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**Intact Actin Filaments are Required for Cytosolic Phospholipase A<sub>2</sub> Translocation but not  
for its Activation by Norepinephrine in Vascular Smooth Muscle Cells**

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**Running title:** cPLA<sub>2</sub> Activation is Independent of its Translocation

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### Abstract

Cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) is activated and translocated to the nuclear envelope by various vasoactive agents including norepinephrine (NE), and releases arachidonic acid (AA) from tissue phospholipids. We previously demonstrated that NE-induced cPLA<sub>2</sub> translocation to the nuclear envelope is mediated via its phosphorylation by calcium/calmodulin dependent kinase-II in rabbit vascular smooth muscle cells (VSMC). Cytoskeletal structures actin and microtubule filaments have been implicated in the trafficking of proteins to various cellular sites. This study was conducted to investigate the contribution of actin and microtubule filaments to cPLA<sub>2</sub> translocation to the nuclear envelope and its activation by NE in rabbit VSMC. NE (10 μM) caused cPLA<sub>2</sub> translocation to the nuclear envelope as determined by immunofluorescence. Cytochalasin D (CD, 0.5 μM) and latrunculin A (LA, 0.5 μM) that disrupted actin filaments, blocked cPLA<sub>2</sub> translocation elicited by NE. On the other hand, disruption of microtubule filaments by colchicine (10 μM) did not block NE-induced cPLA<sub>2</sub> translocation to the nuclear envelope. CD and LA did not inhibit NE-induced increase in cytosolic calcium and cPLA<sub>2</sub> activity, determined from the hydrolysis of L-1-<sup>14</sup>C-arachidonyl phosphatidylcholine and release of AA. Co-immunoprecipitation studies showed an association of actin with cPLA<sub>2</sub>, which was not altered by CD or LA. Far-Western analysis showed that cPLA<sub>2</sub> interacts directly with actin. Our data suggest that NE-induced cPLA<sub>2</sub> translocation to the nuclear envelope requires an intact actin but not microtubule filaments and that cPLA<sub>2</sub> phosphorylation and activation and AA release is independent of its translocation to the nuclear envelope in rabbit VSMC.

## INTRODUCTION

Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) enzymes catalyze the hydrolysis of glycerophospholipids at the *sn*-2 position to release arachidonic acid (AA), a precursor of eicosanoids including prostaglandins, leukotrienes, lipoxins and hydroxyeicosa-tetraenoic and trienoic acids (Funk, 2001; Serhan, 1997; Capdevila et al., 1982). Cytosolic PLA<sub>2</sub> (cPLA<sub>2</sub>), the group IVA of the PLA<sub>2</sub> enzymes, is an 85-kDa protein with a preference for AA over the other fatty acids and is activated by submicromolar calcium (Ca<sup>2+</sup>) and by phosphorylation (Lin et al., 1993; Kudo and Murakami, 2002). Structural analysis of cPLA<sub>2</sub> has shown the presence of an N-terminal Ca<sup>2+</sup>-phospholipid binding domain (C2 domain) and a C-terminal catalytic domain (Dessen, 2000). cPLA<sub>2</sub> can be activated by phosphorylation of one or more sites in the catalytic domain depending on the cell type and stimulating agent (Hirabayashi and Shimizu, 2000). cPLA<sub>2</sub> has been shown to be phosphorylated on Ser<sup>505</sup> by the Mitogen Activated Protein Kinases (MAPKs), Extracellular Regulated Kinases1/2 (ERK1/2) and p38 MAPK (Lin et al., 1993; Kramer, 1996; Borsch-Haubold et al., 1998; Gijon et al., 2000), on Ser<sup>515</sup> by calcium/calmodulin dependent protein kinase-II (CaMK-II; Muthalif et al., 2001), and on Ser<sup>727</sup> by MAPK-interacting kinase I (MNK-I; Hefner et al., 2000).

Several agonists that increase cellular levels of Ca<sup>2+</sup>, including NE, promote translocation of cPLA<sub>2</sub> to the nuclear envelope or its association with cell membranes (Glover et al., 1995; Schievella et al., 1995; de Cavalho et al., 1996; Muthalif et al., 1996). Ca<sup>2+</sup> is believed to be required for binding of cPLA<sub>2</sub> to membranes but not for its catalytic activity (Wijkander and Sundler, 1992; Nalefski et al., 1994). Short duration of increase in intracellular Ca<sup>2+</sup> translocates cPLA<sub>2</sub> to the Golgi, whereas long duration of increase in Ca<sup>2+</sup> causes translocation of cPLA<sub>2</sub> to the Golgi, endoplasmic reticulum (ER) and perinuclear membrane (Evans et al., 2001). These

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findings along with the localization of AA metabolizing enzymes to the nuclear envelope suggest that cPLA<sub>2</sub> releases AA for prostanoid synthesis from the membrane phospholipids around nuclear envelope and adjacent ER (Woods et al., 1993; Regier et al., 1995).

Recently, it has been reported that in MDCK cells translocation of cPLA<sub>2</sub> to the Golgi is independent of cPLA<sub>2</sub> phosphorylation by ERK1/2 MAPK because cPLA<sub>2</sub> mutants (S505A and S727A) were translocated to the Golgi in a similar manner as the wild type cPLA<sub>2</sub> in response to ATP and ionomycin and this was not altered by ERK1/2 MAPK kinase (MEK 1) inhibitor U0126 (Evans et al., 2004). However, in rabbit vascular smooth muscle cells (VSMCs), we found that phosphorylation of cPLA<sub>2</sub> by CaMK-II mediates its translocation to the nuclear envelope in response to NE by a mechanism independent of its catalytic activity and that Ca<sup>2+</sup> alone is insufficient for its translocation (Fatima et al., 2003). The mechanism by which cPLA<sub>2</sub> phosphorylated by CaMK-II translocates to the nuclear envelope and the cytoskeletal components involved in its transport are not known. The cytoskeletal structures actin and microtubule filaments are known to be involved in various cellular activities, including organelle movements, transport of cargo between organelles and intracellular trafficking of proteins (Mukherjee et al., 1997; Hirschberg et al., 1998; Valderrama et al., 2001). For example, disruption of actin filaments by agents such as cytochalasin D (CD) or latrunculin B has been reported to inhibit Golgi-to-ER retrograde protein transport, internalization rate of B cell antigen receptor, and movement from early endosomes to late endosomes/lysosomes (Rogers and Gelfand, 2000; Valderrama et al., 2001). Similarly, inhibitors of microtubule filaments nocodazole or colchicines interfere with the cellular transport of macromolecules to lysosomes or transcytosis (Hirschberg et al., 1998). Therefore, it is possible that actin and microtubules might also be involved in the transport of cPLA<sub>2</sub> to the nuclear envelope, which is linked to AA release.

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To test this hypothesis, we have investigated the effect of inhibitors of actin (CD and Latrunculin A; LA) and microtubule (Colchicine) filament polymerization on cPLA<sub>2</sub> translocation to the nuclear envelope, phosphorylation and its activation and AA release in response to the adrenergic neurotransmitter NE in the rabbit VSMC. The results of this study indicate that intact actin but not microtubule filaments are required for NE-induced cPLA<sub>2</sub> translocation to the nuclear envelope but not for its phosphorylation and activation and release of AA in rabbit VSMC.

## METHODS

*Materials*—M-199 medium, fetal bovine serum, penicillin/amphoterecin, norepinephrine, aprotinin, leupeptin, pepstatin, Triton X-100, Trypan Blue were from Sigma (St. Louis, MO). Cytochalasin D, Latrunculin A, and myristoylated-autocamtide-2-related inhibitory peptide (AIP) were from Biomol (Plymouth Meeting, PA). Ionomycin, 2-[N-(2-hydroxyethyl)-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine (KN-93) and colchicine were from Calbiochem (La Jolla, CA). Angiotensin-II was from Bachem (Torrance, CA). L-1-<sup>14</sup>C-arachidonyl phosphatidylcholine, was from American Radiolabeled Chemicals, Inc., (St. Louis, MO). Anti-cPLA<sub>2</sub>, anti-actin, anti-tubulin, anti-CaMK-II $\alpha$  mouse monoclonal antibodies and anti-phospho-CaMK-II $\alpha$  goat polyclonal antibodies were from Santa Cruz Biotechnology, Inc. (San Diego, CA). Antiphospho-cPLA<sub>2</sub> (Ser<sup>505</sup>) antibody was from Cell Signaling Technology (Beverly, MA). Texas Red- or FITC-conjugated anti-mouse antibody and peroxidase labeled anti-goat antibody were from Vector Laboratories (Burlingame, CA). Rhodamine phalloidin and Fura-2 AM were from Molecular Probes (Eugene, OR). Recombinant cPLA<sub>2</sub> was a kind gift from Dr. J. D. Clark of Wyeth Inc. (Madison, NJ). ECL Plus system was from Amersham Biosciences (Piscataway, NJ). All other reagents were of analytical grade.

*Culture of Rabbit VSMC*— The rabbit aortic smooth muscle cells were isolated and cultured in M-199 medium supplemented with 10% fetal bovine serum and 1% penicillin/amphoterecin under constant exposure to 5% CO<sub>2</sub> (Nebigil and Malik, 1992). Cells from 3-4 passages grown to approximately 70% confluency were used. For confocal microscopic studies, the cells were grown on chamber slides; for determining Ca<sup>2+</sup> levels the cells were grown on cover slips; for CaMK phosphorylation, immunoprecipitation, cPLA<sub>2</sub>

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activity and cPLA<sub>2</sub> phosphorylation the cells were grown in 10 cm<sup>2</sup> dishes; and for AA release the cells were cultured in 6 well plates.

*Culture of MDCK Cells*—MDCK cells were purchased from ATCC and cultured according to the ATCC protocol in MEM medium supplemented with 10% fetal bovine serum and 1% penicillin/amphoterecin under constant exposure to 5% CO<sub>2</sub>. Cells were grown on chamber slides and then used for confocal microscopic studies.

*Confocal Microscopy*—Cells were viewed by confocal fluorescence microscopy (BioRad MRC-1000, Laser Scanning Confocal Imaging system using argon/krypton lamp with a 40 objective lens) with anti-cPLA<sub>2</sub> or anti-CaMK-II $\alpha$  monoclonal antibodies as described earlier (Fatima et al., 2003).

To visualize actin filaments, the cells were fixed for 5 min at 25°C with 4% formaldehyde, washed with PBS (pH 7.4), post-fixed with 95% ethanol at -20°C, washed with PBS, rehydrated with 0.1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) at 25°C, incubated with rhodamine phalloidin (in the dark) for 1 h at 25°C, washed with PBS and visualized under confocal microscope. To view actin filaments and cPLA<sub>2</sub> simultaneously, actin filaments were stained red by rhodamine phalloidin, cPLA<sub>2</sub> was stained green with FITC and the merged images were observed by confocal microscopy.

The microtubule filaments were visualized by using anti-tubulin monoclonal antibodies followed by Texas Red-conjugated IgG.

*Measurement of Cytosolic Ca<sup>2+</sup> Levels*—VSMCs were loaded with Fura-2 AM (5  $\mu$ M for 30 min at 37°C), and the level of cytosolic Ca<sup>2+</sup> was determined as described (Fatima et al., 2003). The effect of NE (10  $\mu$ M) in the presence and absence of actin polymerization inhibitors (CD 0.5  $\mu$ M, LA 0.5  $\mu$ M) or their vehicle on cytosolic Ca<sup>2+</sup> levels was measured.



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*cPLA<sub>2</sub> Assay*—cPLA<sub>2</sub> activity was determined from the hydrolysis of substrate L-1-<sup>14</sup>C-arachidonyl phosphatidylcholine (50 mCi/mmol) using 25 μg protein from cell lysates as described (Muthalif et al., 1996).

*Phosphorylation of cPLA<sub>2</sub>*—Phosphorylation of cPLA<sub>2</sub> in response to NE was measured as described (Lin et al., 1993). Briefly, growth arrested VSMC were labeled with 300 μCi/ml <sup>32</sup>P-orthophosphoric acid for 4 h in plain M-199 medium. The media was removed and the cells were washed with plain M-199 medium and treated with CD (0.5 μM), LA (0.5 μM) or their vehicle for 30 min followed by stimulation with NE (10 μM) or its vehicle for 10 min. The cells were lysed in lysis buffer and cPLA<sub>2</sub> was immunoprecipitated using anti-cPLA<sub>2</sub> monoclonal antibodies. <sup>32</sup>P-labelled cPLA<sub>2</sub> immunoprecipitate was subjected to 10% SDS-PAGE. The gel was dried and the radioactivity detected by autoradiography.

*Immunoprecipitation*—Cells were grown on 100 mm tissue culture dishes to subconfluency and arrested for growth for 48 h. Immunoprecipitation was performed as described (Fatima et al., 2003). Briefly, growth arrested cells were incubated for 30 min in serum-free M-199 medium along with inhibitors and treated with NE (10 μM) for 10 minutes. The cells were lysed in lysis buffer containing protease and phosphatase inhibitors (50 mM Tris (pH 7.4), 150 mM NaCl, 1% Igepal, 0.25% sodium deoxycholate, 1 mM EDTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM sodium fluoride, and 1 mg/ml p-nitrophenyl phosphate). cPLA<sub>2</sub> and actin were immunoprecipitated using anti-cPLA<sub>2</sub> or anti-actin goat polyclonal antibodies, respectively. cPLA<sub>2</sub> and actin immunoprecipitates were subjected to 10% SDS-PAGE followed by immunoblotting.

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*Immunoblotting*—To determine CaMK-II and cPLA<sub>2</sub> activities from their phosphorylation, samples (20  $\mu$ g of protein) were resolved on 10% SDS-polyacrylamide gels and then transferred to a nitrocellulose membrane. After blocking with 2% milk and 2% BSA in 20 mM Tris, pH 7.6, 137 mM NaCl and 0.05% Tween (TBST buffer) for 2 h, the membrane was incubated overnight with anti-phospho-CaMK-II $\alpha$  goat polyclonal or anti-phospho-cPLA<sub>2</sub> (Ser<sup>505</sup>) rabbit polyclonal antibodies at 1:1000 dilution in TBST buffer containing 5% BSA followed by incubation with anti-goat IgG horse radish peroxidase antibody (1:20000 dilution in TBST) for 1 h at 25°C. The immunoreactive protein was detected using the ECL Plus system. CaMK-II $\alpha$  and cPLA<sub>2</sub> protein levels were detected using anti-CaMK-II goat polyclonal and anti-cPLA<sub>2</sub> mouse monoclonal antibodies (Santa Cruz, San Diego, CA) respectively.

For co-immunoprecipitation studies, the immunoprecipitates obtained with anti-cPLA<sub>2</sub> and anti-actin antibodies were subjected to SDS-PAGE analysis followed by immunoblotting using both anti-cPLA<sub>2</sub> and anti-actin mouse monoclonal antibodies (Santa Cruz, San Diego, CA).

*Far-Western Analysis*— To determine if cPLA<sub>2</sub> binds directly to actin, we performed Far-Western analysis. Briefly, pure recombinant cPLA<sub>2</sub> was subjected to SDS-PAGE and transferred to a nitrocellulose membrane, which were blocked with 5% (w/v) BSA in TBST for 2 h at room temperature or overnight at 4°C. The nitrocellulose membranes, with immobilized proteins, were then incubated for 2 h at room temperature with 1  $\mu$ g/ml purified actin. The blots were washed and the bound proteins were immunoblotted with anti-actin antibody followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG. Then blots were washed and developed with ECL Plus Reagent.

*Trypan Blue Cell Viability Test*—Cultured rabbit VSMC were treated with CD or LA (0.5  $\mu$ M each) for 30 min. The cells were washed with Hank's balanced salt solution (HBSS) 3 times

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and trypsinized. 2 ml of M-199 full medium was added to neutralize the trypsin and the cells were transferred into microtubes, and centrifuged at 700 rpm for 5 min at room temperature. 40  $\mu$ l of Trypan Blue dye (vol 1:1) was added to 40  $\mu$ l pellet and left at room temperature for 5 min. 10  $\mu$ l of the resuspended cells were placed on hemocytometer and counted under a light microscope. The cells that were able to exclude the stain were considered viable and the percentage of non-blue cells over total cells was used as an index of viability.

*Measurement of  $^3\text{H}$ -AA Release from VSMC*—The release of AA and its tritiated products from VSMC was measured as described (Muthalif et al., 1996). Briefly, the cells labeled overnight with  $^3\text{H}$ -AA were washed with HBSS and exposed to inhibitors of actin polymerization, CD and LA, in M-199 medium containing bovine serum albumin for 15 min at 37°C. Release of  $^3\text{H}$ -AA and its products into extracellular medium were measured by liquid scintillation spectroscopy. Total radioactivity in the cells was determined after treating the cells with 1 M NaOH overnight.  $^3\text{H}$  released into the medium was expressed as a percentage of the total cellular radioactivity and referred to as fractional release.

*Analysis of Data*—Phosphorylation of pCaMK-II $\alpha$ , cPLA<sub>2</sub> catalytic activity and AA release were expressed as mean  $\pm$  SEM. Data were analyzed by one-way ANOVA; the Newman-Keuls multiple range test was used to determine the difference among multiple groups. The unpaired Student's *t*-test was used to determine the difference between two groups. A value of  $p < 0.05$  was considered statistically significant.

## RESULTS

*NE-induced translocation of cPLA<sub>2</sub> to the nuclear envelope is blocked by actin disruption*—Previous studies from our laboratory have shown that cPLA<sub>2</sub> is translocated from the cytoplasm to the nuclear envelope in response to NE (10  $\mu$ M) (Fatima et al., 2003). CD (0.5  $\mu$ M), a fungal toxin which depolymerizes actin filaments by binding to the barbed end of actin filaments and lowering the amount of ATP-bound monomeric actin or LA (0.5  $\mu$ M), a marine macrolide that sequesters G-actin, thereby shifting the balance toward net disassembly, caused disruption of actin filaments in the VSMC (Fig. 1B) and prevented NE-induced translocation of cPLA<sub>2</sub> to the nuclear envelope (Fig. 1A). The effects of CD and LA were not due to any toxic effect of these agents, because more than 97% of VSMC were viable as indicated by trypan blue exclusion test.

*Disruption of actin filaments does not block NE-induced cPLA<sub>2</sub> translocation by inhibiting calcium influx*—Ca<sup>2+</sup> is required for cPLA<sub>2</sub> activation and translocation from the cytoplasm to the nuclear envelope in response to NE in VSMC (Muthalif et al., 1996). To determine whether inhibitors of actin polymerization block NE-induced cPLA<sub>2</sub> translocation to the nuclear envelope by blocking Ca<sup>2+</sup> influx, the effect of CD and LA on cytosolic Ca<sup>2+</sup> in VSMC was examined. Exposure of VSMC to NE twice at 10 min intervals increased the level of cytosolic Ca<sup>2+</sup>; the increase from the second exposure was slightly less than the first exposure to NE. However, the ratio between the second and first response was consistent among experiments performed on different batches of cells. CD, LA or their vehicles were infused over the cells during the second exposure to NE. The ratio of the increase in cytosolic Ca<sup>2+</sup> between the first and second exposure to NE obtained during the infusion of these agents was not different from the

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corresponding ratio obtained during infusion of their vehicle (supplemental data figure 1). Therefore, blockade of NE-induced cPLA<sub>2</sub> translocation to the nuclear envelope is not due to decreased Ca<sup>2+</sup> influx, but rather by the disruption of actin filaments *per se*.

*NE-induced translocation of cPLA<sub>2</sub> is not blocked by disruption of microtubule filaments—* To determine if microtubule filaments are also required for NE-induced cPLA<sub>2</sub> translocation, to the nuclear envelope, cells were treated with colchicine, an agent that disrupts microtubule filaments. Colchicine (10 μM) caused disruption of microtubule filaments but did not alter NE-induced cPLA<sub>2</sub> translocation to the nuclear envelope in response to NE in VSMC (Fig. 2). Therefore, translocation of cPLA<sub>2</sub> to the nuclear envelope in response to NE is dependent upon intact actin but not microtubule filaments.

*NE promotes the activation and translocation of CaMK-II to the nuclear envelope and the disruption of actin filaments blocks its translocation but not activation—* NE increases cytosolic Ca<sup>2+</sup> that binds to calmodulin and activates CaMK-II (Muthalif et al., 1996). Activated CaMK-II phosphorylates cPLA<sub>2</sub> and translocates it to the nuclear envelope (Fatima et al., 2003). Previous studies from our laboratory have shown that CaMK-II translocates to the nuclear envelope in response to NE in rabbit VSMC (Muthalif et al., 1996). In order to determine whether disruption of actin filaments affects NE-induced CaMK-II translocation and its activity to the nuclear envelope, we examined the effect of CD and LA on CaMK-II phosphorylation and translocation in response to NE in VSMC. NE-induced increase in CaMK-II activity (Fig. 3B), as measured from its phosphorylation by immunoblot analysis using anti-phospho-CaMK-II antibodies, was not reduced and density of phosphorylation is shown in figure 3C; whereas CaMK-II

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translocation, as determined by confocal microscopy (Fig. 3A), was blocked by CD and LA indicating that intact actin filaments are also required for CaMK-II as well as for cPLA<sub>2</sub> translocation to the nuclear envelope.

*Actin polymerization is not disrupted by inhibition of CaMK-II activity*—Disruption of actin filaments with CD or LA did not block NE-induced increase in CaMK-II phosphorylation, an indicative of its activity. Since cPLA<sub>2</sub> translocation to the nuclear envelope in response to NE is mediated by CaMK-II and is dependent on intact actin filaments, we determined whether inhibition of CaMK-II activity disrupts actin filaments. The effect of inhibitors of CaMK-II activity (KN-93 and autocamtide-2-related inhibitory peptide) on actin polymerization was examined. None of these agents caused disruption of actin filaments (supplemental data figure 2). Although they inhibited NE-induced CaMK-II phosphorylation (supplemental data figure 2), which suggests that actin polymerization in VSMC is not dependent on CaMK-II activity.

*cPLA<sub>2</sub> is associated with actin filaments*—In order to determine whether there is any association between cPLA<sub>2</sub> and actin filaments, cPLA<sub>2</sub> immunoprecipitates were probed with anti-actin antibodies and actin immunoprecipitates with anti-cPLA<sub>2</sub> antibodies. Our studies showed that cPLA<sub>2</sub> and actin coimmunoprecipitate, as well as disruption of actin filaments by CD and LA does not affect this association (Fig. 4A and B). There was a possibility that actin disruption may alter the amounts of phosphorylated cPLA<sub>2</sub> bound to actin. To test this, we immunoprecipitated actin and determined the amount of Ser<sup>505</sup> phosphorylated cPLA<sub>2</sub> bound to it by Western blot analysis. Our data shows that the amount of Ser<sup>505</sup> phosphorylated cPLA<sub>2</sub> bound to actin did not change upon actin disruption by CD and LA as compared to their vehicle

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(Fig. 4C). We also performed colocalization studies of actin filaments (stained red with rhodamine phalloidin) and cPLA<sub>2</sub> (stained green with fluorescein) to determine their association by confocal microscopy. We observed colocalization of cPLA<sub>2</sub> with actin filaments (Fig. 4D). The nuclei labeled with propidium iodide are shown in Fig. 4E. Whether phosphorylation of cPLA<sub>2</sub> at Ser<sup>515</sup> by CaMK-II (Muthalif et al., 2001) or other residues alters its association with actin could not be determined because we have not yet been able to generate cPLA<sub>2</sub> antibody specific against this phosphorylation sites of cPLA<sub>2</sub>.

*cPLA<sub>2</sub> binds directly to Actin*—Coimmunoprecipitation and colocalization studies demonstrated the association of cPLA<sub>2</sub> with actin. To determine whether cPLA<sub>2</sub> binds directly to actin, we performed Far-Western analysis with pure recombinant cPLA<sub>2</sub>. Our studies revealed that cPLA<sub>2</sub> binds to actin (Fig. 5).

*NE-induced cPLA<sub>2</sub> phosphorylation and activation and AA release were not altered by actin filaments disruption*—To determine the consequence of actin filaments disruption on cPLA<sub>2</sub> phosphorylation and activity, cells labeled with or without <sup>32</sup>P were treated with CD or LA, exposed to NE, and then lysed. The lysates were assayed for cPLA<sub>2</sub> phosphorylation, measured by <sup>32</sup>P incorporation (Fig. 6A), cPLA<sub>2</sub> activity was determined from the hydrolysis of L-1-<sup>14</sup>C-arachidonyl phosphatidylcholine (Fig. 6B), and fractional AA release was measured by labeling the cells with <sup>3</sup>H-AA (Fig. 6C) as described in experimental procedures. Neither NE-induced cPLA<sub>2</sub> phosphorylation nor activation or AA release was altered by CD or LA. Therefore, it appears that an intact actin filaments provides tracks for the translocation of cPLA<sub>2</sub> to the nuclear envelope, but it is not involved in its phosphorylation or activation process in rabbit VSMC.

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*Translocation of cPLA<sub>2</sub> to the Nuclear Envelope by Angiotensin-II and Ionomycin is also blocked by Actin Disruption in Rabbit VSMC*— To determine if disruption of actin filaments interferes with cPLA<sub>2</sub> translocation elicited by stimuli other than NE, we examined the effect of CD on ionomycin- and angiotensin-II-induced cPLA<sub>2</sub> translocation in the rabbit VSMC. Angiotensin-II (0.1  $\mu$ M) and ionomycin (1  $\mu$ M) caused translocation of cPLA<sub>2</sub> to the nuclear envelope, and this translocation was blocked by the disruption of actin filaments with CD (0.5  $\mu$ M; supplemental data figure 3). These observations indicate that translocation of cPLA<sub>2</sub> to the nuclear envelope by agents other than NE also require intact actin filaments in rabbit VSMC.

*Translocation of cPLA<sub>2</sub> to the Nuclear Envelope by ATP is blocked by Actin Disruption in MDCK Cells*—Disruption of actin filaments blocked the translocation of cPLA<sub>2</sub> to the nuclear envelope in rabbit VSMC. To determine if an intact actin is also essential for the translocation of cPLA<sub>2</sub> in other cells, we examined the effect of CD cPLA<sub>2</sub> translocation in response to ATP MDCK. In these cells where ATP is known to cause cPLA<sub>2</sub> translocation to the nuclear envelope (Evans et al., 2002), disruption of actin filaments with CD (0.5  $\mu$ M) blocked ATP (100  $\mu$ M)-induced cPLA<sub>2</sub> translocation to the nuclear envelope (Fig. 7). Therefore, it appears that intact actin filaments are also required for translocation of cPLA<sub>2</sub> to the perinuclear region in cells other than rabbit VSMC.



## DISCUSSION

This study demonstrates a novel role of actin filaments in NE-induced translocation of cPLA<sub>2</sub> to the nuclear envelope in VSMC. Moreover, it shows that phosphorylation and activation of cPLA<sub>2</sub> in response to NE is independent of its translocation to the nuclear envelope. This conclusion is based on our findings that disruption of actin filaments in VSMC with CD or LA prevented cPLA<sub>2</sub> translocation to the nuclear envelope in response to NE. This effect of CD or LA was not due to any cytotoxic effect of these agents as indicated by trypan blue exclusion test. Moreover, the role of actin filaments in the translocation of cPLA<sub>2</sub> to the nuclear envelope appears to be specific to this cytoskeletal structure, because disruption of microtubule filaments by colchicine in VSMC did not prevent NE-induced translocation of cPLA<sub>2</sub> to the nuclear envelope.

Ca<sup>2+</sup> is essential for the binding of cPLA<sub>2</sub> to phospholipid vesicles or membranes (Channon and Leslie, 1990; Wijkander and Sundler, 1992; Nalefski et al., 1994). This enzyme contains an N-terminal C2 domain that binds Ca<sup>2+</sup> and promotes attachment of cPLA<sub>2</sub> to membranes (Zhang et al., 1996; Nalefski and Falke, 1998; Xu et al., 1998). Deletion of C2 domain, but not the C-terminal domain, prevents the binding of cPLA<sub>2</sub> to membranes (Nalefski et al., 1994). NE promotes influx of extracellular Ca<sup>2+</sup> and translocation of cPLA<sub>2</sub> to the nuclear envelope in VSMC (Nebigil and Malik, 1992; Muthalif et al., 1996). Moreover, in the absence of extracellular Ca<sup>2+</sup>, NE or ionomycin failed to cause translocation of cPLA<sub>2</sub> to the nuclear envelope in rabbit VSMC (Muthalif et al., 1996; Fatima et al., 2003). Therefore, it is possible that CD and LA block cPLA<sub>2</sub> translocation to the nuclear envelope by interfering with the influx of extracellular Ca<sup>2+</sup>. CD has been reported to inhibit voltage activated L-type Ca<sup>2+</sup> current in A7r5 vascular smooth muscle cell line (Nakamura et al., 2000). However, in the present study, it

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appears to be unlikely because neither CD nor LA decreased NE-induced rise in cytosolic  $\text{Ca}^{2+}$  in VSMC. The effect of CD to inhibit  $\text{Ca}^{2+}$  current in A7r5 cells and not to decrease cytosolic  $\text{Ca}^{2+}$  levels in rabbit VSMC could be due to differences in the phenotype of these cells and/or the differences in  $\text{Ca}^{2+}$  channels involved in  $\text{Ca}^{2+}$  influx.

Previously, we have shown that NE-induced cPLA<sub>2</sub> translocation to the nuclear envelope is mediated through its phosphorylation by the  $\text{Ca}^{2+}$ /calmodulin dependent CaMK-II in VSMC (Fatima et al., 2003). Therefore, CD and LA could block cPLA<sub>2</sub> translocation by inhibiting CaMK-II activity in the VSMC. Our findings that CD or LA did not alter CaMK-II activity, as determined by its phosphorylation in response to NE, suggest that the effect of CD or LA to block NE-induced cPLA<sub>2</sub> translocation to the nuclear envelope is not due to a decrease in CaMK-II activity in VSMC. CaMK-II is a Ser/Thr kinase that phosphorylates cPLA<sub>2</sub> at Ser<sup>515</sup> (Muthalif et al., 2001). Previously we have shown that CaMK-II also translocates to the nuclear envelope in response to NE in a  $\text{Ca}^{2+}$ -dependent manner (Muthalif et al., 1996). Although CD or LA did not inhibit CaMK-II activity, they blocked NE-induced translocation of CaMK-II around the nucleus. This indicates that intact actin filaments are also required for the transport of CaMK-II, as well as for cPLA<sub>2</sub> to the nuclear envelope. Since CD and LA did not alter phosphorylation of either CaMK-II or cPLA<sub>2</sub>, it appears that these enzymes are phosphorylated/activated before translocation to the nuclear envelope. Since CaMK-II in some cells has been implicated in actin polymerization (Borbiev et al., 2003), it is possible that activated CaMK-II by maintaining the integrity of actin filaments allows the transport of cPLA<sub>2</sub> to the nuclear envelope in response to NE in VSMC. However, our demonstration that inhibition of CaMK-II activity with KN-93 and autocamtide inhibitory peptide did not cause disruption of

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actin filaments, suggest that the integrity of actin filaments is not dependent upon CaMK-II activity in rabbit VSMC.

The role of actin in cPLA<sub>2</sub> transport to the nuclear envelope in response to NE was also indicated from our co-immunoprecipitation experiments showing that actin is associated with cPLA<sub>2</sub>. Since anti-actin antibody does not differentiate between polymerized (F-actin) and depolymerised ( monomeric G-actin) form of actin, we cannot differentiate which of these form of actin binds to cPLA<sub>2</sub>. Our finding that CD or LA did not alter this association suggests that cPLA<sub>2</sub> most likely binds to both forms of actin. Moreover, NE also failed to alter the association of cPLA<sub>2</sub> and actin. Therefore, it appears that actin is not directly involved in the transport of cPLA<sub>2</sub> to the nuclear envelope but rather intact actin serves as a track for its movement to the nuclear envelope. Whether one or more motor proteins involved in the movement of organelles during the process of endocytosis and transcytosis in various cell systems (Allan and Schroer, 1999), also participate in the transport of cPLA<sub>2</sub> on actin filaments, remains to be determined. The requirement of intact actin filaments for cPLA<sub>2</sub> translocation to the nuclear envelope in VSMC was not unique to the action of NE because cPLA<sub>2</sub> translocation elicited by angiotensin II and ionomycin was also inhibited by disruption of actin filaments with CD. Moreover, it appears that intact actin is also required for cPLA<sub>2</sub> translocation in other cells as indicated by our demonstration that disruption actin filaments with CD prevented localization of cPLA<sub>2</sub> to the perinuclear region in response to ATP in MDCK cells.

cPLA<sub>2</sub> can also bind with other cellular proteins, including vimentin (Nakatani et al., 2000), calpain light chain, p11, a Ca<sup>2+</sup> binding protein, annexin I, annexin V and the nuclear protein PLIP and most of these proteins inhibit the activity of cPLA<sub>2</sub> or AA release (see review Kudo and Murakami, 2002). The molecular mechanism of interaction of these proteins with

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cPLA<sub>2</sub> and their contribution to cPLA<sub>2</sub> translocation to the perinuclear region is not known. cPLA<sub>2</sub> which colocalizes with vimentin, an intermediate filament protein, in the perinuclear region of human embryonic kidney (HEK) 293 cells in response to A23187 has been shown to bind to C2 domain of cPLA<sub>2</sub> (Nakatani et al., 2002). In our study the Far-Western analysis showed that the full length cPLA<sub>2</sub> binds directly to actin. It is possible that actin also binds to C2 domain of cPLA<sub>2</sub>. However, studies with various cPLA<sub>2</sub> mutants including cPLA<sub>2</sub> deleted of its C2 domain, would be required to address this issue.

The translocation of cPLA<sub>2</sub> to the nuclear envelope by various agents together with the localization of AA-metabolizing enzymes to this region of the cells has led to the proposition that cPLA<sub>2</sub> releases AA for prostanoid synthesis from the membrane phospholipids around the nuclear envelope and/or adjacent ER (Woods et al., 1993; Regier et al., 1995). However, in MDCK cells ATP and ionomycin caused phosphorylation, activation and translocation of cPLA<sub>2</sub> to the nuclear envelope but the inhibitor of MAPK kinase (MEK) U0126 reduced cPLA<sub>2</sub> activity, as measured by release of AA, without altering its phosphorylation or translocation (Evans et al., 2002). On the other hand, phosphorylation of cPLA<sub>2</sub> in VSMC by CaMK-II but not its catalytic activity was required for its translocation to the nuclear envelope (Fatima et al., 2003). Since ERK1/2 MAPK phosphorylation in VSMC is mediated by AA metabolites generated subsequent to activation of cPLA<sub>2</sub> by CaMK-II (Muthalif et al., 1998) and the inhibition of cPLA<sub>2</sub> catalytic activity with methyl arachidonyl fluorophosphate attenuated ERK1/2 MAPK activity without blocking cPLA<sub>2</sub> translocation (Fatima et al., 2003), it appears that phosphorylation of cPLA<sub>2</sub> by ERK1/2 in VSMC (Muthalif et al., 1998), like in MDCK cells (Evans et al., 2002), is not required for cPLA<sub>2</sub> translocation to nuclear envelope. The translocation of cPLA<sub>2</sub> to the nuclear envelope in response to NE is also independent of p38 MAPK in rabbit VSMC (Fatima et al.,

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2001). These observations along with the results of the present study that disruption of actin filaments with CD or LA blocked NE-induced cPLA<sub>2</sub> translocation but not its phosphorylation, activity and AA release, suggest that NE may also release AA from tissue phospholipids of cellular membranes other than the nuclear envelope/endoplasmic reticulum in rabbit VSMC. Our findings also raise an important question regarding the functional significance of cPLA<sub>2</sub> translocation to the nuclear envelope in VSMC. It is possible that cPLA<sub>2</sub> translocated to the nuclear envelope releases AA locally, which directly or through its metabolite(s) participates in some nuclear function and does not contribute to the fraction of AA released from the VSMC. Whether the interruption of cPLA<sub>2</sub> translocation to the nuclear envelope by disruption of actin filaments affect VSMC functions such as migration, proliferation or contractility and AA metabolism remains to be determined.

In conclusion, the present study demonstrates that intact actin, but not microtubule, filaments is required for cPLA<sub>2</sub> translocation to the nuclear envelope in response to NE in VSMC. Moreover, phosphorylation and activation of cPLA<sub>2</sub> are not dependent upon its translocation to the nuclear envelope; activated cPLA<sub>2</sub> may release AA from the nuclear membrane as well as cell membrane in VSMC in response to NE.

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### FOOTNOTES

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## FIGURE LEGENDS

**FIG. 1.** cPLA<sub>2</sub> accumulation around the nuclear envelope in response to norepinephrine (NE) in VSMC is lost when actin filaments are disrupted by inhibitors of actin filament polymerization. VSMC were treated with inhibitors of actin polymerization cytochalasin D (CD, 0.5  $\mu$ M) and latrunculin A (LA, 0.5  $\mu$ M) or their vehicle (VEH). (A) Distribution of cPLA<sub>2</sub> ( $n=5$ ), (B) density of cPLA<sub>2</sub> fluorescence around nuclear envelope ( $n=5$ ) and (C) distribution of actin filaments ( $n=5$ ) was visualized by confocal microscopy (40 X magnification) in VSMC treated with NE (10  $\mu$ M) or its vehicle (V) in the presence of CD, LA or their vehicle (VEH). \* Value significantly different from vehicle (VEH).

**FIG. 2.** cPLA<sub>2</sub> translocation to the nuclear envelope is not altered by disruption of microtubule filaments with colchicine. Rabbit VSMC were pretreated with colchicine (10  $\mu$ M, 30 min) and then stimulated with NE (10  $\mu$ M, 10 min) or its vehicle (V). cPLA<sub>2</sub> translocation and microtubule filaments were observed by confocal microscopy (40 X magnification) as described under Experimental Procedures ( $n=5$ ).

**FIG. 3.** Translocation of CaMK-II to the nuclear envelope in response to NE in VSMC is lost when actin filaments are disrupted by inhibitors of actin filament polymerization. VSMC were treated with CD (0.5  $\mu$ M), LA (0.5  $\mu$ M,) or their vehicle (VEH) and exposed to NE (10  $\mu$ M, 10 min) or its vehicle (V). (A) CaMK-II translocation to the nuclear envelope was visualized by confocal microscopy (40 X magnification) ( $n=5$ ) and (B) CaMK-II phosphorylation was detected

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using anti-phospho-CaMK-II $\alpha$  antibody (upper panels) and the CaMK-II protein levels (lower panels) were detected using anti-CaMK-II $\alpha$  goat antibodies in cells treated with NE (10  $\mu$ M) or its vehicle (V) as described under Experimental Procedures ( $n=3$ ). (C) Density of CaMK-II phosphorylation was quantified using NIH Image 1.63 program. \* Value significantly different from vehicle (VEH).

**FIG. 4.** cPLA<sub>2</sub> and actin are associated with each other. Coimmunoprecipitation experiments show that cPLA<sub>2</sub> is associated with actin and disruption of actin filaments by CD (0.5  $\mu$ M) and LA (0.5  $\mu$ M) have no affect on their association. VSMC were treated with CD (0.5  $\mu$ M, 30 min), LA (0.5  $\mu$ M, 30 min), or their vehicle (VEH) followed by treatment with NE (10  $\mu$ M, 10 min) or its vehicle (V). (A) cPLA<sub>2</sub> was immunoprecipitated from cell lysates by incubating with cPLA<sub>2</sub>-specific polyclonal antibody followed by incubation with protein A agarose with SDS-sample buffer. The cPLA<sub>2</sub> and actin proteins were detected by immunoblotting with the cPLA<sub>2</sub>-specific monoclonal antibody (upper panel) and with anti-actin monoclonal antibody (lower panel), respectively ( $n=3$ ). (B) Actin was immunoprecipitated from the cell lysate as above using an actin-specific polyclonal antibody and immunoblotting with the actin-specific monoclonal antibody (upper panel) and anti-cPLA<sub>2</sub> monoclonal antibody (lower panel) ( $n=3$ ). (C) Actin was immunoprecipitated from cell lysates by incubating with an actin-specific polyclonal antibody followed by incubation with protein A agarose with SDS-sample buffer. The phospho-cPLA<sub>2</sub> and cPLA<sub>2</sub> proteins were detected by immunoblotting with the phospho-cPLA<sub>2</sub>-specific polyclonal antibody (upper panel) and cPLA<sub>2</sub>-specific monoclonal antibody (lower panel), respectively ( $n=3$ ). (D) Confocal microscopic examination of the VSMC show the association (Yellow) of cPLA<sub>2</sub> (Green) with actin (Red) as indicated by their specific



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monoclonal antibodies ( $n=5$ ). (E) Propidium iodide staining of VSMC treated with CD ( $0.5 \mu\text{M}$ , 30 min) or its vehicle (VEH) and exposed to norepinephrine ( $10 \mu\text{M}$ ; NE) or its vehicle (V), and visualized under confocal microscope (40X magnification) ( $n=5$ ).

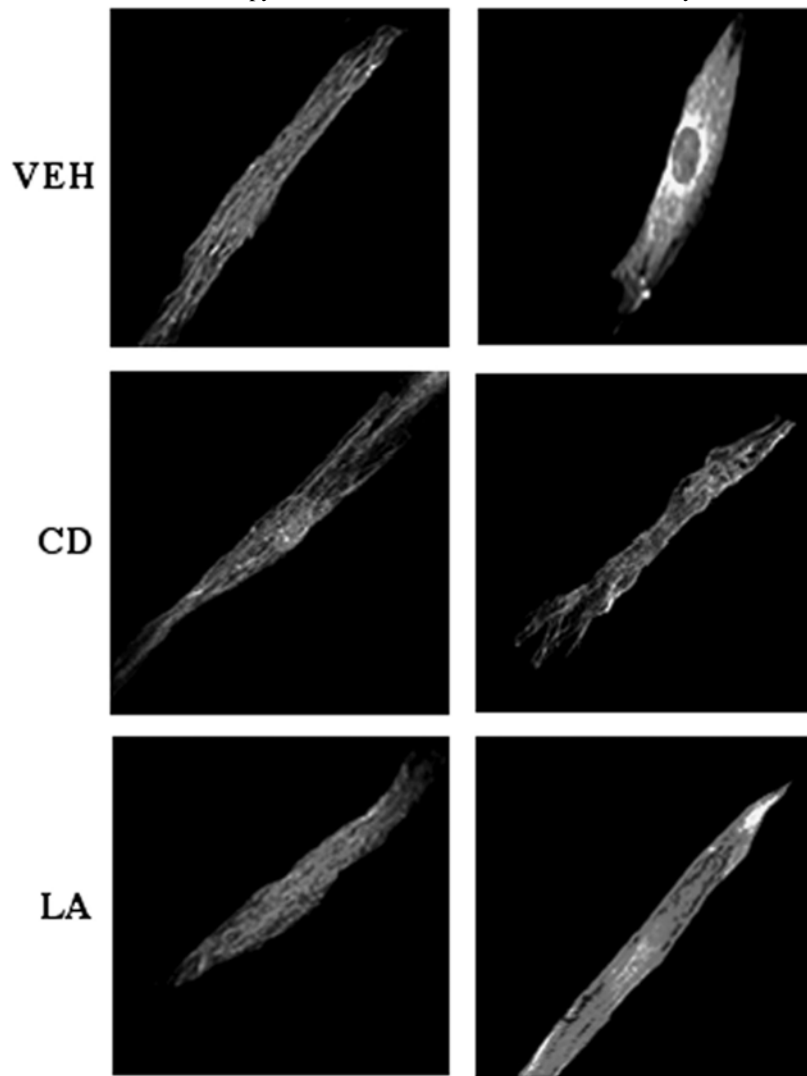
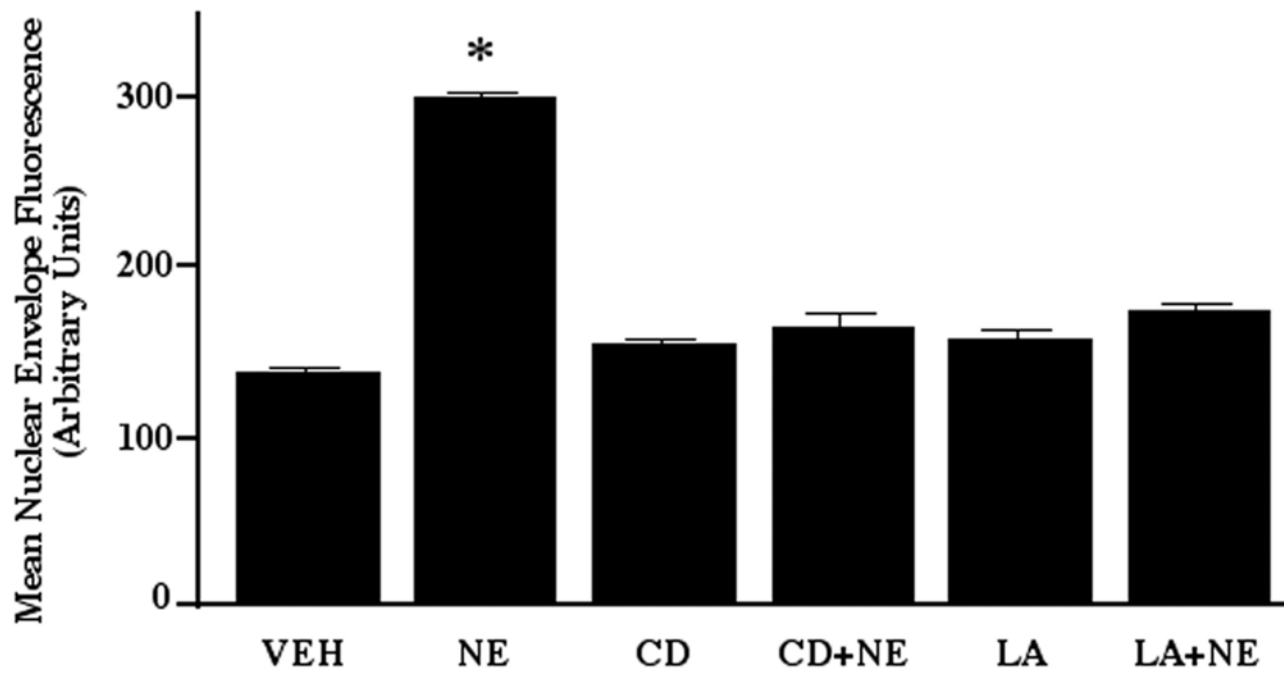
**FIG. 5.** Actin binds directly to cPLA<sub>2</sub>. Far-Western analysis was performed on recombinant cPLA<sub>2</sub> (85 kDa) as described under Experimental Procedures. (A) Actin binding to cPLA<sub>2</sub> was detected using anti-actin antibody and (B) cPLA<sub>2</sub> protein was detected using anti-cPLA<sub>2</sub> monoclonal antibody ( $n=3$ ).

**FIG. 6.** Disruption of actin filament polymerization does not inhibit NE-induced cPLA<sub>2</sub> phosphorylation, activity or arachidonic acid release. VSMC were treated with CD ( $0.5 \mu\text{M}$ ), LA ( $0.5 \mu\text{M}$ ), or their vehicle (VEH) and then exposed to NE ( $10 \mu\text{M}$ , 10 min) or its vehicle (V). (A) Phosphorylation of cPLA<sub>2</sub> was determined by <sup>32</sup>P incorporation ( $n=3$ ); (B) cPLA<sub>2</sub> activity was determined from the hydrolysis of substrate L-1-<sup>14</sup>C-arachidonyl phosphatidylcholine. The absolute value for vehicle of cPLA<sub>2</sub> was  $2160.5 \pm 43.6$  CPM and (C) fractional arachidonic acid release after labeling the cells was measured with <sup>3</sup>H-AA as described under Experimental Procedures. The absolute (medium/total) value for vehicle was  $2.31 \pm 0.13$  CPM ( $n=5$ ). \* Value significantly different from vehicle (VEH).

**FIG. 7.** Translocation of cPLA<sub>2</sub> to the nuclear envelope in response to ATP in MDCK cells is lost when actin filaments are disrupted by an inhibitor of actin filament polymerization CD. MDCK cells were treated with CD ( $0.5 \mu\text{M}$ ), or its vehicle (VEH) and exposed to ATP ( $100 \mu\text{M}$ )

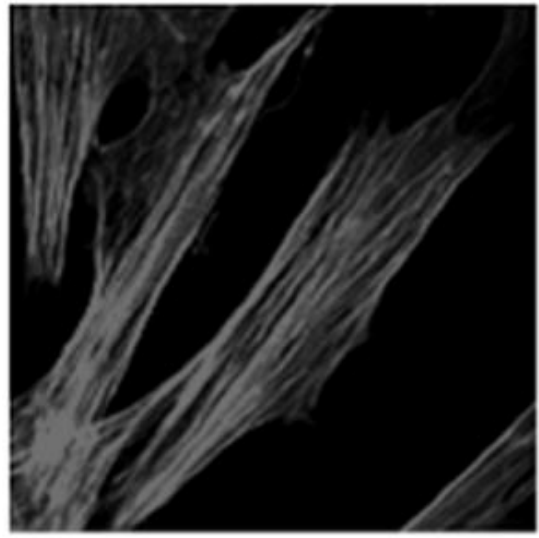
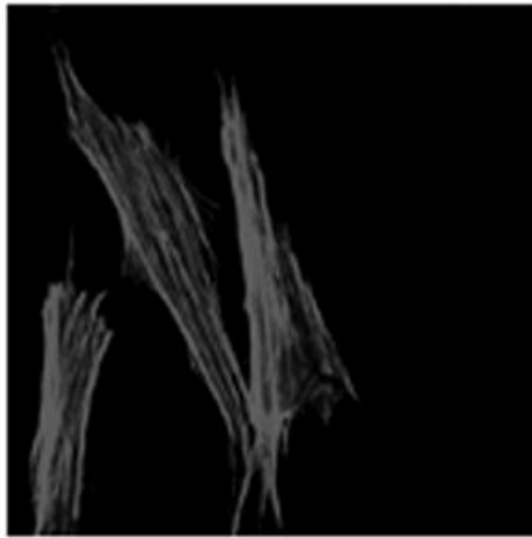
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or its vehicle (VEH). cPLA<sub>2</sub> translocation to the nuclear envelope and actin filament polymerization was visualized by confocal microscopy (40 X magnification) (*n*=5).

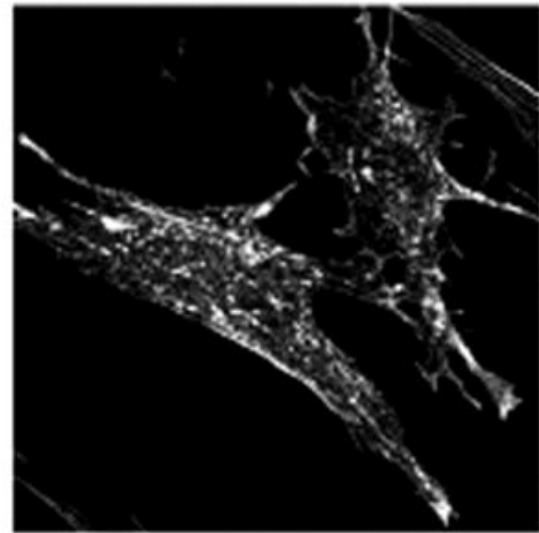
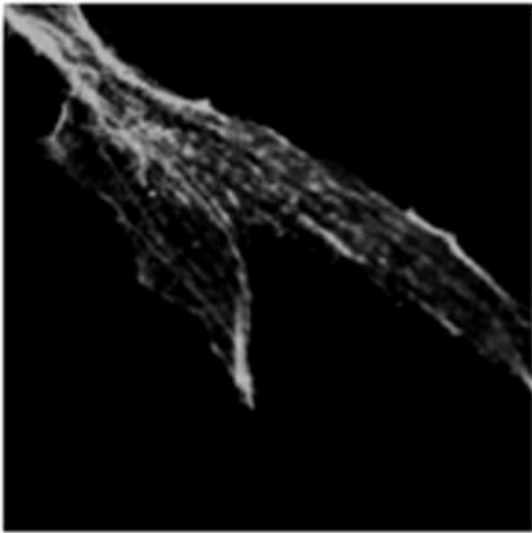
**A****B**

**Figure 1A-B**

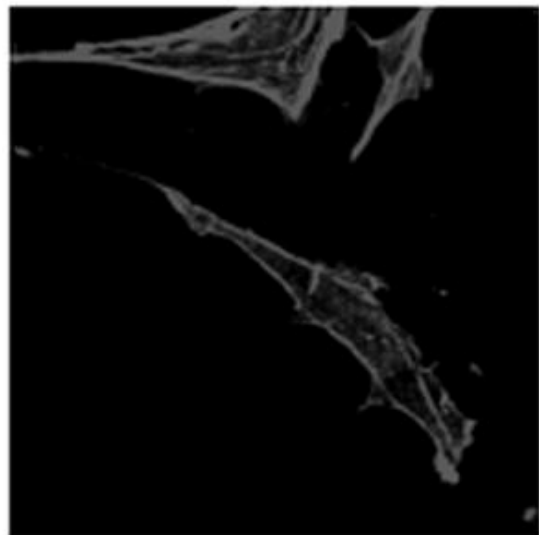
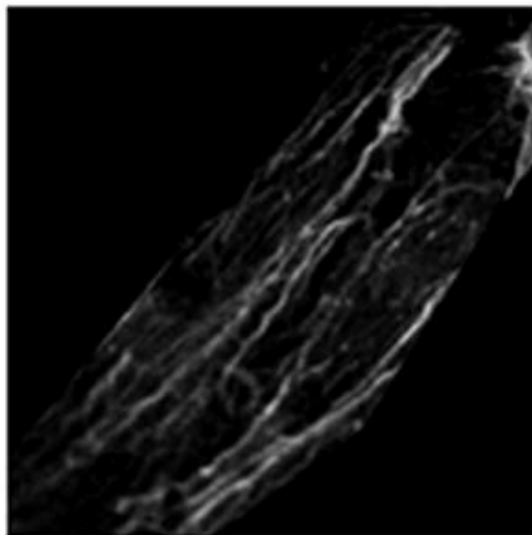
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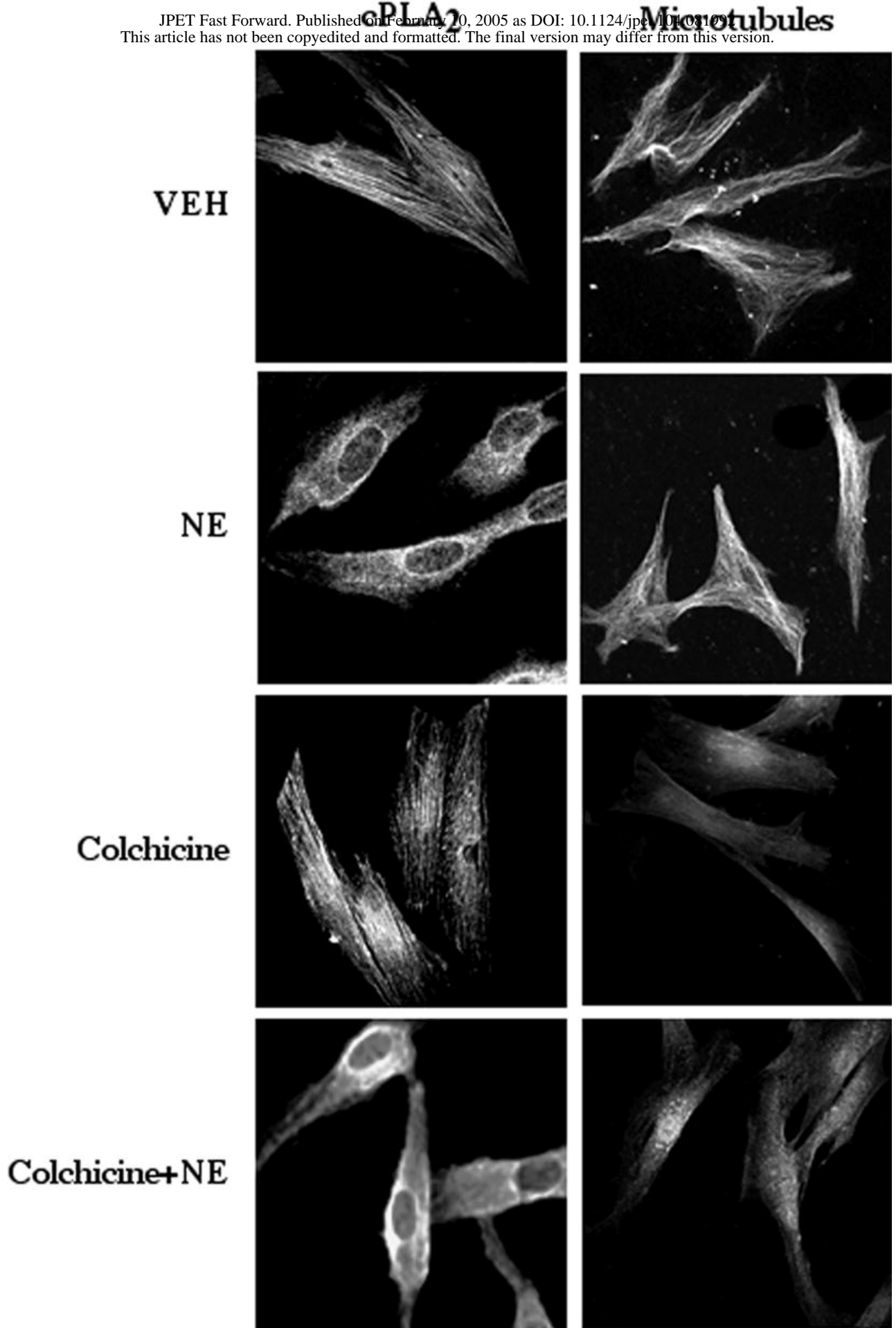
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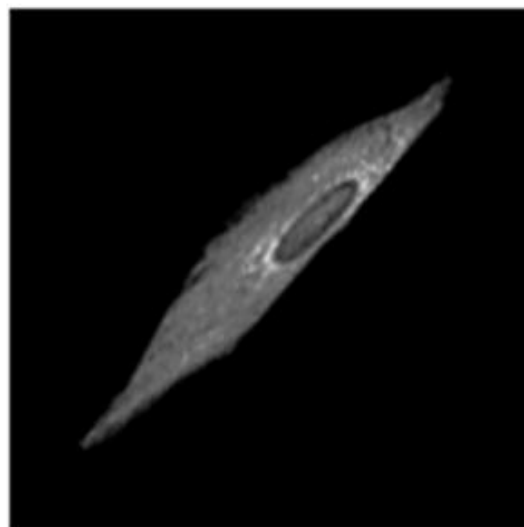
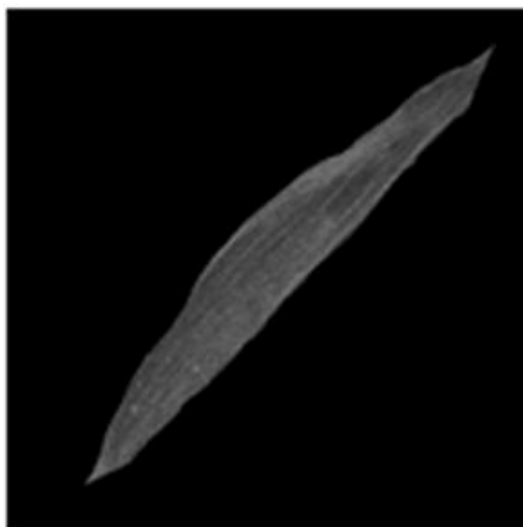
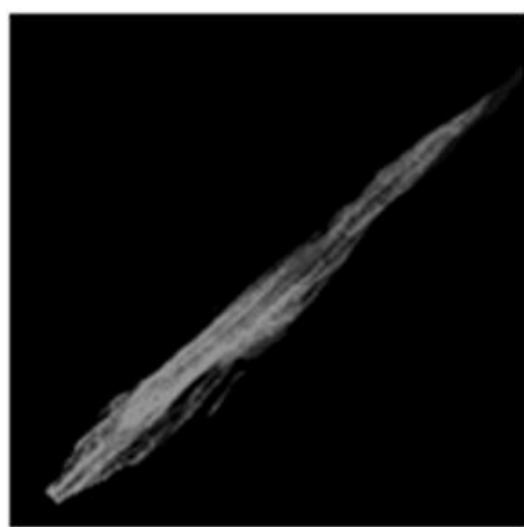
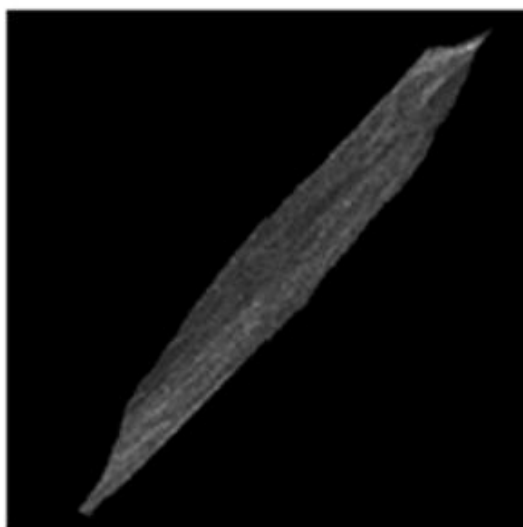
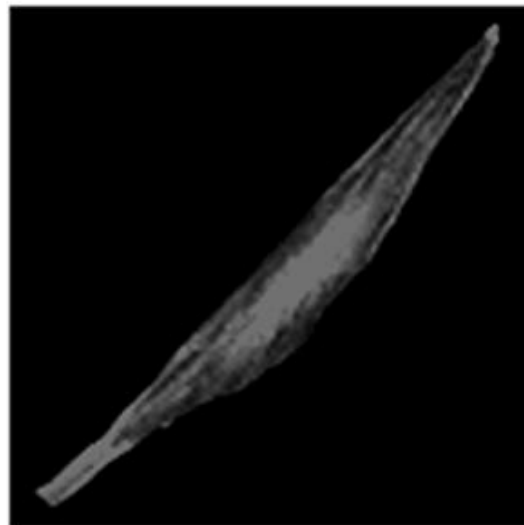
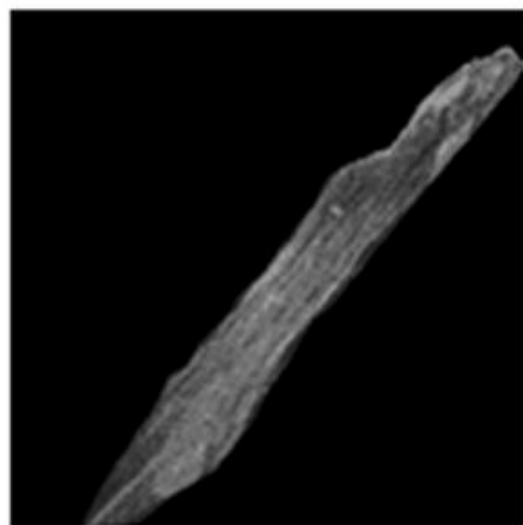
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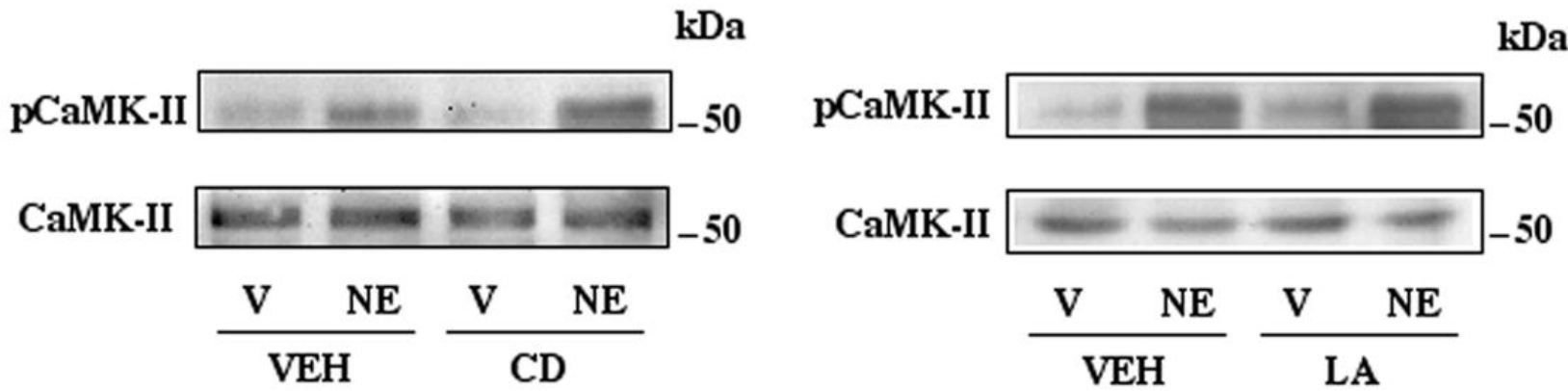
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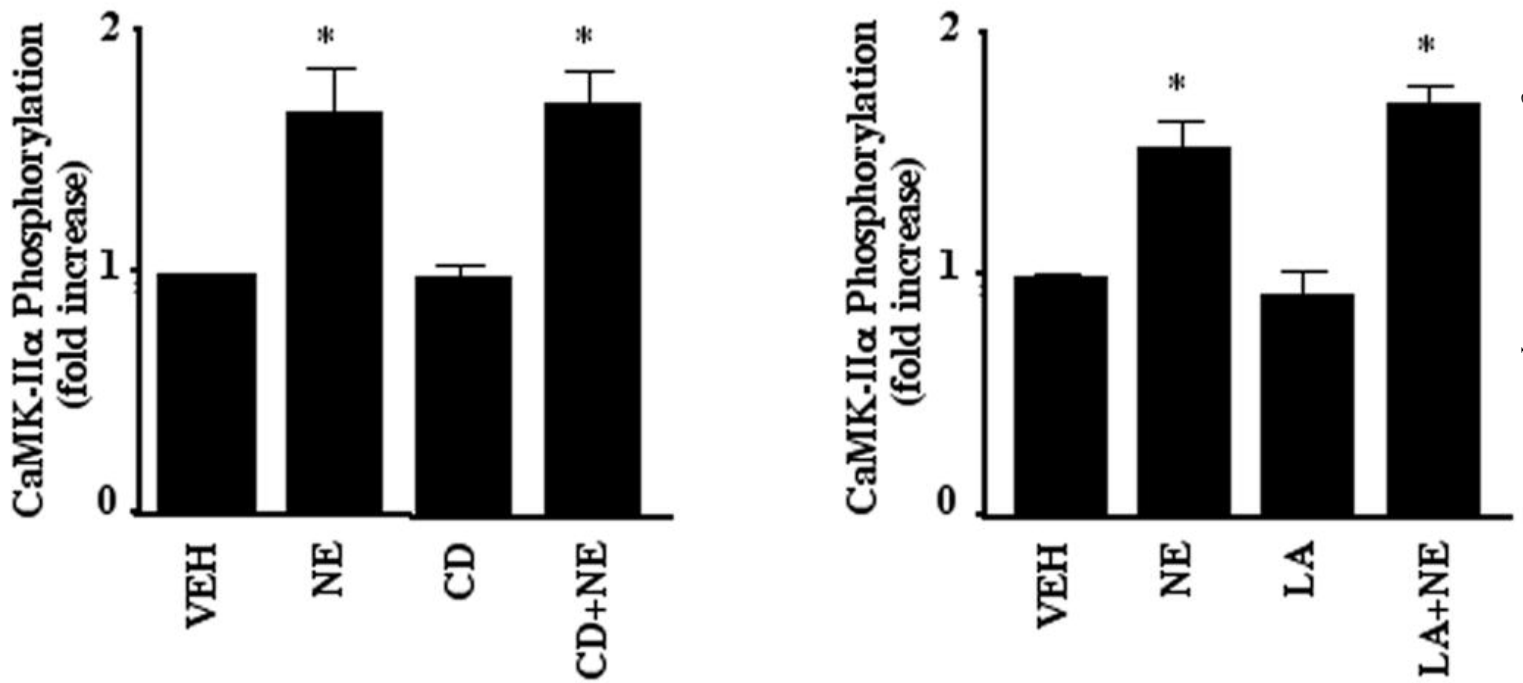
**Figure 2**

**V****NE****VEH****CD****LA****Figure 3 A**

**B**



**C**



**Figure 3 B & C**

A

IP: cPLA<sub>2</sub>

IB: cPLA<sub>2</sub>



IB: Actin

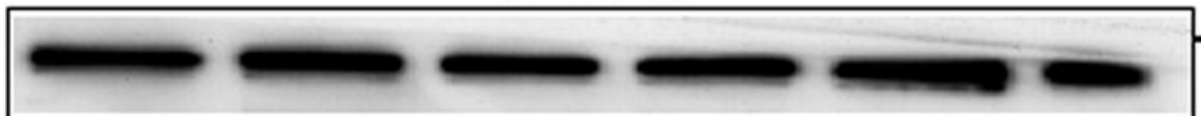


V NE V NE V NE  
VEH CD LA

B

IP: Actin

IB: cPLA<sub>2</sub>



IB: Actin



V NE V NE V NE  
VEH CD LA

C

IP: Actin

IB: pcPLA<sub>2</sub>



IB: cPLA<sub>2</sub>



V NE V NE V NE  
VEH CD LA

kDa

105

50

kDa

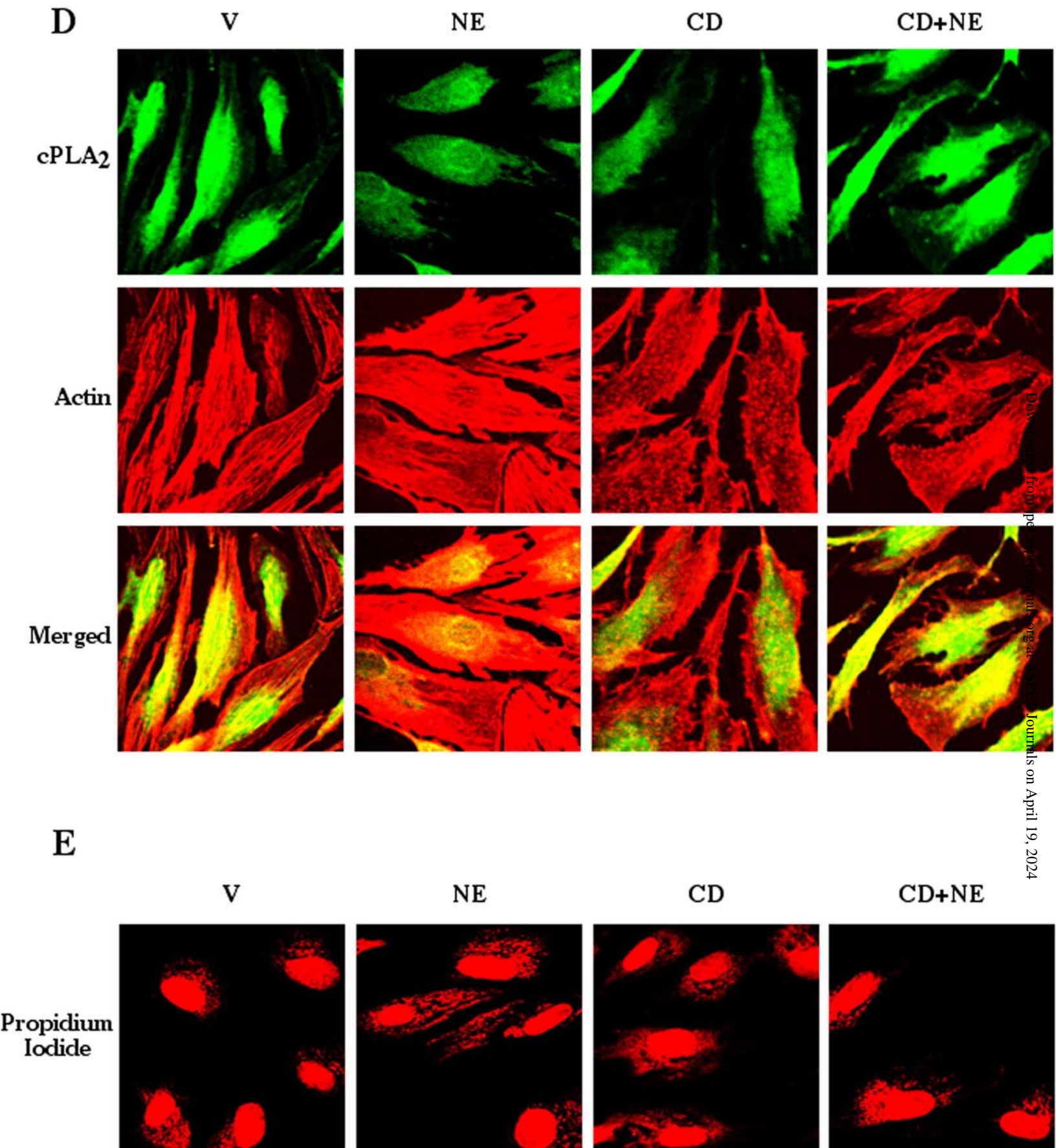
105

50

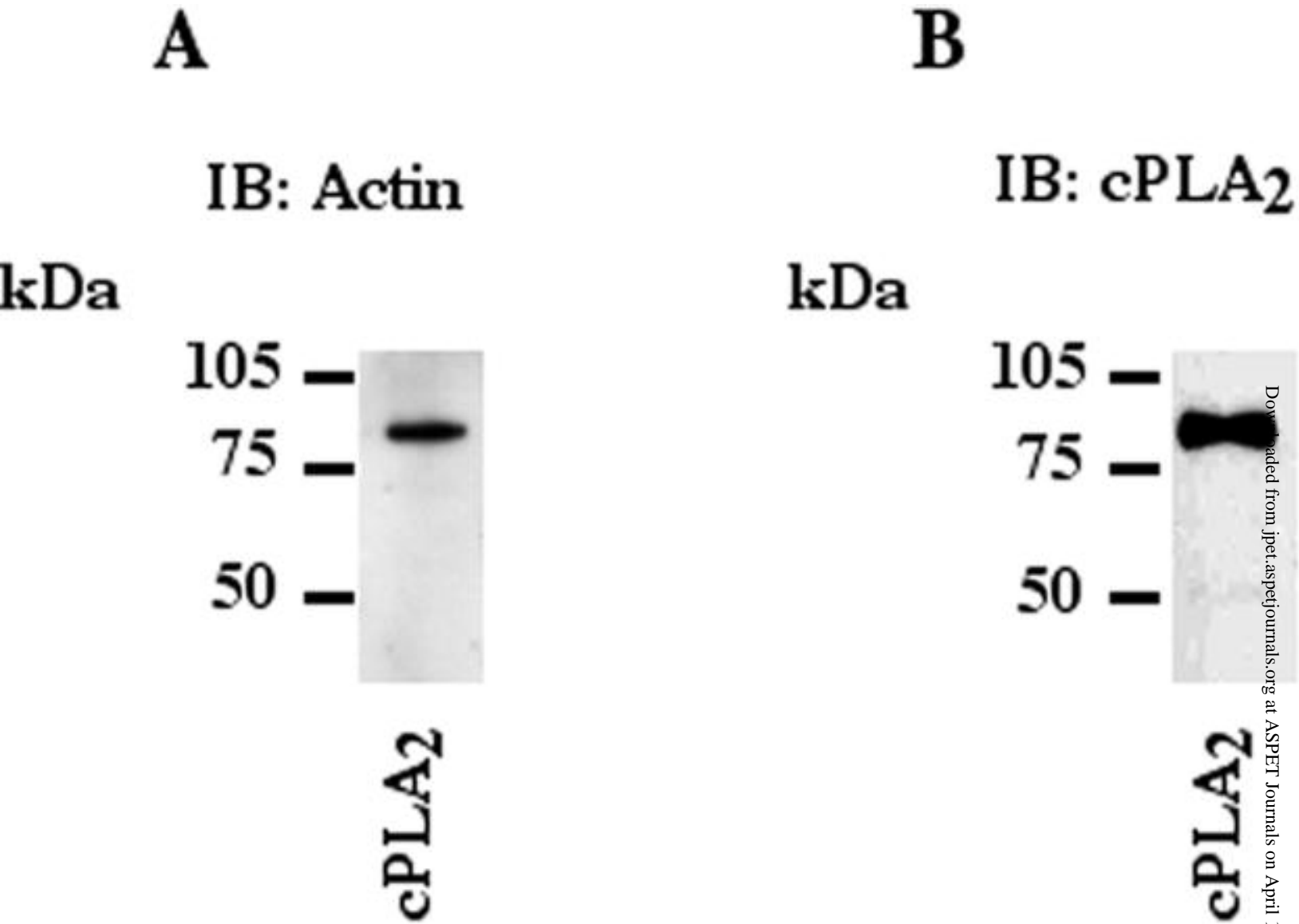
Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

Figure 4 A-C

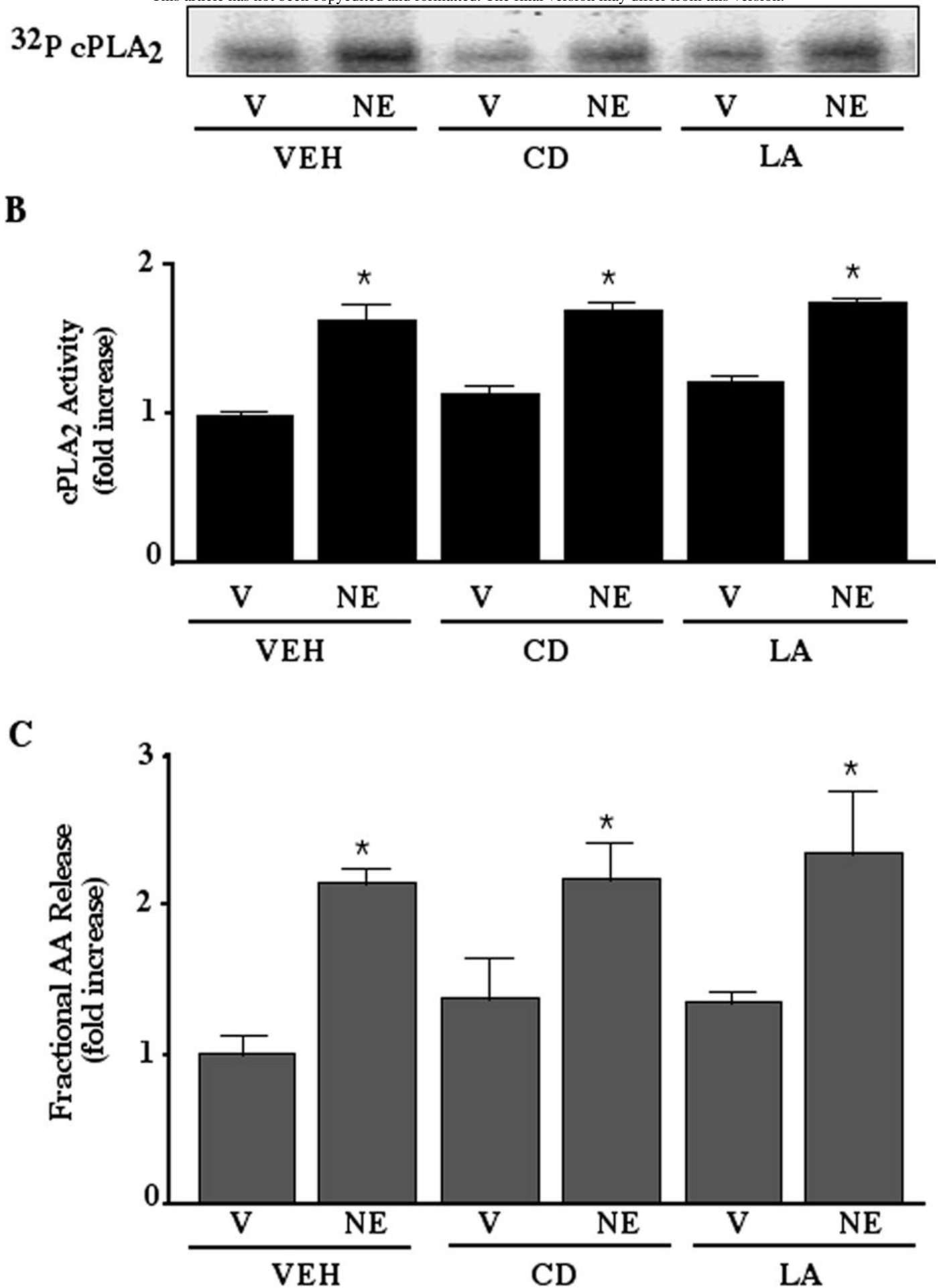




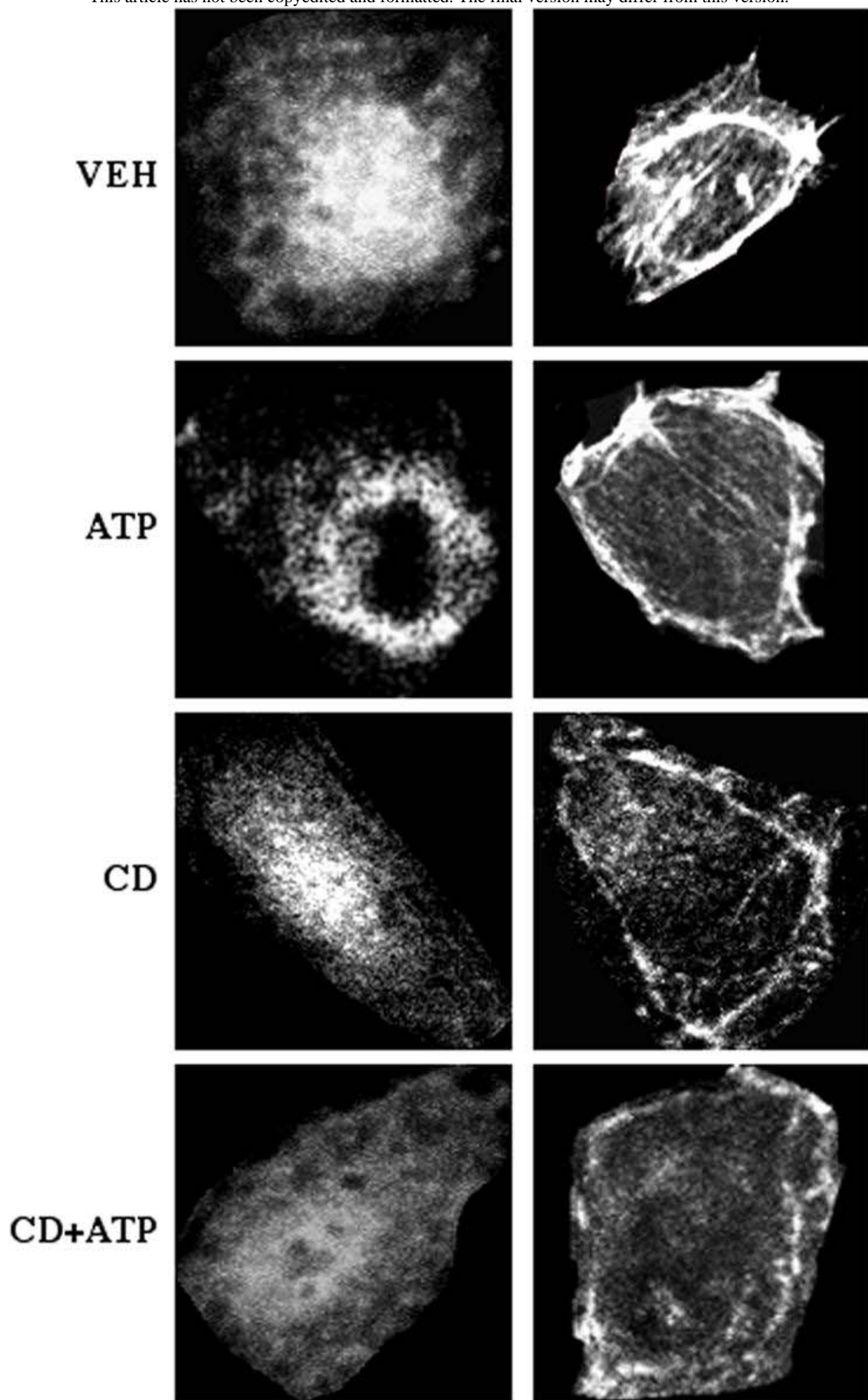
**Figure 4 D-E**



**Figure 5**



**Figure 6**



**Figure 7**