribution of fluorescence.

**Phosphorylation of ectopically expressed ECFPcPLA2 wt and its mutants S515A and S505A in response to NE in VSMCs**

To further establish whether NE phosphorylates cPLA2 at S515 and S505, we performed experiments on VSMCs transduced with AdECFPcPLA2 wt and its mutants S515A and S505A. NE (10 μM) increased the phosphorylation of ECFPcPLA2 wt at S515 and S505 (~130 kDa) at the same time points as endogenous cPLA2 (~110 kDa) (Fig. 6A). In VSMCs expressing the endogenous cPLA2 S515A mutant, NE failed to cause phosphorylation of the ectopic protein ECFPcPLA2 S515A at S515, because serine was mutated to alanine and reduced the phosphorylation of the ectopic protein at S505 (Fig. 6C, D). In the same cells, it also reduced the phosphorylation of the endogenous cPLA2 at S515 and S505 (Fig. 6G, H). In VSMCs expressing the endogenous cPLA2 S505A mutant, NE increased phosphorylation at S515 but not at S505 in the ectopic protein (Fig. 6K, L). In the same cells, the phosphorylation of endogenous cPLA2 protein at S505 but not at S515 elicited by NE was also reduced (Fig. 6O, P). Our data suggest that the expression of ECFPcPLA2 S515A has a dominant negative effect, and its effect of reducing the phosphorylation of cPLA2 at S505 supports our findings that the phosphorylation of endogenous cPLA2 at S515 and S505 in response to NE is attenuated by the CaMKII inhibitor KN-93 but not by its inactive analog KN-92, whereas treatment of VSMCs with the ERK1/2 inhibitor U0126 attenuates the phosphorylation of endogenous cPLA2 at S505 but not at S515 (Fig. 5A–D).

**NE-induced [3H]AA release is inhibited in VSMCs expressing ECFPcPLA2 S515A, ECFPcPLA2 S505A, and ECFPcPLA2 S515A/S505A mutants in VSMCs**

To determine the functional significance of cPLA2 phosphorylation at S515 and S505, we examined the effect of NE on the release of AA in VSMCs transduced with AdECFPcPLA2 wt and its mutants AdECFPcPLA2 S515A, AdECFPcPLA2 S505A, and AdECFPcPLA2 S515A/S505A and empty virus (AdECFP). Transduction of VSMCs with 60 MOI AdECFP alone increased the basal [3H]AA release, which was further increased by doubling its concentration (120 MOI), although it was not significantly different from that obtained with 60 MOI (10–15%) (data not shown). Transduction with AdECFPcPLA2 wt or its mutants did not further increase the basal release of [3H]AA. NE significantly increased the release of [3H]AA in nontransduced VSMCs as well as those transduced with AdECFP alone; the increase in [3H]AA release by NE between these groups was not significant. However, in VSMCs transduced with 60 MOI ECFPcPLA2 wt, the effect of NE at increasing [3H]AA was significantly greater than.
that observed in VSMCs transduced with AdECFP (Fig. 7). The amount of $[^3H]$AA released in response to NE was proportional to the increase in the titer of AdECFPcPLA$_2$ wt. In cells transduced with 120 MOI of AdECFPcPLA$_2$ wt, the NE-induced release of $[^3H]$AA was doubled compared with that in cells that were transduced with 60 MOI of this mutant (Fig. 7). In contrast to cells expressing ECFPcPLA$_2$ wt, in cells transduced with 60 or 120 MOI AdECFPcPLA$_2$S515A or AdECFPcPLA$_2$S505A/S515A, the release of $[^3H]$AA was reduced below that obtained in cells transduced with AdECFP alone, whereas in cells transduced with the S505A mutant it was not different from that observed in those transduced with AdECFP (Fig. 7). This suggests that mutants S515A and S505A diminish the functional ability of cPLA$_2$ to release $[^3H]$AA. Moreover, these observations indicate that, although the phosphorylation of cPLA$_2$ at S505 is dependent upon its phosphorylation at S515, it is necessary for NE-induced $[^3H]$AA release in cells expressing ECFPcPLA$_2$ wt.

**DISCUSSION**

This study extends our previous in vitro data by demonstrating 1) that NE promotes the phosphorylation of cPLA$_2$ at S515 and S505 in VSMCs, 2) that phosphorylation on S505 is dependent on S515, and 3) that phosphorylation of these Ser residues of cPLA$_2$ is important for the NE-stimulated AA release in VSMCs. Phosphorylation of cPLA$_2$ on S505 and S727 is important for AA release, because the expression of cPLA$_2$ mutated at these sites failed to increase agonist-induced AA release from mammalian cell models (5, 12). Phosphopeptide analysis of cPLA$_2$ expressed in insect cells (Sf9) (11), and of cPLA$_2$ from platelets (13, 23), HeLa cells (13), and CHO cells (12), revealed a similar degree of phosphorylation on S505 and S727 (11–13, 23). Purified rat brain CaMKII phosphorylates recombinant cPLA$_2$ on S515 (18). In the present in vivo study in rabbit VSMCs, NE stimulated the phosphorylation of cPLA$_2$ at S515 via activation of CaMKII, which in turn resulted in the activation of ERK1/2, the phosphorylation of cPLA$_2$ at S505, and AA release.

We previously reported that cPLA$_2$ activation by ERK1/2 in response to NE and angiotensin II is mediated in large part by the activation of CaMKII in rabbit VSMCs (3, 15, 17). ERK1/2 activation in response to angiotensin II, thrombin, and ionomycin is also dependent to a significant degree on CaMKII activation in rat VSMCs (16). Activation of cPLA$_2$ by CaMKII initiates the production of
AA metabolites, mainly hydroxyeicosatetraenoic acids that activate ERK1/2, which amplifies the activity of cPLA2 and releases additional AA (15). Phosphorylation of cPLA2 at S505 has been reported to increase cPLA2 activity (5). We have found that the phosphorylation of cPLA2 at S505 by ERK1/2 occurred at 5 min after cPLA2 phosphorylation at S515 by CaMKII, indicating that S505 phosphorylation is dependent on S515 phosphorylation in response to NE. Supporting this view was our demonstration that the inhibitor of CaMKII, KN-93, but not its inactive analog, KN-92, attenuated the phosphorylation of ERK1/2 and cPLA2 at S505, whereas the ERK1/2 inhibitor U0126 diminished the phosphorylation of ERK1/2 and of cPLA2 at S505 but not at S515 in rabbit VSMCs.

To investigate the function of the site-specific phosphorylation of cPLA2, phosphorylation site mutants were expressed in VSMCs and examined for their distribution, phosphorylation, and ability to release [3H]AA in response to NE. cPLA2 is known to be translocated from the cytosol to membranes in response to Ca2+ ionophore and various agents, including NE, that increase cellular levels of Ca2+ (3, 5–7, 15). It has been reported that the short duration of intracellular Ca2+ concentration transients translocates cPLA2 to the Golgi, whereas long intracellular Ca2+ concentration transients cause its translocation to the Golgi, endoplasmic reticulum, and perinuclear envelope (24). In the present study, transduction of AdECFPcPLA2 wt and its mutants AdECFPcPLA2-S515A, AdECFPcPLA2-S505A, and AdECFPcPLA2-S515A/S505A in VSMCs resulted in uniform distribution of ECFPcPLA2 and its mutants into the cytoplasm. However, upon stimulation with NE, ECFPcPLA2 wt and its mutants translocated mainly to the perinuclear region. These observations suggest that the phosphorylation of cPLA2 at S505 and S515 is not required for its translocation to the nuclear envelope. In MDCK cells, cPLA2 mutants S505A and S727A were translocated to the Golgi in a similar manner as wild-type cPLA2 in response to ATP and ionomycin (7). However, in VSMCs, the translocation of cPLA2 to the nuclear envelope in response to NE was blocked by the CaMKII inhibitor KN-93, which attenuates the phosphorylation of cPLA2 at S515 (25). This suggests that inhibition of CaMKII by KN93 blocks some additional signaling event triggered by CaMKII activation that is required for cPLA2 translocation. Expressing phosphorylation site mutants of cPLA2 allowed us to specifically determine the role of site-specific phosphorylation without interfering with NE-induced CaMKII activation.

Our demonstration that NE stimulated the phosphorylation of ECFPcPLA2 wt, but not that of ECFPcPLA2 S515A mutant in vivo, expressed in VSMCs, supports our in vitro finding that cPLA2 is phosphorylated at S515 by CaMKII (18). In VSMCs expressing the ECFPcPLA2-S515A mutant, the phosphorylation at S505 of this mutant and that of ERK1/2 in response to NE was reduced, suggesting that the phosphorylation of cPLA2 at S505 is in part dependent upon its phosphorylation at S515. Moreover, the fact that ECFPcPLA2 wt, but not S505A, expressed in VSMCs was phosphorylated at S505, which was attenuated by the ERK1/2 inhibitor U0126, supports the view that phosphorylation at S505 consequent to phosphorylation at S515 by CaMKII is mediated by ERK1/2. The phosphorylation of recombinant cPLA2 by purified rat brain CaMKII at S515 increased its ability to hydrolyze AA from phosphatidylcholine in vitro (18). Because in VSMCs ectopically expressing ECFPcPLA2 wt the release of [3H]AA in response to NE was significantly greater than in VSMCs transduced with AdECFP alone or in nontransduced cells, and the increase in the release of [3H]AA was related to the titer of the virus, it appears that NE also causes the activation of ectopically expressed ECFPcPLA2 wt. This was further supported by our observations that the NE-induced increase in [3H]AA release in VSMCs expressing ECFPcPLA2-S515A, S505A, or S515/S505A was inhibited, suggesting that their functional inactivity is caused by a mutation. From these observations, it follows that the activation of ectopically expressed cPLA2 in VSMCs is also mediated via CaMKII and ERK1/2. Because in cells expressing ECFPcPLA2-S515A the ability of NE to increase [3H]AA release was reduced below that obtained in cells transduced with AdECFP alone, it appears that the expression of ECFPcPLA2-S515A in VSMCs inhibits the activity of endogenous cPLA2 by exerting a dominant negative effect. This may be attributable to the binding of CaMKII to the increased amount of cPLA2 in VSMCs (18).

The mechanism by which NE releases [3H]AA to a lesser degree in VSMCs expressing cPLA2 mutants than in those

![Fig. 8. Proposed pathway of cPLA2 phosphorylation on S515 and S505 by NE in VSMCs. NE activates CaMKII by binding to α-adrenergic receptor (α-AR), which phosphorylates cPLA2 at S515 and releases AA and one or more AA metabolites [hydroxyeicosatetraenoic acids (HETEs)] generated via lipoxigenase (LO), and cytochrome P450 (CYP450) activates ERK1/2, which phosphorylates cPLA2 at S505 and leads to its further activation and additional AA release.](image-url)
expressing ECFPcPLA2 wt is not known. It has been reported that the mechanism by which S505 phosphorylation increases the activity of cPLA2 is to allow its sustained interaction with membrane in response to transient Ca\textsuperscript{2+} increase (26). However, our data indicate that the translocation of phosphorylation site mutants and ECFPcPLA2 wt were similar in response to NE. This is consistent with a previous report that the translocation properties of cPLA2 mutated at S505 and wild-type cPLA2 are similar in response to transient, physiological calcium increases (7). Because phosphorylation sites are near the flexible hinge region of cPLA2, it has also been suggested that phosphorylation may optimize the conformation of the catalytic domain and enhance cPLA2 activity (27). Our data are consistent with a role for phosphorylation in regulating catalytic activity, because the VSMCs expressing phosphorylated site-mutated cPLA2 release less AA.

In conclusion, the present study demonstrates that NE promotes AA release by promoting cPLA2 phosphorylation at S515 and S505 in vivo in VSMCs. NE stimulates cPLA2 phosphorylation at S515 via the activation of CaMKII, and phosphorylation at S505 by ERK1/2 is dependent upon its phosphorylation at S515 (Fig. 8). Activation of cPLA2 by its phosphorylation by CaMKII appears to be unique to VSMCs. Because the inhibition of CaMKII and cPLA2 has been shown to decrease VSMC growth (28) and neointimal growth in balloon-injured rat carotid artery (29, 30), the selective inactivation of cPLA2 phosphorylation at S515 to reduce its activity could be an important target for the development of agents to treat restenosis caused by vascular injury.

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