cPLA$_2$ 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 A$_2$ (cPLA$_2$) 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 cPLA$_2$ at serine-515 (S515) and increases its activity in vitro. We have studied the sites of cPLA$_2$ 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 cPLA$_2$ antibodies and by mutagenesis of S515 and S505 to alanine. NE increased the phosphorylation of cPLA$_2$ at S515, followed by phosphorylation of ERK1/2 and consequently phosphorylation of cPLA$_2$ at S505. The CaMKII inhibitor 2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzene-sulfonyl)]amino-N-(4-chlorocinnamyl)-methylbenzylamine attenuated cPLA$_2$ 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 cPLA$_2$ 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 cPLA$_2$ and AA release. ▌ These data suggest that the NE-stimulated phosphorylation of cPLA$_2$ 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. cPLA$_2$ 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.

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 A$_2$ (cPLA$_2$) (2, 3), a group IV phospholipase A$_2$ member (4). NE-induced cPLA$_2$ activation and AA release require calcium (Ca^{2+}), calmodulin (CaM), and calmodulin-dependent kinase II (CaMKII) in VSMCs (2, 3). cPLA$_2$ activity has been reported to be regulated by phosphorylation by extracellular regulated kinase 1/2 (ERK1/2), p42/p44 mitogen-activated protein kinase, and Ca^{2+}-dependent translocation to the nuclear envelope, allowing its access to arachidonyl-containing phospholipid substrate at the *sn*-2 position (6). ERK1/2 phosphorylates cPLA$_2$ at serine-505 (S505) in several cell types (5–7). However, the phosphorylation and activation of cPLA$_2$ 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 cPLA$_2$ (S431, S454, S505, and S727) suggested cPLA$_2$ as a substrate for other kinases (11). Studies in platelets, HeLa cells, and CHO cells have shown that cPLA$_2$ is phosphorylated on S505 and S727 (12). S727 is phosphorylated by MNK1 or a closely related isoform, a protein

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 CaMKII 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 and fused with the enhanced cyan fluorescent protein (ECFP) at the N-terminal end of cPLA₂ and AA release in rabbit VSMCs. Our results indicate that NE stimulates phosphorylation on S515 and the activation of endogenous cPLA₂ and exogenously expressed enhanced cyan fluorescent protein cytosolic phospholipase A₂ wild type (ECFPcPLA₂ wt). Moreover, phosphorylation of cPLA₂ on S505 is dependent on its phosphorylation at S515 elicited by CaMKII, and both sites of phosphorylation are required for AA release in response to NE in VSMCs.

EXPERIMENTAL PROCEDURES

Materials

Arterenol NE and ampicillin were from Sigma (St. Louis, MO); the CaMKII inhibitor 2-[N-(2-hydroxyethyl)]N-(4-methoxybenzene-sulfonyl)] 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). [³H]AA (0.1 µCi/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 G, and 100 µg/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 liposomic 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 S515A/S505A were prepared as described (20, 21). These plasmids contained coding sequence (methionine-1 to alanine-749) of the human cPLA₂ gene (GenBank accession number M72393). This gene was inserted into multiple cloning sites HindIII/SmaI 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 Eco47III isoschizomer AfeI, and SmaI 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 Maxi-prep kit (Qiagen, Valencia, CA).

The new construct was confirmed by digestion with a unique PacI 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′-GTCACT-CAAAGGAGCCAGTGGATAAGATG-3′; for mutation of S515 to alanine (S515A), forward primer 5′-GACTTTGCCACACAG-GACGCCTTTGATGATGATGAACTG-3′ and reverse primer 5′-CAGTTCATCATCATCAAAGGCGTCCTGTGTGGCAAAGTC-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 $CsCl_2$ gradient and dialysis in PBS. Viral particle concentration was determined by measuring optical density at 260 nm and by tissue culture infection dose 50 (20).

Transduction of rabbit VSMCs with adenoviral ECFPcPLA$_2$ wt and its mutants

Adenoviruses carrying cDNA ECFPcPLA$_2$ 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 ECFPcPLA$_2$ 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 40× magnification and 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-cPLA$_2$ antibody (Santa Cruz Biotechnology). The transduction efficiency of VSMCs with adenoviral enhanced cyan fluorescent protein cytosolic phospholipase A$_2$ wild type (AdECFPcPLA$_2$ wt) and its mutants, as determined by fluorescence microscopy, was >95% and was similar for both AdECFPcPLA$_2$ wt and its mutants.

Preparation of anti-phospho-S515 cPLA$_2$ antibody

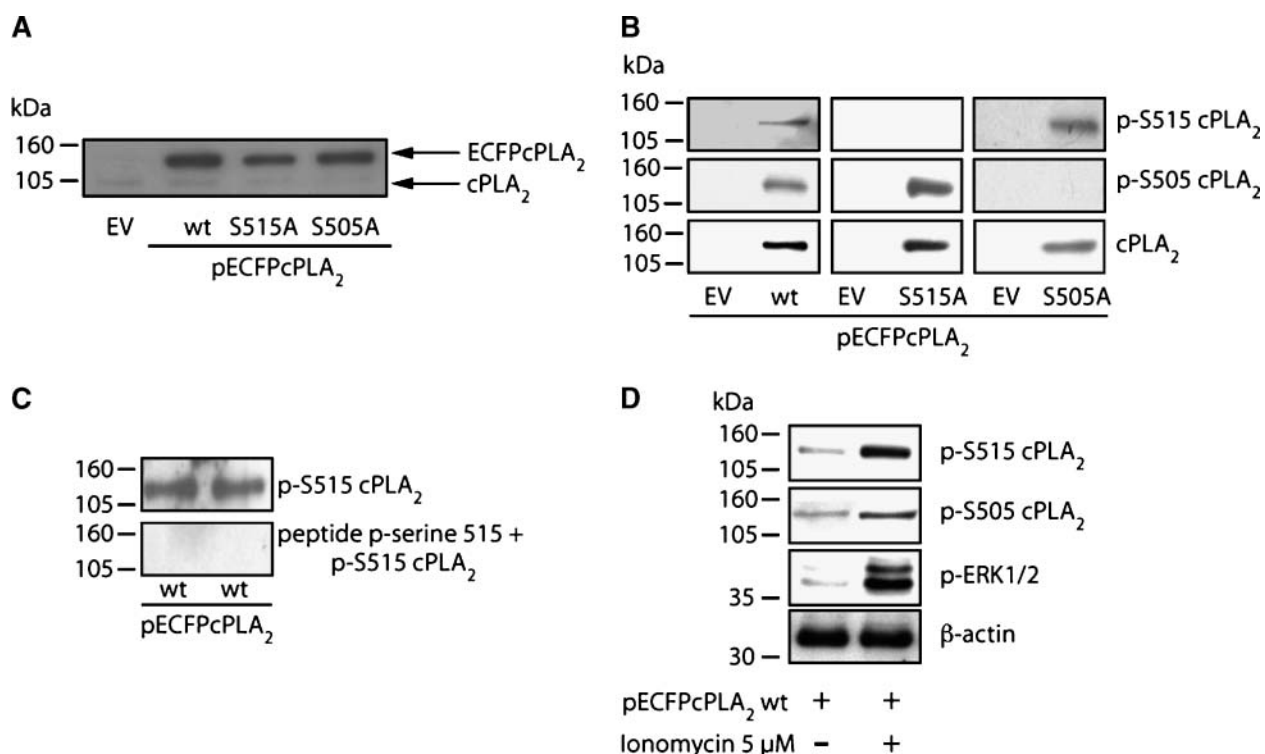Anti-phospho-S515 antibody was produced against human cPLA$_2$ sequence amino acids (510–520) by Quality Controlled

Fig. 1. Specificity of the anti-phospho-serine-515 (S515) cytosolic phospholipase A$_2$ (p-S515 cPLA$_2$) antibody determined in HEK293 cells transfected with plasmid pacAd5CMV carrying enhanced cyan fluorescent protein cytosolic phospholipase A$_2$ wild type (pECFPcPLA$_2$ wt) and mutants S515A and S505A or empty vector (EV). Cells were transfected with these plasmids using Effectene transfection reagent (Qiagen); at 48 h after transfection, the cells were lysed, the concentration of the protein in total cell lysates was measured, and proteins were separated by SDS-PAGE. After electrophoresis, the proteins were transferred to nitrocellulose membranes and the phosphorylation of the proteins was detected using anti-p-S515 cPLA$_2$ antibody. A: Blot of the proteins from lysates of HEK293 cells transfected with empty vector, pECFPcPLA$_2$ wt, and its mutants S515A and S505A and probed with anti-cPLA$_2$ antibody. The blot shows the expression of ectopic protein ECFPcPLA$_2$ wt and mutants S505A and S515A (~130 kDa) and a very faint band of endogenous cPLA$_2$ (~100 kDa) under basal nonstimulated conditions. B: Blots of the proteins from lysates of HEK293 cells transfected with empty vector, pECFPcPLA$_2$ wt, and its mutants S515A and S505A and probed with anti-p-S515 and anti-p-S505 cPLA$_2$ antibodies, showing constitutive phosphorylation on S515 (upper panel) and on S505 (middle panel). The bottom panel shows the total amount of cPLA$_2$ as determined by stripping the membranes and reprobing the blots with anti-cPLA$_2$ antibody. C: Western blots (probed with anti-p-S515 cPLA$_2$ antibody) of the competition assay showing constitutively phosphorylated S515 cPLA$_2$ in HEK293 cells expressing pECFPcPLA$_2$ (upper panel). Anti-p-S515 cPLA$_2$ antibody and the phospho-S515 peptide were added together in the same blocking solution (lower panel). D: Western blots showing the expression of pECFPcPLA$_2$ wt in HEK293 cells with and without stimulation with ionomycin. Cells were transfected with pECFPcPLA$_2$ wt and stimulated with ionomycin (5 µM) for 10 min or without stimulation. The cells were lysed and proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with primary anti-p-S515 (panel 1), anti-p-S505 (panel 2), anti-p-extracellular regulated kinase 1/2 (ERK1/2; panel 3), and β-actin (panel 4) antibodies. Ionomycin increased the phosphorylation of pECFPcPLA$_2$ wt at S515 (~5-fold), at S505 (~2-fold), and of ERK1/2 (~7-fold) (right side) compared with that observed in unstimulated cells (left side). Equivalent loading was confirmed with β-actin antibody. All the blots shown are representative of three experiments performed in different batches of HEK293 cells.

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 ECFPcPLA$_2$ wt and mutants S515A, S505A, and S505A/S515A ECFPcPLA$_2$. 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 cPLA$_2$ antibody prepared against human cPLA$_2$ peptide sequence

(FATQDpSFDDDE) was able to recognize the rabbit cPLA$_2$ peptide sequence (DFTQEpSFDDDE).

Microscopic imaging of fluorescent protein localization in VSMCs transduced with AdECFPcPLA$_2$

To determine the localization of ECFPcPLA$_2$ 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. AdECFPcPLA$_2$ 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.
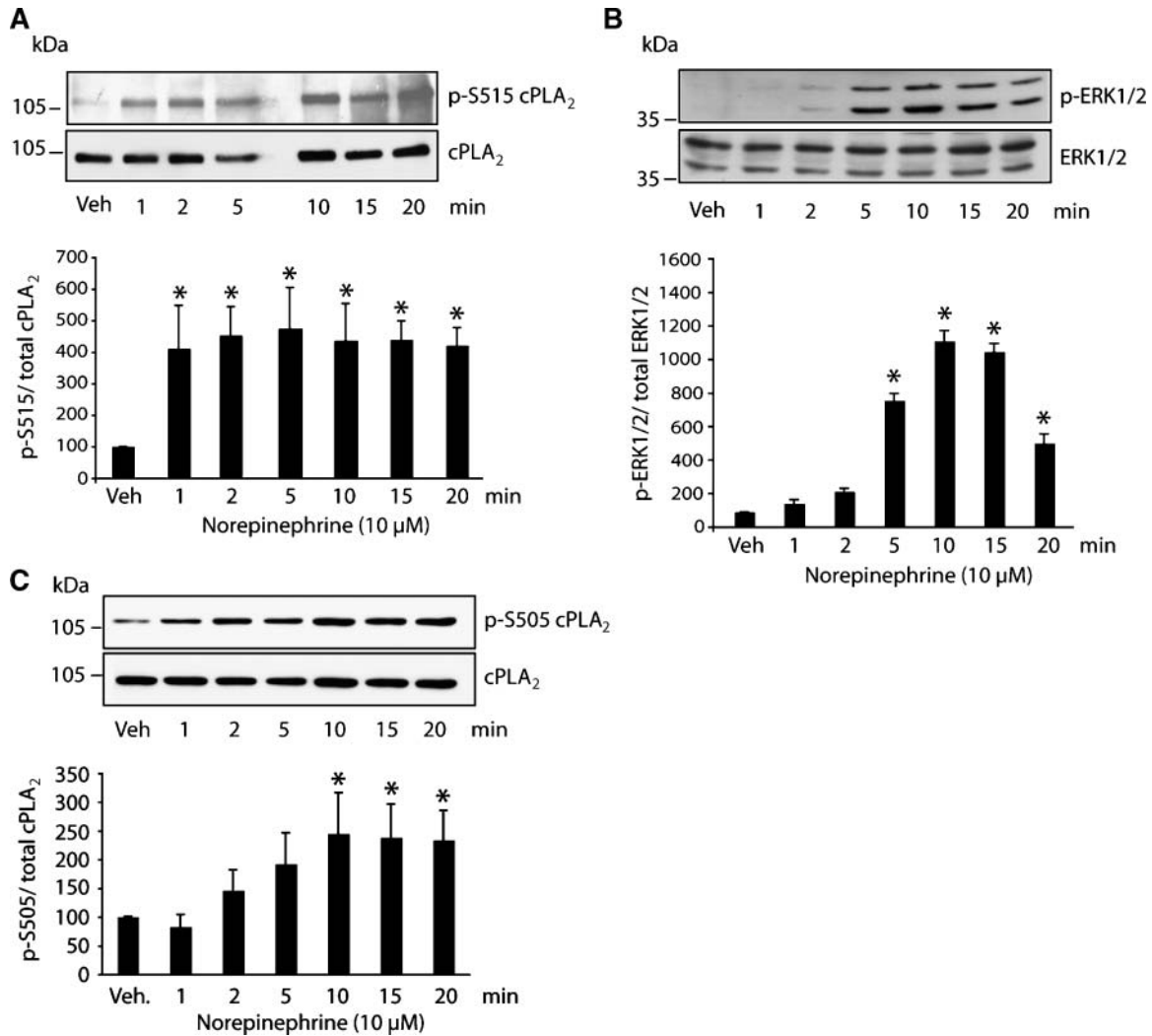

Fig. 2. Time course of the phosphorylation of endogenous cPLA$_2$ at S515 and S505 and of ERK1/2 in rabbit vascular smooth muscle cells (VSMCs). Cells arrested in serum-free M199 medium for 48 h were exposed to norepinephrine (NE; 10 μM) for 1, 2, 5, 10, 15, and 20 min. Proteins were separated by SDS-PAGE, 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) cPLA$_2$ 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-cPLA$_2$ and anti-ERK1/2 antibodies to determine total cPLA$_2$ 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 cPLA$_2$; B, lower panel, phospho-ERK1/2; C, lower panel, phospho-S505 cPLA$_2$). 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-
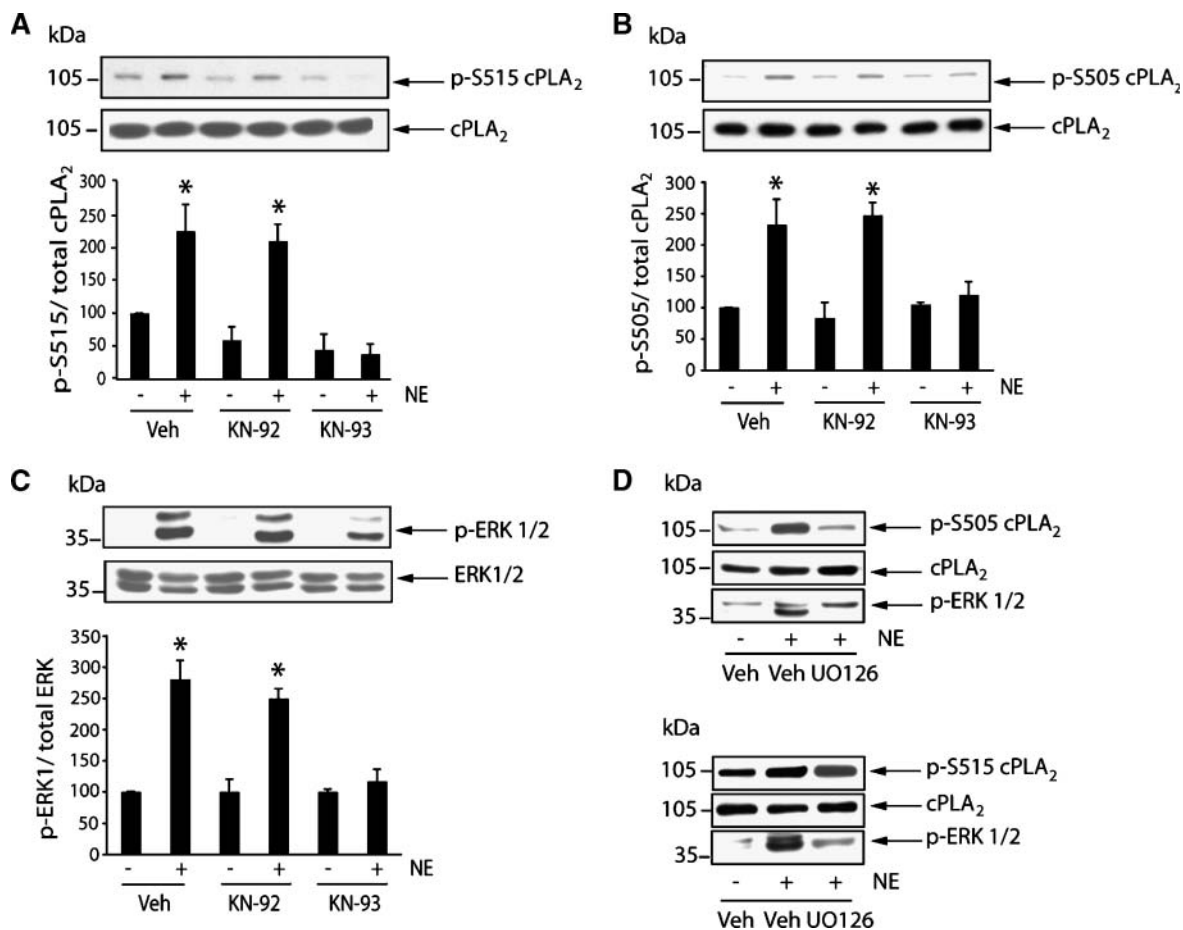

Fig. 3. The calmodulin kinase II (CaMKII) inhibitor 2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzene-sulfonyl)]amino-N-(4-chlorocinnamyl)-methylbenzylamine (KN-93), but not 2-[N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine (KN-92), attenuates the NE-induced phosphorylation of endogenous cPLA₂ at S515 and S505 and of ERK1/2 in VSMCs. Quiescent VSMCs were pretreated with KN-93 or KN-92 (30 μM) for 30 min before exposure of cells to NE (10 μM) for 10 min. Proteins were separated by SDS-PAGE, and the membranes were probed with anti-p-S515 and anti-p-S505 cPLA₂ and anti-p-ERK1/2 antibodies. The blots were stripped and reprobed with anti-cPLA₂ and anti-ERK1/2 antibodies. 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. In cells pretreated with KN-93, but not its inactive analog KN-92, the phosphorylation of cPLA₂ on S515 (A) and S505 (B) as well as that of ERK1/2 (C) was decreased. Treatment of cells with the ERK1/2 inhibitor UO126 (30 min, 10 μM) decreased the NE-induced phosphorylation of cPLA₂ at S505 and of ERK1/2 but did not decrease the phosphorylation S515 (D). Values are means ± SEM from at least three different experiments with the same treatment in triplicate in different batches of cells. Asterisks indicate values significantly different from the corresponding values obtained in the presence of vehicle of NE ($P < 0.05$).

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 ECFPcPLA$_2$ 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 AdECFPcPLA$_2$ 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 cPLA$_2$ antibody.

[³H]AA release

VSMCs were plated on 24-well plates at a density of 10^5 cells per well and infected with viral particles carrying AdECFPcPLA$_2$
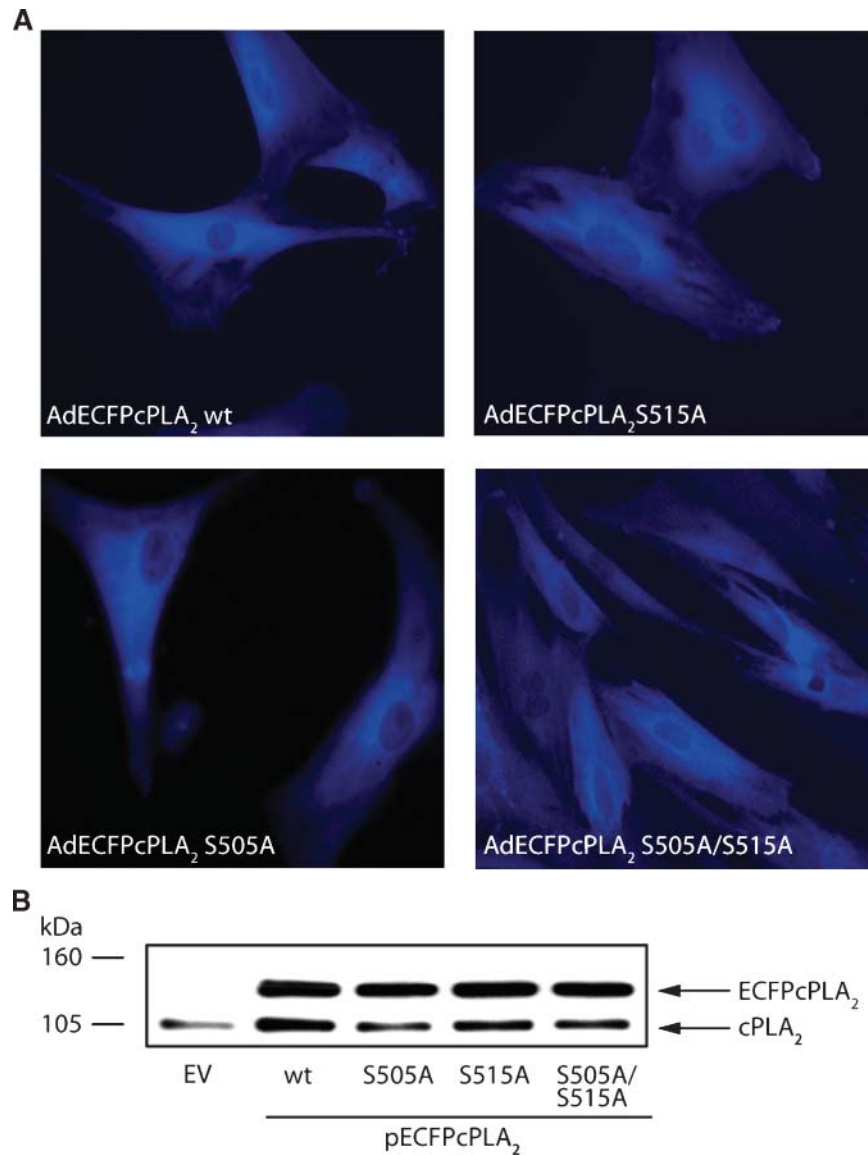

Fig. 4. Expression of exogenous ECFPcPLA$_2$ wt and its mutants S515A, S505A, and S505A/S515A in VSMCs. The cells were transduced with adenovirus (Ad) carrying ECFPcPLA$_2$ wt and its mutants S515A, S505A, and S505A/S515A [60 multiplicity of infection (MOI) viral particles per cell] or empty vector (EV) for 48 h in serum-free M199 medium. After this period, fluorescence images were taken at 60× magnification or expression of wild-type cPLA$_2$ and its mutants was determined by Western blot analysis using cPLA$_2$ antibody. A: Fluorescence micrographs showing the distribution of fluorescence in live cells transduced with AdECFPcPLA$_2$ wt and its mutants. The fluorescence is uniformly distributed throughout the cytoplasm. B: Western blot of lysates of VSMCs transduced with AdECFPcPLA$_2$ wt and its mutants S515A, S505A, and S505A/S515A and probed with anti-cPLA$_2$ antibody. The upper bands show the ectopically expressed ECFPcPLA$_2$ wt and its mutants (∼130 kDa), and the lower bands show the endogenous cPLA$_2$ (∼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 [^{3}H]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. [^{3}H]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 AdECFPcPLA$_2$ wt and its mutants, we counted the number of viable cells at 72 h after transduction with AdECFPcPLA$_2$ 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 [^{3}H]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 cPLA$_2$, phospho-S505/total cPLA$_2$ and phospho-ERK1/2/total ERK1/2 ratios were calculated from the
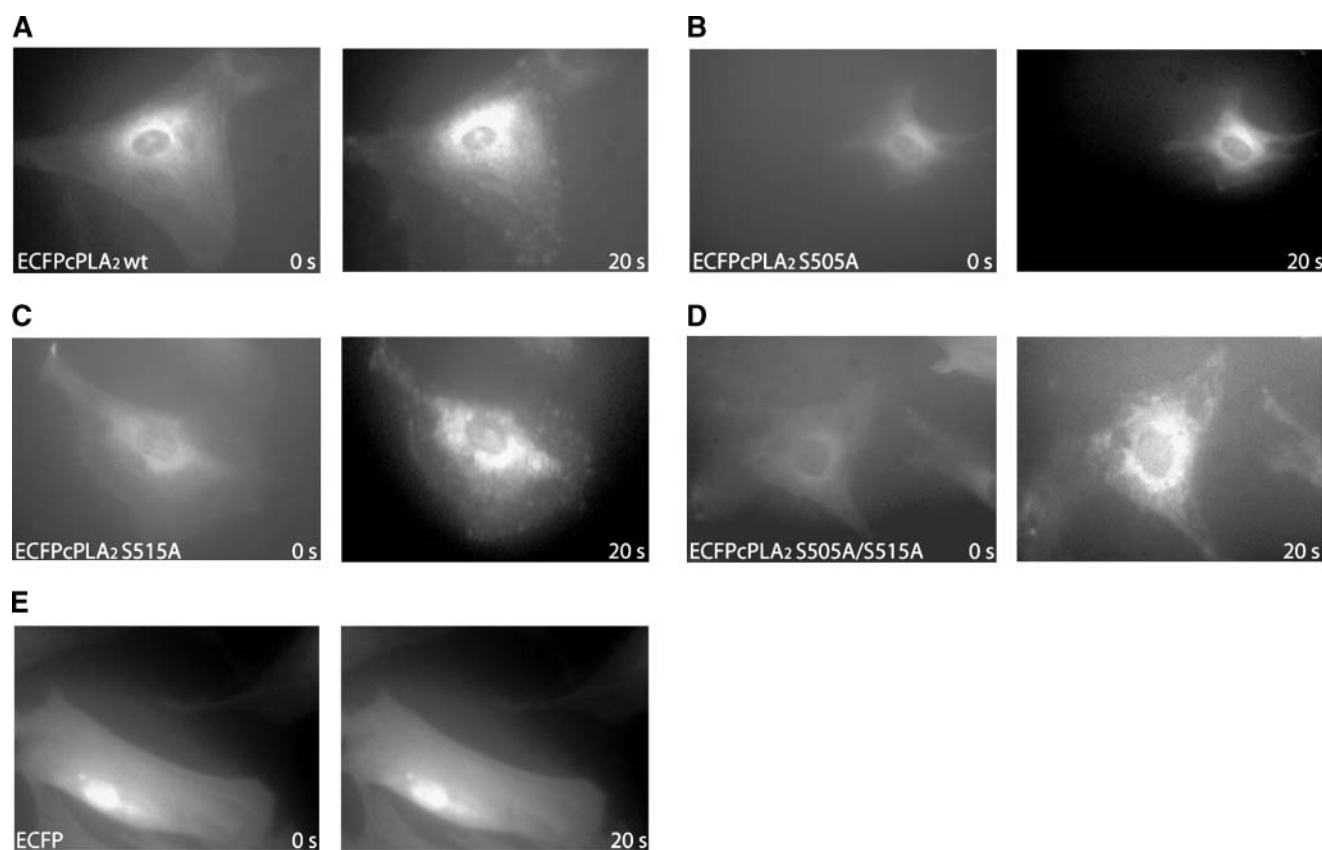

Fig. 5. Distribution of ectopically expressed ECFPcPLA$_2$ wt and its mutants S515A, S505A, and S505A/S515A in response to NE as measured by time-lapse fluorescence imaging. Rabbit VSMCs were transduced with AdECFPcPLA$_2$ wt and its mutants S505A, S515A, S505A/S515A (60 MOI of viral particles) or AdECFP (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 ECFPcPLA$_2$ wt and its mutants S515A, S505A, and S505A/S515A and AdECFP alone. A–D: ECFPcPLA$_2$ 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 the ground-glass appearance of the fluorescence in the cytoplasm without accumulation of the fluorescence perinuclearly. In VSMCs expressing ECFPcPLA$_2$ 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 ECFPcPLA$_2$ (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.
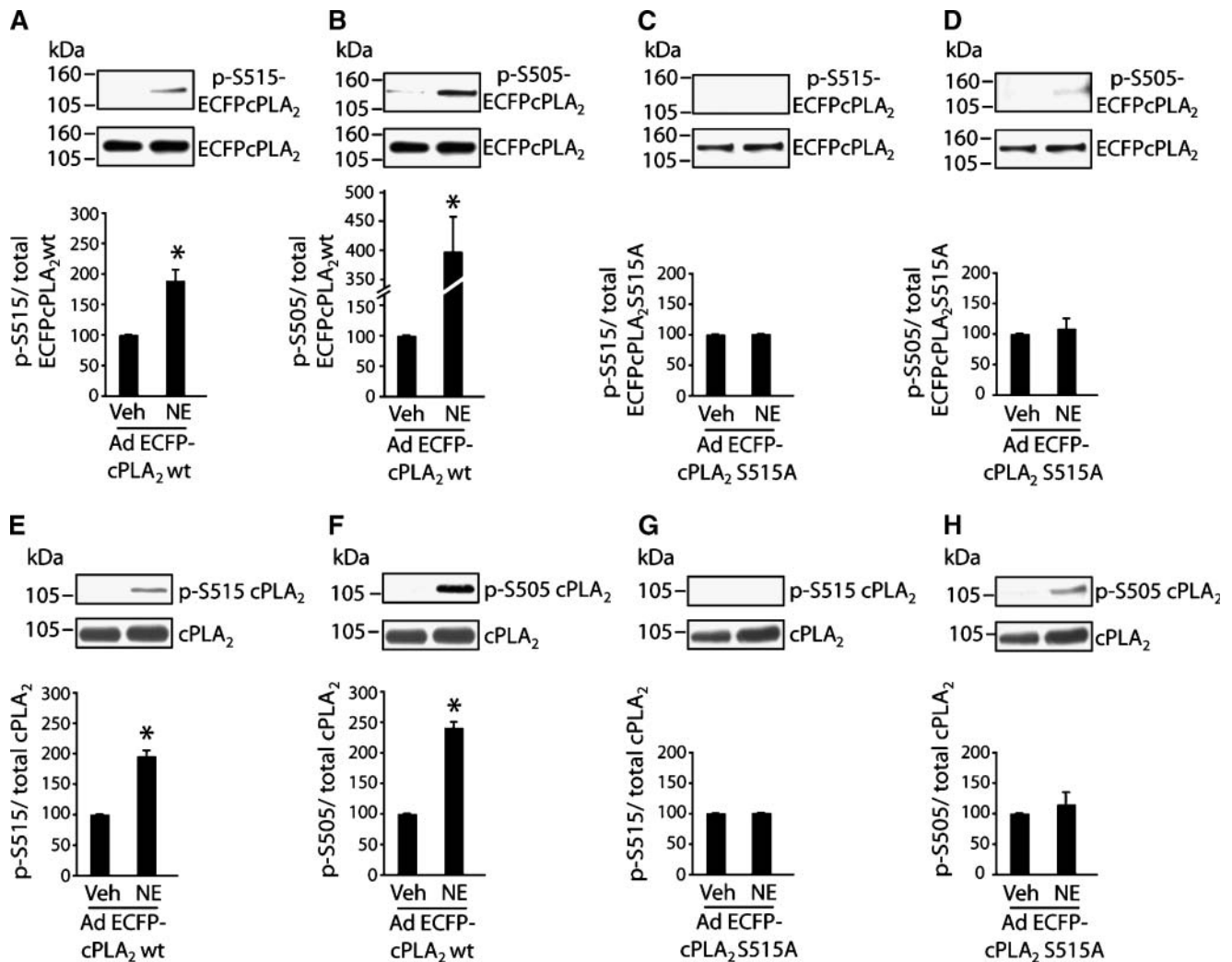
Fig. 6. Transduction of VSMCs with AdECFPcPLA₂S515A mutant inhibits the phosphorylation of ectopically expressed cPLA₂ at S505 and of endogenous cPLA₂ at S515 and S505. At 48 h after transduction with AdECFPcPLA₂ wt, AdECFPcPLA₂S515A, or AdECFPcPLA₂S505A (60 MOI of viral particles), VSMCs were treated 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 (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 S515 was not reduced but phosphorylation at S505 of ectopically expressed protein ECFPcPLA₂ was absent (K, L). The phosphorylation of endogenous cPLA₂ at S515 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 ($P < 0.05$).

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 plas-mid 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
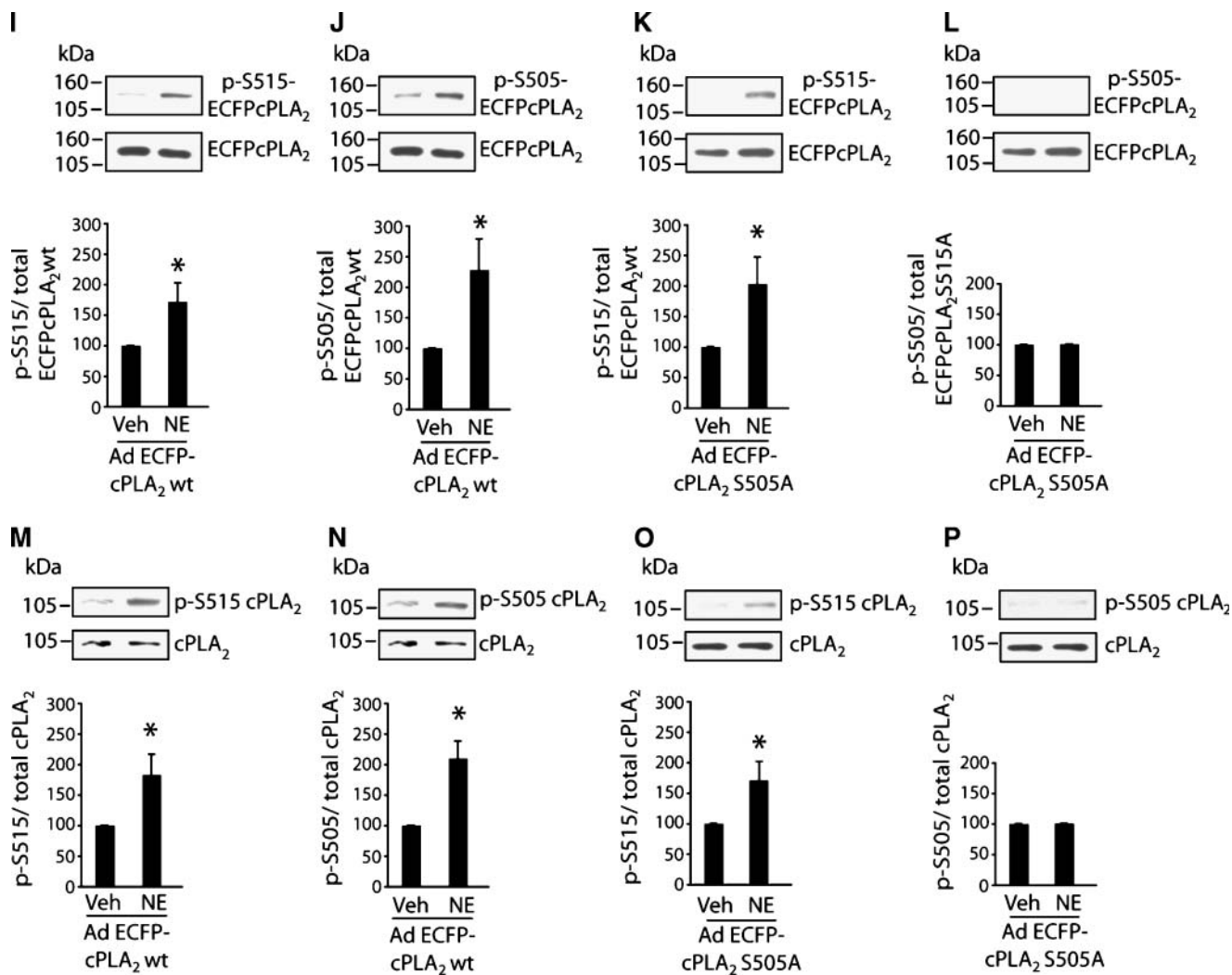
Fig. 6.—*Continued.*

with pECFPcPLA$_2$ wt and pECFPcPLA$_2$S505A mutant but not pECFPcPLA$_2$S515A (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 pECFPcPLA$_2$ wt and pECFPcPLA$_2$S515A mutant but not pECFPcPLA$_2$S505A (Fig. 1B, middle panel). The level of total cPLA$_2$ in HEK293 cells transfected with these mutants was detected by probing the blots with anti-cPLA$_2$ (Fig. 1B, lower panel). These results show that anti-phospho-S515 antibody and anti-phospho-S505 antibody are specific in recognizing phospho-S515 cPLA$_2$ and phospho-S505 cPLA$_2$, 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 pECFPcPLA$_2$ wt (Fig. 1C). This further confirms the specificity of antiphospho-S515 cPLA$_2$ antibody. In HEK293 cells expressing pECFPcPLA$_2$ 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 pECFPcPLA$_2$ 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 Ca^{2+}.

Phosphorylation of endogenous cPLA$_2$ 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 cPLA$_2$ at S515 and leads to the phosphorylation and activation of ERK1/2 and, in turn, the phosphorylation of cPLA$_2$ at S505 in vivo in VSMCs, we examined the time course of phosphorylation of endogenous cPLA$_2$ 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 cPLA$_2$ at S515, as detected by anti-phospho-S515 antibody. CaMKII was rapidly activated (<1 min) by NE (unpub-

lished data) and phosphorylated cPLA$_2$ 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 cPLA$_2$, as determined by specific anti-phospho-S505 cPLA$_2$ antibody (Fig. 2C). The phosphorylation of both ERK1/2 and cPLA$_2$ at S505 by NE followed the phosphorylation of cPLA$_2$ at S515. KN-93, which inhibits CaMKII activity in VSMCs, but not its inactive analog KN-92 (3), attenuated the phosphorylation of cPLA$_2$ 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 cPLA$_2$ elicited in response to NE (Fig. 3D), indicating that the phosphorylation of cPLA$_2$ at S505 depends upon its phosphorylation at S515 by CaMKII in VSMCs.

Expression and distribution of exogenous AdECFPcPLA$_2$ wt and AdECFPcPLA$_2$ S505A, S515, and S505A/S515A mutants in VSMCs

To further establish that NE phosphorylates cPLA$_2$ at S515, which in turn leads to the phosphorylation of cPLA$_2$ at S505, we made fusion constructs of ECFPcPLA$_2$ 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 ECFPcPLA$_2$ wt and its mutants was determined by fluorescence microscopy and by Western blot analysis. Fluorescence microscopic examination showed the expression of ECFPcPLA$_2$ 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-cPLA$_2$ antibody also showed higher expression of ECFPcPLA$_2$ wt and its mutants (Fig. 4B, upper bands; ~130 kDa) compared with endogenous cPLA$_2$ (Fig. 4B, lower bands; ~100 kDa) in VSMCs transduced with AdECFPcPLA$_2$ wt and its mutants (S505A, S515A, and S505A/S515A).

Translocation of ECFP, ECFPcPLA$_2$ wt, and its mutants S515A, S505A, and S505A/S515A in response to NE in VSMCs

To determine whether ECFPcPLA$_2$ wt and its mutants (S505A, S515A, and S505A/S515A) translocate from the cytosol to the membranes, VSMCs were transduced with AdECFPcPLA$_2$ wt and mutants (S505A, S515A, and S505A/S515A). Fluorescence images were obtained at 72 h after transduction. AdECFPcPLA$_2$ 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 ECFPcPLA$_2$ 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 ECFPcPLA$_2$ wt and its mutants S515A and S505A in response to NE in VSMCs

To further establish whether NE phosphorylates cPLA$_2$ at S515 and S505, we performed experiments on VSMCs transduced with AdECFPcPLA$_2$ wt and its mutants S515A and S505A. NE (10 μM) increased the phosphorylation of ECFPcPLA$_2$ wt at S515 and S505 (~130 kDa) at the same time points as endogenous cPLA$_2$ (~110 kDa) (**Fig. 6A, B, E, F, I, J, M, N**). In VSMCs expressing the ECFPcPLA$_2$S515A mutant, NE failed to cause phosphorylation of the ectopic protein ECFPcPLA$_2$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 cPLA$_2$ at S515 and S505 (Fig. 6G, H). In VSMCs expressing the ECFPcPLA$_2$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 cPLA$_2$ protein at S505 but not at S515 elicited by NE was also reduced (Fig. 6O, P). Our data suggest that the expression of ECFPcPLA$_2$S515A has a dominant negative effect, and its effect of reducing the phosphorylation of cPLA$_2$ at S505 supports our findings that the phosphorylation of endogenous cPLA$_2$ 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 cPLA$_2$ at S505 but not at S515 (Fig. 3A–D).

NE-induced [^{3}H]AA release is inhibited in VSMCs expressing ECFPcPLA$_2$S515A, ECFPcPLA$_2$S505A, and ECFPcPLA$_2$S515A/S505A mutants in VSMCs

To determine the functional significance of cPLA$_2$ phosphorylation at S515 and S505, we examined the effect of NE on the release of AA in VSMCs transduced with AdECFPcPLA$_2$ wt and its mutants AdECFPcPLA$_2$-S515A, AdECFPcPLA$_2$S505A, and AdECFPcPLA$_2$S515A/S505A and empty virus (AdECFP). Transduction of VSMCs with 60 MOI AdECFP alone increased the basal [^{3}H]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 AdECFPcPLA$_2$ wt or its mutants did not further increase the basal release of [^{3}H]AA. NE significantly increased the release of [^{3}H]AA in nontransduced VSMCs as well as those transduced with AdECFP alone; the increase in [^{3}H]AA release by NE between these groups was not significant. However, in VSMCs transduced with 60 MOI ECFPcPLA$_2$ wt, the effect of NE at increasing [^{3}H]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 AdECFPcPLA₂ wt. In cells transduced with 120 MOI of AdECFPcPLA₂ 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 ECFPcPLA₂ wt, in cells transduced with 60 or 120 MOI AdECFPc-PLA₂S515A or AdECFPcPLA₂S505A/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 cPLA₂ to release [³H]AA. Moreover, these observations indicate that, although the phosphorylation of cPLA₂ at S505 is dependent upon its phosphorylation at S515, it is necessary for NE-induced [³H]AA release in cells expressing ECFPcPLA₂ wt.

DISCUSSION

This study extends our previous in vitro data by demonstrating *1*) that NE promotes the phosphorylation of cPLA₂ at S515 and S505 in VSMCs, *2*) that phosphorylation on S505 is dependent on S515, and *3*) that phosphorylation of these Ser residues of cPLA₂ is important for the NE-stimulated AA release in VSMCs. Phosphorylation of cPLA₂ on S505 and S727 is important for AA release, because the expression of cPLA₂ mutated at these sites failed to increase agonist-induced AA release from mammalian cell models (5, 12). Phosphopeptide analysis of cPLA₂ expressed in insect cells (Sf9) (11), and of cPLA₂ 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 cPLA₂ on S515 (18). In the present in vivo study in rabbit VSMCs, NE stimulated the phosphorylation of cPLA₂ at S515 via activation of CaMKII, which in turn resulted in the activation of ERK1/2, the phosphorylation of cPLA₂ at S505, and AA release.

We previously reported that cPLA₂ 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 cPLA₂ by CaMKII initiates the production of
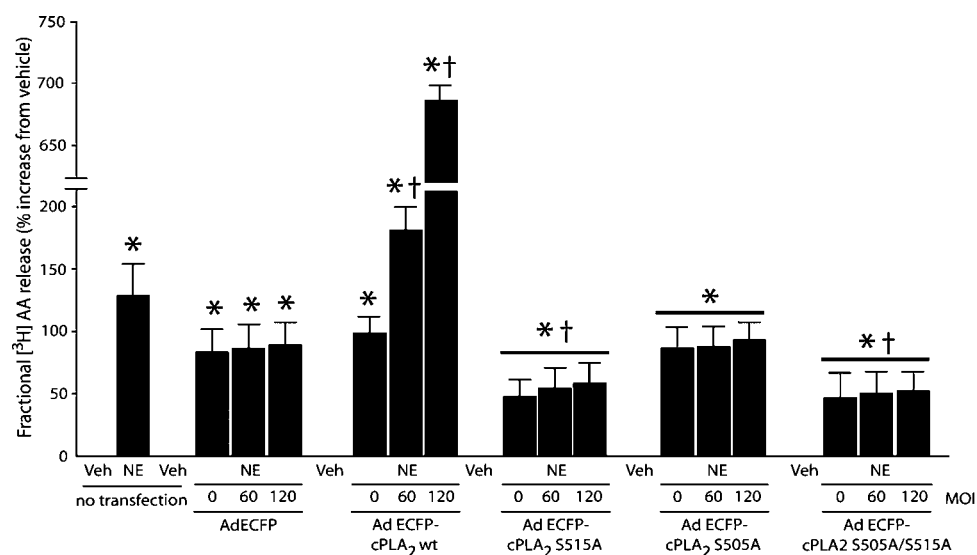

Fig. 7. NE-induced [³H]arachidonic acid (AA) release is inhibited in cells transduced with AdECFPcPLA₂ mutants S515A, S505A, and double mutant S505A/S515A. Cells were transduced with AdECFPcPLA₂ wt and its mutants S515A, S505A, and double mutant S505A/S515A or empty vector (AdECFP) at 60 and 120 MOI viral particles in serum-free M199 medium for 48 h. The expression of ECFPcPLA₂ wt and its mutants was determined by fluorescence microscopy at 24 h after transduction. Cells were then labeled with 0.1 μCi/ml [³H]AA overnight. After that, the cells were washed with 0.1% BSA HBSS to remove any unincorporated [³H]AA and treated with NE (10 μM) for 20 min. Supernatant was collected, the cells were lysed in 1 N NaOH overnight at 4°C, and the cell lysates were collected to measure radioactivity by scintillation counting. The amount of radioactivity in the medium was calculated as fractional release of that present in cell lysates and medium together (taken as total). The increase in the release of [³H]AA produced by NE in nontransduced cells and cells transduced with AdECFP alone, with AdECFPcPLA₂ wt, or with its mutants was expressed as a percentage of the radioactivity released in the presence of vehicle (Veh) of NE (basal), which was taken as 100% in each group. Values are means ± SEM. ECFPcPLA₂ wt (n = 10), S515A (n = 18), S505A (n = 5), and S505A/S515A (n = 9). Asterisks indicate values significantly different from the corresponding values obtained in the presence of vehicle of NE ($P < 0.05$); daggers indicate values significantly different from the corresponding values obtained in cells transduced with similar MOI of empty virus AdECFP ($P < 0.05$).

AA metabolites, mainly hydroxyeicosatetraenoic acids that activate ERK1/2, which amplifies the activity of cPLA$_2$ and releases additional AA (15). Phosphorylation of cPLA$_2$ at S505 has been reported to increase cPLA$_2$ activity (5). We found that the phosphorylation of cPLA$_2$ at S505 by ERK1/2 occurred at 5 min after cPLA$_2$ 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 cPLA$_2$ at S505, whereas the ERK1/2 inhibitor U0126 diminished the phosphorylation of ERK1/2 and of cPLA$_2$ at S505 but not at S515 in rabbit VSMCs.

To investigate the function of the site-specific phosphorylation of cPLA$_2$, phosphorylation site mutants were expressed in VSMCs and examined for their distribution, phosphorylation, and ability to release [^{3}H]AA in response to NE. cPLA$_2$ is known to be translocated from the cytosol to membranes in response to Ca^{2+} ionophore and various agents, including NE, that increase cellular levels of Ca^{2+} (3, 5–7, 15). It has been reported that the short duration of intracellular Ca^{2+} concentration transients translocates cPLA$_2$ to the Golgi, whereas long intracellular Ca^{2+} concentration transients cause its translocation to the Golgi, endoplasmic reticulum, and perinuclear membrane (24). In the present study, transduction of AdECFPcPLA$_2$ wt and its mutants AdECFPcPLA$_2$S515A, AdECFPcPLA$_2$S505A, and AdECFPcPLA$_2$S515A/S505A in VSMCs resulted in uniform distribution of ECFPcPLA$_2$ and its mutants into the cytoplasm. However, upon stimulation with NE, ECFPcPLA$_2$ wt and its mutants translocated mainly to the perinuclear region. These observations suggest that the phosphorylation of cPLA$_2$ at S505 and S515 is not required for its translocation to the nuclear envelope. In MDCK cells, cPLA$_2$ mutants S505A and S727A were translocated to the Golgi in a similar manner as wild-type cPLA$_2$ in response to ATP and ionomycin (7). However, in VSMCs, the translocation of cPLA$_2$ to the nuclear envelope in response to NE was blocked by the CaMKII inhibitor KN-93, which attenuates the phosphorylation of cPLA$_2$ at S515 (25). This suggests that inhibition of CaMKII by KN93 blocks some additional signaling event triggered by CaMKII activation that is required for cPLA$_2$ translocation. Expressing phosphorylation site mutants of cPLA$_2$ 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 ECFPcPLA$_2$ wt, but not that of ECFPcPLA$_2$-S515A mutant in vivo, expressed in VSMCs, supports our in vitro finding that cPLA$_2$ is phosphorylated at S515 by CaMKII (18). In VSMCs expressing the ECFPcPLA$_2$-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 cPLA$_2$ at S505 is in part dependent upon its phosphorylation at S515. Moreover, the fact that ECFPcPLA$_2$ 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 cPLA$_2$ by purified rat brain CaMKII at S515 increased its ability to hydrolyze AA from phosphatidylcholine in vitro (18). Because in VSMCs ectopically expressing ECFPcPLA$_2$ wt the release of [^{3}H]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 [^{3}H]AA was related to the titer of the virus, it appears that NE also causes the activation of ectopically expressed ECFPcPLA$_2$ wt. This was further supported by our observations that the NE-induced increase in [^{3}H]AA release in VSMCs expressing ECFPcPLA$_2$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 cPLA$_2$ in VSMCs is also mediated via CaMKII and ERK1/2. Because in cells expressing ECFPcPLA$_2$S515A the ability of NE to increase [^{3}H]AA release was reduced below that obtained in cells transduced with AdECFP alone, it appears that the expression of ECFPcPLA$_2$S515A in VSMCs inhibits the activity of endogenous cPLA$_2$ by exerting a dominant negative effect. This may be attributable to the binding of CaMKII to the increased amount of cPLA$_2$ in VSMCs (18).

The mechanism by which NE releases [^{3}H]AA to a lesser degree in VSMCs expressing cPLA$_2$ mutants than in those
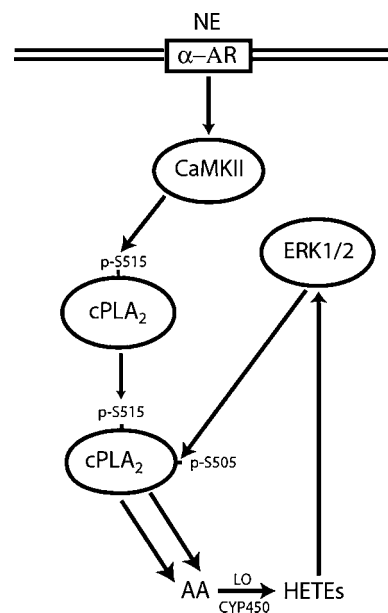

Fig. 8. Proposed pathway of cPLA$_2$ phosphorylation on S515 and S505 by NE in VSMCs. NE activates CaMKII by binding to α-adrenergic receptor (α-AR), which phosphorylates cPLA$_2$ at S515 and releases AA and one or more AA metabolites [hydroxyeicosatetraenoic acids (HETEs)] generated via lipoxygenase (LO), and cytochrome P450 (CYP450) activates ERK1/2, which phosphorylates cPLA$_2$ at S505 and leads to its further activation and additional AA release.

expressing ECFPcPLA$_2$ wt is not known. It has been reported that the mechanism by which S505 phosphorylation increases the activity of cPLA$_2$ is to allow its sustained interaction with membrane in response to transient Ca^{2+} increase (26). However, our data indicate that the translocation of phosphorylation site mutants and ECFPcPLA$_2$ wt were similar in response to NE. This is consistent with a previous report that the translocation properties of cPLA$_2$ mutated at S505 and wild-type cPLA$_2$ are similar in response to transient, physiological calcium increases (7). Because phosphorylation sites are near the flexible hinge region of cPLA$_2$, it has also been suggested that phosphorylation may optimize the conformation of the catalytic domain and enhance cPLA$_2$ activity (27). Our data are consistent with a role for phosphorylation in regulating catalytic activity, because the VSMCs expressing phosphorylated site-mutated cPLA$_2$ release less AA.

In conclusion, the present study demonstrates that NE promotes AA release by promoting cPLA$_2$ phosphorylation at S515 and S505 in vivo in VSMCs. NE stimulates cPLA$_2$ 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 cPLA$_2$ by its phosphorylation by CaMKII appears to be unique to VSMCs. Because the inhibition of CaMKII and cPLA$_2$ has been shown to decrease VSMC growth (28) and neointimal growth in balloon-injured rat carotid artery (29, 30), the selective inactivation of cPLA$_2$ 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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