JPET Fast Forward. Published on February 10, 2005 as DOI: 10.1124/jpet.104.081992

JPET #81992

Intact Actin Filaments are Required for Cytosolic Phospholipase A_2 Translocation but not for its Activation by Norepinephrine in Vascular Smooth Muscle Cells

Soghra Fatima, Fariborz A. Yaghini, Zoran Pavicevic, Shailaja Kalyankrishna, Nauzanene Jafari, Elizabeth Luong, Anne Estes and Kafait U. Malik

Department of Pharmacology, College of Medicine, University of Tennessee Health Science Center, Memphis, TN 38163

Running title: $cPLA_2$ Activation is Independent of its Translocation

To whom correspondence should be addressed:

Kafait U. Malik, Ph.D., D.Sc.

Department of Pharmacology,

College of Medicine,

874 Union Avenue

University of Tennessee Health Science Center,

Memphis, TN 38163

Phone: (901) 448-6075

Fax: (901) 448-7206

E-Mail: kmalik@utmem.edu

Number of pages: 33

Number of tables: 0

Number of figures: 7

Number of references: 40

Number of words in the Abstract: 249

Number of words in the Introduction: 698

Number of words in the Discussion: 1498

Recommended section assignment: Cardiovascular

Non-standard abbreviations: bovine serum albumin (BSA), calcium/calmodulin dependent protein kinase-II (CaMK-II), cytosolic phospholipase A_2 ($cPLA_2$), endoplasmic reticulum (ER), extracellular regulated kinases 1/2 (ERK1/2), mitogen activated protein kinase (MAPK), phosphate buffered saline (PBS), vascular smooth muscle cells (VSMC)

Abstract

Cytosolic phospholipase A_2 ($cPLA_2$) 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 $cPLA_2$ 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 $cPLA_2$ translocation to the nuclear envelope and its activation by NE in rabbit VSMC. NE (10 μM) caused $cPLA_2$ 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 $cPLA_2$ translocation elicited by NE. On the other hand, disruption of microtubule filaments by colchicine (10 μM) did not block NE-induced $cPLA_2$ translocation to the nuclear envelope. CD and LA did not inhibit NE-induced increase in cytosolic calcium and $cPLA_2$ activity, determined from the hydrolysis of L-1-^{14}C-arachidonyl phosphatidylcholine and release of AA. Co-immunoprecipitation studies showed an association of actin with $cPLA_2$, which was not altered by CD or LA. Far-Western analysis showed that $cPLA_2$ interacts directly with actin. Our data suggest that NE-induced $cPLA_2$ translocation to the nuclear envelope requires an intact actin but not microtubule filaments and that $cPLA_2$ phosphorylation and activation and AA release is independent of its translocation to the nuclear envelope in rabbit VSMC.

JPET Fast Forward. Published on February 10, 2005 as DOI: 10.1124/jpet.104.081992
This article has not been copyedited and formatted. The final version may differ from this version.

INTRODUCTION

Phospholipase A_2 (PLA_2) 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_2 ($cPLA_2$), the group IVA of the PLA_2 enzymes, is an 85-kDa protein with a preference for AA over the other fatty acids and is activated by submicromolar calcium (Ca^{2+}) and by phosphorylation (Lin et al., 1993; Kudo and Murakami, 2002). Structural analysis of $cPLA_2$ has shown the presence of an N-terminal Ca^{2+}-phospholipid binding domain (C2 domain) and a C-terminal catalytic domain (Dessen, 2000). $cPLA_2$ 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). $cPLA_2$ has been shown to be phosphorylated on Ser^{505} 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^{515} by calcium/calmodulin dependent protein kinase-II (CaMK-II; Muthalif et al., 2001), and on Ser^{727} by MAPK-interacting kinase I (MNK-I; Hefner et al., 2000).

Several agonists that increase cellular levels of Ca^{2+}, including NE, promote translocation of $cPLA_2$ 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^{2+} is believed to be required for binding of $cPLA_2$ to membranes but not for its catalytic activity (Wijkander and Sundler, 1992; Nalefski et al., 1994). Short duration of increase in intracellular Ca^{2+} translocates $cPLA_2$ to the Golgi, whereas long duration of increase in Ca^{2+} causes translocation of $cPLA_2$ to the Golgi, endoplasmic reticulum (ER) and perinuclear membrane (Evans et al., 2001). These

JPET Fast Forward. Published on February 10, 2005 as DOI: 10.1124/jpet.104.081992
This article has not been copyedited and formatted. The final version may differ from this version.

findings along with the localization of AA metabolizing enzymes to the nuclear envelope suggest that $cPLA_2$ 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 $cPLA_2$ to the Golgi is independent of $cPLA_2$ phosphorylation by ERK1/2 MAPK because $cPLA_2$ mutants (S505A and S727A) were translocated to the Golgi in a similar manner as the wild type $cPLA_2$ 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 $cPLA_2$ 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^{2+} alone is insufficient for its translocation (Fatima et al., 2003). The mechanism by which $cPLA_2$ 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 $cPLA_2$ to the nuclear envelope, which is linked to AA release.

To test this hypothesis, we have investigated the effect of inhibitors of actin (CD and Latrunculin A; LA) and microtubule (Colchicine) filament polymerization on $cPLA_2$ 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 $cPLA_2$ 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-methoxybenzenesulfonly)]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-^{14}C-arachidonyl phosphatidylcholine, was from American Radiolabeled Chemicals, Inc., (St. Louis, MO). Anti-cPLA$_2$, anti-actin, anti-tubulin, anti-CaMK-IIα mouse monoclonal antibodies and anti-phospho-CaMK-IIα goat polyclonal antibodies were from Santa Cruz Biotechnology, Inc. (San Diego, CA). Antiphospho-cPLA$_2$ (Ser^{505}) 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$_2$ 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_2 (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^{2+} levels the cells were grown on cover slips; for CaMK phosphorylation, immunoprecipitation, cPLA$_2$

activity and $cPLA_2$ phosphorylation the cells were grown in 10 cm^2 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_2. 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-$cPLA_2$ or anti-CaMK-IIα 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 $cPLA_2$ simultaneously, actin filaments were stained red by rhodamine phalloidin, $cPLA_2$ 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^{2+} *Levels*—VSMCs were loaded with Fura-2 AM (5 μM for 30 min at 37˚C), and the level of cytosolic Ca^{2+} was determined as described (Fatima et al., 2003). The effect of NE (10 μM) in the presence and absence of actin polymerization inhibitors (CD 0.5 μM, LA 0.5 μM) or their vehicle on cytosolic Ca^{2+} levels was measured.

cPLA$_2$ Assay—cPLA$_2$ activity was determined from the hydrolysis of substrate L-1-^{14}C-arachidonyl phosphatidylcholine (50 mCi/mmol) using 25 μg protein from cell lysates as described (Muthalif et al., 1996).

Phosphorylation of cPLA$_2$—Phosphorylation of cPLA$_2$ in response to NE was measured as described (Lin et al., 1993). Briefly, growth arrested VSMC were labeled with 300 μCi/ml ^{32}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$_2$ was immunoprecipitated using anti-cPLA$_2$ monoclonal antibodies. ^{32}P-labelled cPLA$_2$ immunoprecipiate 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$_2$ and actin were immunoprecipitated using anti-cPLA$_2$ or anti-actin goat polyclonal antibodies, respectively. cPLA$_2$ and actin immunoprecipitates were subjected to 10% SDS-PAGE followed by immunoblotting.

Immunoblotting—To determine CaMK-II and $cPLA_2$ activities from their phosphorylation, samples (20 μg 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-IIα goat polyclonal or anti-phospho-$cPLA_2$ (Ser^{505}) 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-IIα and $cPLA_2$ protein levels were detected using anti-CaMK-II goat polyclonal and anti-$cPLA_2$ mouse monoclonal antibodies (Santa Cruz, San Diego, CA) respectively.

For co-immunoprecipitation studies, the immunoprecipitates obtained with anti-$cPLA_2$ and anti-actin antibodies were subjected to SDS-PAGE analysis followed by immunoblotting using both anti-$cPLA_2$ and anti-actin mouse monoclonal antibodies (Santa Cruz, San Diego, CA).

Far-Western Analysis— To determine if $cPLA_2$ binds directly to actin, we performed Far-Western analysis. Briefly, pure recombinant $cPLA_2$ was subjected to SDS-PAGE and transferred to a nitrocellulase 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 μg/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 μM each) for 30 min. The cells were washed with Hank's balanced salt solution (HBSS) 3 times

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 μl of Trypan Blue dye (vol 1:1) was added to 40 μl pellet and left at room temperature for 5 min. 10 μl 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}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}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}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}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-IIα, $cPLA_2$ catalytic activity and AA release were expressed as mean ± 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 $cPLA_2$ to the nuclear envelope is blocked by actin disruption—Previous studies from our laboratory have shown that $cPLA_2$ is translocated from the cytoplasm to the nuclear envelope in response to NE (10 μM) (Fatima et al., 2003). CD (0.5 μM), 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 μM), a marine macrolide that sequesters G-actin, thereby shifting the balance toward net dissembly, caused disruption of actin filaments in the VSMC (Fig. 1B) and prevented NE-induced translocation of $cPLA_2$ 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 $cPLA_2$ translocation by inhibiting calcium influx—Ca^{2+} is required for $cPLA_2$ 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 $cPLA_2$ translocation to the nuclear envelope by blocking Ca^{2+} influx, the effect of CD and LA on cytosolic Ca^{2+} in VSMC was examined. Exposure of VSMC to NE twice at 10 min intervals increased the level of cytosolic Ca^{2+}; 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^{2+} between the first and second exposure to NE obtained during the infusion of these agents was not different from the

JPET Fast Forward. Published on February 10, 2005 as DOI: 10.1124/jpet.104.081992
This article has not been copyedited and formatted. The final version may differ from this version.

corresponding ratio obtained during infusion of their vehicle (supplemental data figure 1). Therefore, blockade of NE-induced $cPLA_2$ translocation to the nuclear envelope is not due to decreased Ca^{2+} influx, but rather by the disruption of actin filaments *per se*.

NE-induced translocation of $cPLA_2$ is not blocked by disruption of microtubule filaments— To determine if microtubule filaments are also required for NE-induced $cPLA_2$ 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 $cPLA_2$ translocation to the nuclear envelope in response to NE in VSMC (Fig. 2). Therefore, translocation of $cPLA_2$ 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^{2+} that binds to calmodulin and activates CaMK-II (Muthalif et al., 1996). Activated CaMK-II phosphorylates $cPLA_2$ 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

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 $cPLA_2$ 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 $cPLA_2$ 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.

$cPLA_2$ is associated with actin filaments—In order to determine whether there is any association between $cPLA_2$ and actin filaments, $cPLA_2$ immunoprecipitates were probed with anti-actin antibodies and actin immunoprecipitates with anti-$cPLA_2$ antibodies. Our studies showed that $cPLA_2$ 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 $cPLA_2$ bound to actin. To test this, we immunoprecipitated actin and determined the amount of Ser^{505} phosphosphorylated $cPLA_2$ bound to it by Western blot analysis. Our data shows that the amount of Ser^{505} phosphorylated $cPLA_2$ bound to actin did not change upon actin disruption by CD and LA as compared to their vehicle

(Fig. 4C). We also performed colocalization studies of actin filaments (stained red with rhodamine phalloidin) and $cPLA_2$ (stained green with fluorescein) to determine their association by confocal microscopy. We observed colocalization of $cPLA_2$ with actin filaments (Fig. 4D). The nuclei labeled with propidium iodide are shown in Fig. 4E. Whether phosphorylation of $cPLA_2$ at Ser^{515} 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 $cPLA_2$ antibody specific against this phosphorylation sites of $cPLA_2$.

$cPLA_2$ binds directly to Actin—Coimmunoprecipitation and colocalization studies demonstrated the association of $cPLA_2$ with actin. To determine whether $cPLA_2$ binds directly to actin, we performed Far-Western analysis with pure recombinant $cPLA_2$. Our studies revealed that $cPLA_2$ binds to actin (Fig. 5).

NE-induced $cPLA_2$ phosphorylation and activation and AA release were not altered by actin filaments disruption—To determine the consequence of actin filaments disruption on $cPLA_2$ phosphorylation and activity, cells labeled with or without ^{32}P were treated with CD or LA, exposed to NE, and then lysed. The lysates were assayed for $cPLA_2$ phosphorylation, measured by ^{32}P incorporation (Fig. 6A), $cPLA_2$ activity was determined from the hydrolysis of L-1-^{14}C-arachidonyl phosphatidylcholine (Fig. 6B), and fractional AA release was measured by labeling the cells with ^{3}H-AA (Fig. 6C) as described in experimental procedures. Neither NE-induced $cPLA_2$ 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 $cPLA_2$ to the nuclear envelope, but it is not involved in its phosphorylation or activation process in rabbit VSMC.

Translocation of $cPLA_2$ 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 $cPLA_2$ translocation elicited by stimuli other than NE, we examined the effect of CD on ionomycin- and angiotensin-II-induced $cPLA_2$ translocation in the rabbit VSMC. Angiotensin-II (0.1 μM) and ionomycin (1 μM) caused translocation of $cPLA_2$ to the nuclear envelope, and this translocation was blocked by the disruption of actin filaments with CD (0.5 μM; supplemental data figure 3). These observations indicate that translocation of $cPLA_2$ to the nuclear envelope by agents other than NE also require intact actin filaments in rabbit VSMC.

Translocation of $cPLA_2$ to the Nuclear Envelope by ATP is blocked by Actin Disruption in MDCK Cells—Disruption of actin filaments blocked the translocation of $cPLA_2$ to the nuclear envelope in rabbit VSMC. To determine if an intact actin is also essential for the translocation of $cPLA_2$ in other cells, we examined the effect of CD $cPLA_2$ translocation in response to ATP MDCK. In these cells where ATP is known to cause $cPLA_2$ translocation to the nuclear envelope (Evans et al., 2002), disruption of actin filaments with CD (0.5 μM) blocked ATP (100 μM)-induced $cPLA_2$ translocation to the nuclear envelope (Fig. 7). Therefore, it appears that intact actin filaments are also required for translocation of $cPLA_2$ 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 $cPLA_2$ to the nuclear envelope in VSMC. Moreover, it shows that phosphorylation and activation of $cPLA_2$ 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 $cPLA_2$ 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 $cPLA_2$ 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 $cPLA_2$ to the nuclear envelope.

Ca^{2+} is essential for the binding of $cPLA_2$ 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^{2+} and promotes attachment of $cPLA_2$ 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 $cPLA_2$ to membranes (Nalefski et al., 1994). NE promotes influx of extracellular Ca^{2+} and translocation of $cPLA_2$ to the nuclear envelope in VSMC (Nebigil and Malik, 1992; Muthalif et al., 1996). Moreover, in the absence of extracellular Ca^{2+}, NE or ionomycin failed to cause translocation of $cPLA_2$ to the nuclear envelope in rabbit VSMC (Muthalif et al., 1996; Fatima et al., 2003). Therefore, it is possible that CD and LA block $cPLA_2$ translocation to the nuclear envelope by interfering with the influx of extracellular Ca^{2+}. CD has been reported to inhibit voltage activated L-type Ca^{2+} current in A7r5 vascular smooth muscle cell line (Nakamura et al., 2000). However, in the present study, it

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

Previously, we have shown that NE-induced $cPLA_2$ translocation to the nuclear envelope is mediated through its phosphorylation by the Ca^{2+}/calmodulin dependent CaMK-II in VSMC (Fatima et al., 2003). Therefore, CD and LA could block $cPLA_2$ 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 $cPLA_2$ 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 $cPLA_2$ at Ser^{515} (Muthalif et al., 2001). Previously we have shown that CaMK-II also translocates to the nuclear envelope in response to NE in a 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 $cPLA_2$ to the nuclear envelope. Since CD and LA did not alter phosphorylation of either CaMK-II or $cPLA_2$, 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 $cPLA_2$ 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

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

The role of actin in $cPLA_2$ transport to the nuclear envelope in response to NE was also indicated from our co-immunoprecipitation experiments showing that actin is associated with $cPLA_2$. 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 $cPLA_2$. Our finding that CD or LA did not alter this association suggests that $cPLA_2$ most likely binds to both forms of actin. Moreover, NE also failed to alter the association of $cPLA_2$ and actin. Therefore, it appears that actin is not directly involved in the transport of $cPLA_2$ 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 $cPLA_2$ on actin filaments, remains to be determined. The requirement of intact actin filaments for $cPLA_2$ translocation to the nuclear envelope in VSMC was not unique to the action of NE because $cPLA_2$ 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 $cPLA_2$ translocation in other cells as indicated by our demonstration that disruption actin filaments with CD prevented localization of $cPLA_2$ to the perinuclear region in response to ATP in MDCK cells.

$cPLA_2$ can also bind with other cellular proteins, including vimentin (Nakatani et al., 2000), calpain light chain, p11, a Ca^{2+} binding protein, annexin I, annexin V and the nuclear protein PLIP and most of these proteins inhibit the activity of $cPLA_2$ or AA release (see review Kudo and Murakami, 2002). The molecular mechanism of interaction of these proteins with

$cPLA_2$ and their contribution to $cPLA_2$ translocation to the perinuclear region is not known. $cPLA_2$ 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 $cPLA_2$ (Nakatani et al., 2002). In our study the Far-Western analysis showed that the full length $cPLA_2$ binds directly to actin. It is possible that actin also binds to C2 domain of $cPLA_2$. However, studies with various $cPLA_2$ mutants including $cPLA_2$ deleted of its C2 domain, would be required to address this issue.

The translocation of $cPLA_2$ 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 $cPLA_2$ 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 $cPLA_2$ to the nuclear envelope but the inhibitor of MAPK kinase (MEK) U0126 reduced $cPLA_2$ activity, as measured by release of AA, without altering its phosphorylation or translocation (Evans et al., 2002). On the other hand, phosphorylation of $cPLA_2$ 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 $cPLA_2$ by CaMK-II (Muthalif et al., 1998) and the inhibition of $cPLA_2$ catalytic activity with methyl arachidonyl fluorophosphate attenuated ERK1/2 MAPK activity without blocking $cPLA_2$ translocation (Fatima et al., 2003), it appears that phosphorylation of $cPLA_2$ by ERK1/2 in VSMC (Muthalif et al., 1998), like in MDCK cells (Evans et al., 2002), is not required for $cPLA_2$ translocation to nuclear envelope. The translocation of $cPLA_2$ to the nuclear envelope in response to NE is also independent of p38 MAPK in rabbit VSMC (Fatima et al.,

2001). These observations along with the results of the present study that disruption of actin filaments with CD or LA blocked NE-induced $cPLA_2$ 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 $cPLA_2$ translocation to the nuclear envelope in VSMC. It is possible that $cPLA_2$ 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 $cPLA_2$ 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 $cPLA_2$ translocation to the nuclear envelope in response to NE in VSMC. Moreover, phosphorylation and activation of $cPLA_2$ are not dependent upon its translocation to the nuclear envelope; activated $cPLA_2$ may release AA from the nuclear membrane as well as cell membrane in VSMC in response to NE.

ACKNOWLEDGEMENTS

We thank Dr. Lauren M. Cagen for the editorial comments.

REFERENCES

Allan VJ and Schroer TA (1999) Membrane motors. *Curr Opin Cell Biol* **11**: 476-482.

Ayscough K (1998) Use of latrunculin-A, an actin monomer-binding drug. *Methods Enzymol* **298**: 18-25.

Borbiev T, Verin AD, Birukova A, Liu F, Crow MT and Garcia JG (2003) Role of CaM kinase II and ERK activation in thrombin-induced endothelial cell barrier dysfunction. *Am J Physiol Lung Cell Mol Physiol* **285**: L43–L54.

Borsch-Haubold AG, Bartoli F, Asselin J, Dudler T, Kramer RM, Apitz-Castro R, Watson SP and Gelb MH (1998) Identification of the phosphorylation sites of cytosolic phospholipase A_2 in agonist-stimulated human platelets and HeLa cells.*J Biol Chem* **273**: 4449-4458.

Capdevila J, Marnett LJ, Chacos N, Prough RA and Estabrook RW (1982) Cytochrome P-450-dependent oxygenation of arachidonic acid to hydroxyicosatetraenoic acids. *Proc Natl Acad Sci USA* **79**: 767-770.

Channon JY and Leslie CC (1990) A calcium-dependent mechanism for associating a soluble arachidonoyl-hydrolyzing phospholipase A_2 with membrane in the macrophage cell line RAW 264.7. *J Biol Chem* **265**: 5409-5413.

de Carvalho MG, McCormack AL, Olson E, Ghomashchi F, Gelb MH, Yates JR 3rd and Leslie CC (1996) Identification of phosphorylation sites of human 85-kDa cytosolic phospholipase A_2 expressed in insect cells and present in human monocytes. *J Biol Chem* **271**; 6987-6997.

Dessen A (2000) Structure and mechanism of human cytosolic phospholipase A_2. *Biochim Biophys Acta* **1488**: 40-47.

Evans JH, Fergus DJ and Leslie CC (2002) Inhibition of the MEK1/ERK pathway reduces arachidonic acid release independently of $cPLA_2$ phosphorylation and translocation. *BMC Biochem* **3(1)**: 30.

Evans JH, Gerber SH, Murray D and Leslie CC (2004) The calcium binding loops of the cytosolic phospholipase A_2 C2 domain specify targeting to Golgi and ER in live cells. *Mol Biol Cell* **15**: 371-383.

Evans JH, Spencer DM, Zweifach A and Leslie CC (2001) Intracellular calcium signals regulating cytosolic phospholipase A_2 translocation to internal membranes. *J Biol Chem* **276**: 30150-30160.

Fatima S, Khandekar Z, Parmentier JH and Malik KU (2001) Cytosolic phospholipase A_2 activation by the p38 kinase inhibitor SB203580 in rabbit aortic smooth muscle cells. *J Pharmacol Exp Ther* **298**: 331-338.

Fatima S, Yaghini FA, Ahmed A, Khandekar Z and Malik KU (2003) CaM kinase IIalpha mediates norepinephrine-induced translocation of cytosolic phospholipase A_2 to the nuclear envelope. *J Cell Sci* **116**: 353-365.

Funk CD (2002) Prostaglandins and leukotrienes: Advances in eicosanoids biology. *Science* **294**: 1871-1875.

Gijon MA, Spencer DM, Siddiqi AR, Bonventre JV and Leslie CC (2000) Cytosolic phospholipase A_2 is required for macrophage arachidonic acid release by agonists that Do and Do not mobilize calcium. Novel role of mitogen-activated protein kinase pathways in cytosolic phospholipase A2 regulation. *J Biol Chem* **275**: 20146-20156.

Glover S, de Carvalho MS, Bayburt T, Jonas M, Chi E, Leslie CC and Gelb MH (1995) Translocation of the 85-kDa phospholipase A_2 from cytosol to the nuclear envelope in rat basophilic leukemia cells stimulated with calcium ionophore or IgE/antigen. *J Biol Chem* **270**: 15359-15367.

Hamel E (1996) Antimitotic natural products and their interactions with tubulin. *Med Res Rev* **16**: 207-231.

Hefner Y, Borsch-Haubold AG, Murakami M, Wilde JI, Pasquet S, Schieltz D, Ghomashchi F, Yates JR 3rd, Armstrong CG, Paterson A, Cohen P, Fukunaga R, Hunter T, Kudo I, Watson

SP and Gelb MH (2000) Serine 727 phosphorylation and activation of cytosolic phospholipase A_2 by MNK1-related protein kinases. *J Biol Chem* **275**: 37542-37551.

Hirabayashi T and Shimizu T (2000) Localization and regulation of cytosolic phospholipase A_2. *Biochim Biophys Acta* **1488**: 124-138.

Hirschberg K, Miller CM, Ellenberg J, Presley JF, Siggia ED, Phair RD and Lippincott-Schwartz J (1998) Kinetic analysis of secretory protein traffic and characterization of golgi to plasma membrane transport intermediates in living cells. *J Cell Biol* **143**: 1485-1503.

Kramer RM, Roberts EF, Um SL, Borsch-Haubold AG, Watson SP, Fisher MJ and Jakubowski JA (1996) p38 mitogen-activated protein kinase phosphorylates cytosolic phospholipase A_2 ($cPLA_2$) in thrombin-stimulated platelets. Evidence that proline-directed phosphorylation is not required for mobilization of arachidonic acid by $cPLA_2$. *J Biol Chem* **271**: 27723-27729.

Kudo I and Murakami M (2002) Phospholipase A_2 enzymes. *Prostaglandins and other lipid mediators*. **68-69**: 3-58.

Lin LL, Wartmann M, Lin AY, Knopf JL, Seth A and Davis RJ (1993) $cPLA_2$ is phosphorylated and activated by MAP kinase. *Cell* **72**: 269-278.

Mukherjee S, Ghosh RN and Maxfield FR (1997) Endocytosis. *Physiol Rev* **77**: 759-803.

Muthalif MM, Benter IF, Karzoun N, Fatima S, Harper J, Uddin MR and Malik KU (1998) 20-Hydroxyeicosatetraenoic acid mediates calcium/calmodulin-dependent protein kinase II-induced mitogen-activated protein kinase activation in vascular smooth muscle cells. *Proc Natl Acad Sci U S A* **95**: 12701-12706.

Muthalif MM, Benter IF, Uddin MR and Malik KU (1996) Calcium/calmodulin-dependent protein kinase IIalpha mediates activation of mitogen-activated protein kinase and cytosolic phospholipase A_2 in norepinephrine-induced arachidonic acid release in rabbit aortic smooth muscle cells. *J Biol Chem* **271**: 30149-30157.

Muthalif MM, Hefner Y, Canaan S, Harper J, Zhou H, Parmentier JH, Aebersold R, Gelb MH and Malik KU (2001) Functional interaction of calcium-/calmodulin-dependent protein kinase II and cytosolic phospholipase A_2. *J Biol Chem* **276**: 39653-39660.

Nakamura M, Sunagawa M, Kosugi T and Sperelakis N (2000) Actin filament disruption inhibits L-type $Ca^{(2+)}$ channel current in cultured vascular smooth muscle cells. *Am J Physiol Cell Physiol* **279**: C480-C487.

Nakatani Y, Tanioka T, Sunaga S, Murakami M and Kudo I (2000) Identification of a cellular protein that functionally interacts with the C2 domain of cytosolic phospholipase A_2 alpha. *J Biol Chem* **275**: 1161-1168.

Nalefski EA and Falke JJ (1998) Location of the membrane-docking face on the Ca^{2+}-activated C2 domain of cytosolic phospholipase A_2. *Biochemistry* **37**: 17642-17650.

Nalefski EA, Sultzman LA, Martin DM, Kriz RW, Towler PS, Knopf JL and Clark JD (1994) Delineation of two functionally distinct domains of cytosolic phospholipase A_2, a regulatory $Ca^{(2+)}$-dependent lipid-binding domain and a $Ca^{(2+)}$-independent catalytic domain. *J Biol Chem* **269**: 18239-18249.

Nebigil C and Malik KU (1992) Prostaglandin synthesis elicited by adrenergic stimuli is mediated via alpha-2C and alpha-1A adrenergic receptors in cultured smooth muscle cells of rabbit aorta. *J Pharmacol Exp Ther* **260**: 849-858.

Regier MK, Otto JC, DeWitt DL and Smith WL (1995) Localization of prostaglandin endoperoxide synthase-1 to the endoplasmic reticulum and nuclear envelope is independent of its C-terminal tetrapeptide-PTEL. *Arch Biochem Biophys* **317**: 457-463.

Rogers SL and Gelfand VI (2000) Membrane trafficking, organelle transport, and the cytoskeleton. *Curr Opin Cell Biol* **12**: 57-62.

Schievella AR, Regier MK, Smith WL and Lin LL (1995) Calcium-mediated translocation of cytosolic phospholipase A_2 to the nuclear envelope and endoplasmic reticulum. *J Biol Chem* **270**: 30749-30754.

Serhan CN (1997) Lipoxins and novel aspirin-triggered 15-epi-lipoxins (ATL): a jungle of cell-cell interactions or a therapeutic opportunity? *Prostaglandins* **53**: 107-137.

Valderrama F, Duran JM, Babia T, Barth H, Renau-Piqueras J and Egea G (2001) Actin microfilaments facilitate the retrograde transport from the Golgi complex to the endoplasmic reticulum in mammalian cells. *Traffic* **2**: 717-726.

Wijkander J and Sundler R (1992) Macrophage arachidonate-mobilizing phospholipase A_2: role of Ca^{2+} for membrane binding but not for catalytic activity. *Biochem Biophys Res Commun* **184**: 118-124.

Woods JW, Evans JF, Ethier D, Scott S, Vickers PJ, Hearn L, Heibein JA, Charleson S and Singer II. (1993) 5-lipoxygenase and 5-lipoxygenase-activating protein are localized in the nuclear envelope of activated human leukocytes. *J Exp Med* **178**: 1935-1946.

Xu GY, McDonagh T, Yu HA, Nalefski EA, Clark JD and Cumming DA (1998) Solution structure and membrane interactions of the C2 domain of cytosolic phospholipase A_2. *J Mol Biol* **280**: 485-500.

Zhang H, Wendel B, Van Wyk V and Nigam S (1996) Identification and molecular characterization of the CalB domain of the cytosolic phospholipase A_2 ($cPLA_2$) in human neutrophils. *Adv Exp Med Biol* **416**: 305-308.

FOOTNOTES

a) This work was supported by NHLBI Grant 19134-29 from the National Institutes of Health and from the Centers for Connective Tissue Diseases and Vascular Biology. Dr. Fariborz A. Yaghini was supported by NIH-training grant 2T32HL007641-16 from HLBI.

b) Reprint request should be addressed to:

Kafait U. Malik, D.Sc., Ph.D.

Department of Pharmacology,

College of Medicine,

874 Union Avenue

University of Tennessee Health Science Center,

Memphis, TN 38163

FIGURE LEGENDS

FIG. 1. $cPLA_2$ 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 μM) and latrunculin A (LA, 0.5 μM) or their vehicle (VEH). (A) Distribution of $cPLA_2$ (n=5), (B) density of $cPLA_2$ 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 μM) or its vehicle (V) in the presence of CD, LA or their vehicle (VEH). * Value significantly different from vehicle (VEH).

FIG. 2. $cPLA_2$ translocation to the nuclear envelope is not altered by disruption of microtubule filaments with colchicine. Rabbit VSMC were pretreated with colchicine (10 μM, 30 min) and then stimulated with NE (10 μM, 10 min) or its vehicle (V). $cPLA_2$ translocation and .microtubule filamants 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 μM), LA (0.5 μM,) or their vehicle (VEH) and exposed to NE (10 μM, 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

using anti-phospho-CaMK-IIα antibody (upper panels) and the CaMK-II protein levels (lower panels) were detected using anti-CaMK-IIα goat antibodies in cells treated with NE (10 μM) 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. $cPLA_2$ and actin are associated with each other. Coimmunoprecipitation experiments show that $cPLA_2$ is associated with actin and disruption of actin filaments by CD (0.5 μM) and LA (0.5 μM) have no affect on their association. VSMC were treated with CD (0.5 μM, 30 min), LA (0.5 μM, 30 min), or their vehicle (VEH) followed by treatment with NE (10 μM, 10 min) or its vehicle (V). (A) $cPLA_2$ was immunoprecipitated from cell lysates by incubating with $cPLA_2$-specific polyclonal antibody followed by incubation with protein A agarose with SDS-sample buffer. The $cPLA_2$ and actin proteins were detected by immunoblotting with the $cPLA_2$-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-$cPLA_2$ 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-$cPLA_2$ and $cPLA_2$ proteins were detected by immunoblotting with the phospho-$cPLA_2$-specific polyclonal antibody (upper panel) and $cPLA_2$-specific monoclonal antibody (lower panel), respectively (n=3). (D) Confocal microscopic examination of the VSMC show the association (Yellow) of $cPLA_2$ (Green) with actin (Red) as indicated by their specific

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

FIG. 5. Actin binds directly to $cPLA_2$. Far-Western analysis was performed on recombinant $cPLA_2$ (85 kDa) as described under Experimental Procedures. (A) Actin binding to $cPLA_2$ was detected using anti–actin antibody and (B) $cPLA_2$ protein was detected using anti-$cPLA_2$ monoclonal antibody (*n*=3).

FIG. 6. Disruption of actin filament polymerization does not inhibit NE-induced $cPLA_2$ phosphorylation, activity or arachidonic acid release. VSMC were treated with CD (0.5 μM), LA (0.5 μM), or their vehicle (VEH) and then exposed to NE (10 μM, 10 min) or its vehicle (V). (A) Phosphorylation of $cPLA_2$ was determined by ^{32}P incorporation (*n*=3); (B) $cPLA_2$ activity was determined from the hydrolysis of substrate L-1-^{14}C-arachidonyl phosphatidylcholine. The absolute value for vehicle of $cPLA_2$ was 2160.5 ± 43.6 CPM and (C) fractional arachidonic acid release after labeling the cells was measured with ^{3}H-AA as described under Experimental Procedures. The absolute (medium/total) value for vehicle was 2.31± 0.13 CPM (*n*=5). * Value significantly different from vehicle (VEH).

FIG. 7. Translocation of $cPLA_2$ 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 μM), or its vehicle (VEH) and exposed to ATP (100 μM)

or its vehicle (VEH). $cPLA_2$ translocation to the nuclear envelope and actin filament polymerization was visualized by confocal microscopy (40 X magnification) (*n*=5).

JPET Fast Forward. Published on February 10, 2005 as DOI: 10.1124/jpet.104.081992
This article has not been copyedited and formatted. The final version may differ from this version.

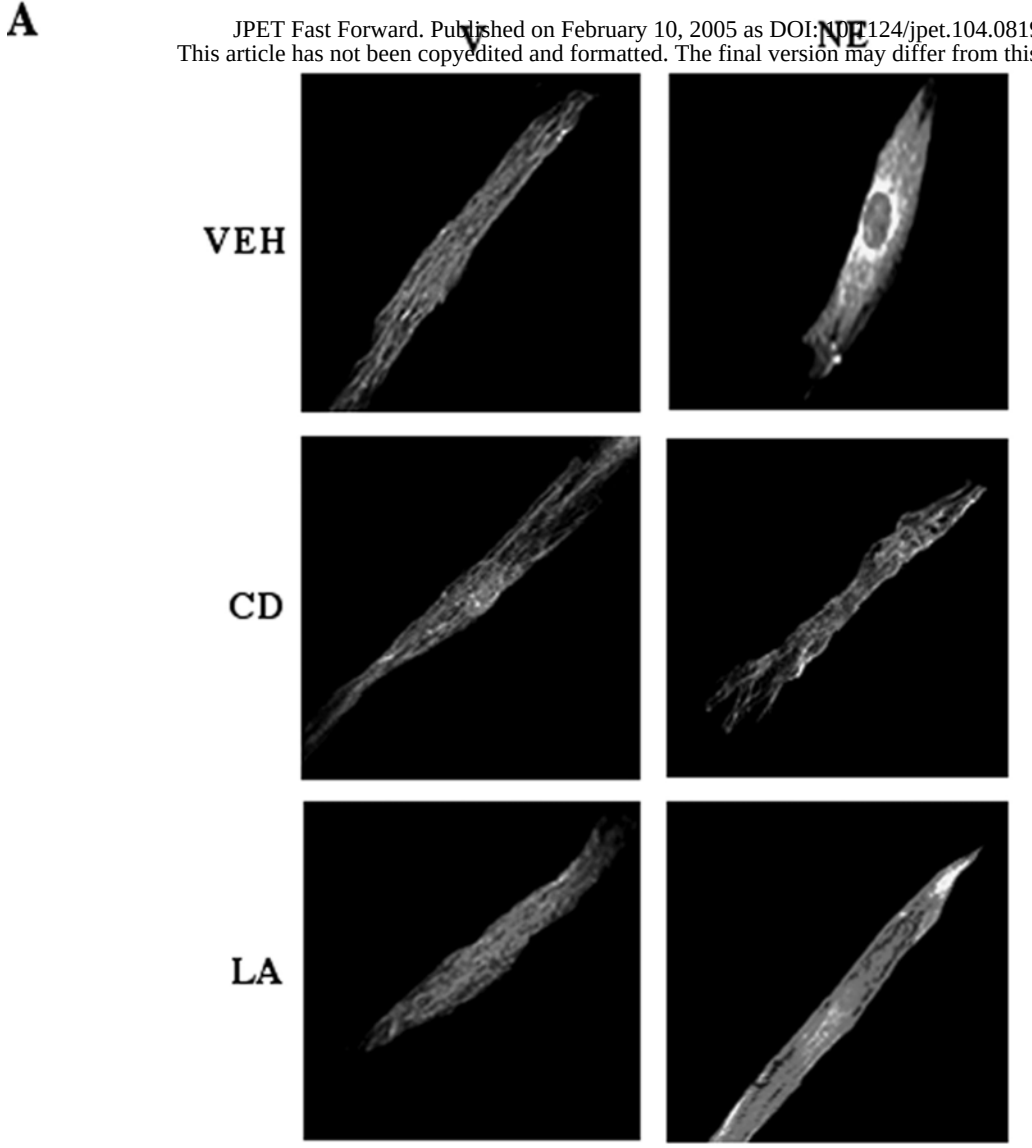


Figure 1A-B

JPET Fast Forward. Published on February 10, 2005 as DOI: 10.1124/jpet.104.081992
This article has not been copyedited and formatted. The final version may differ from this version.

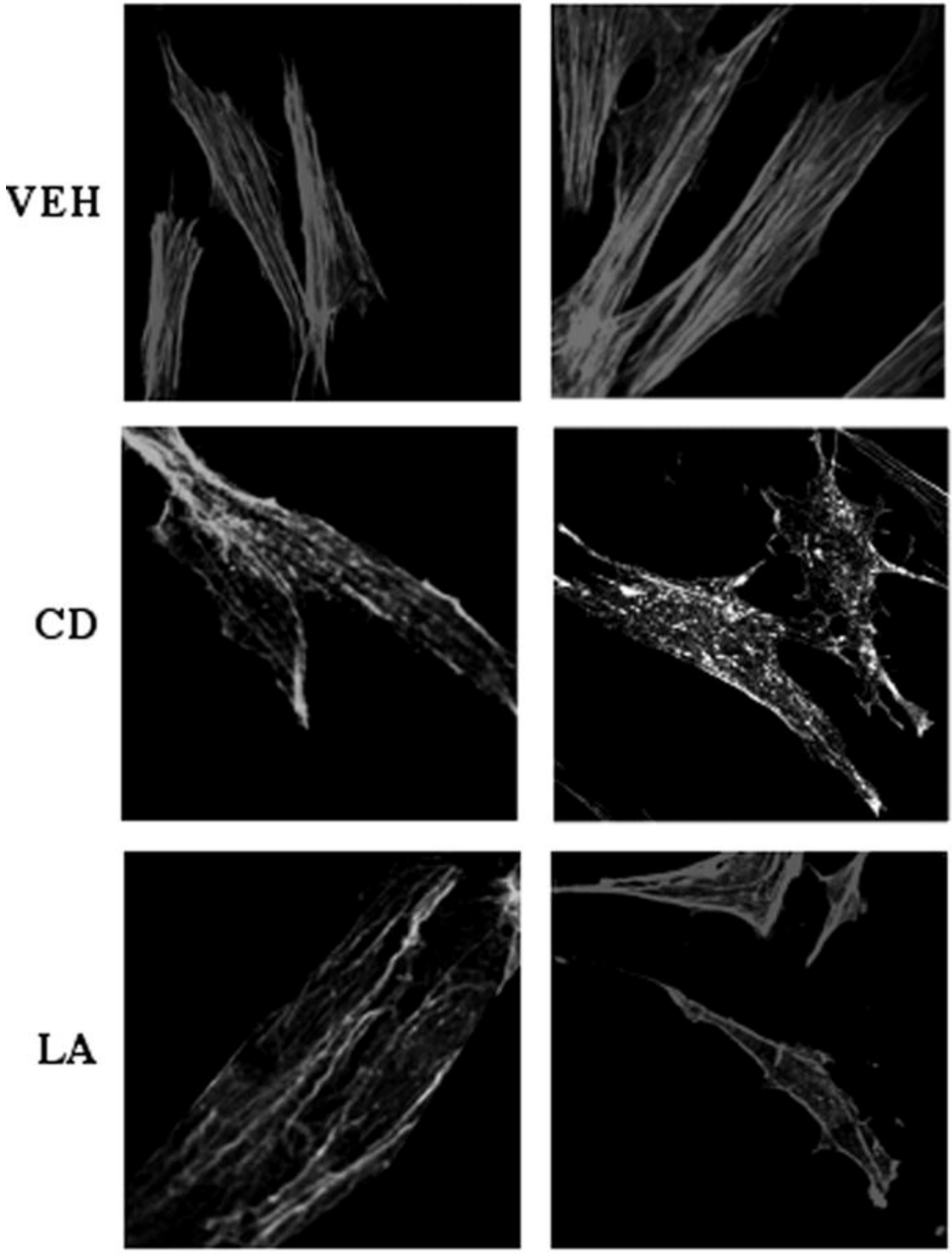


Figure 1C

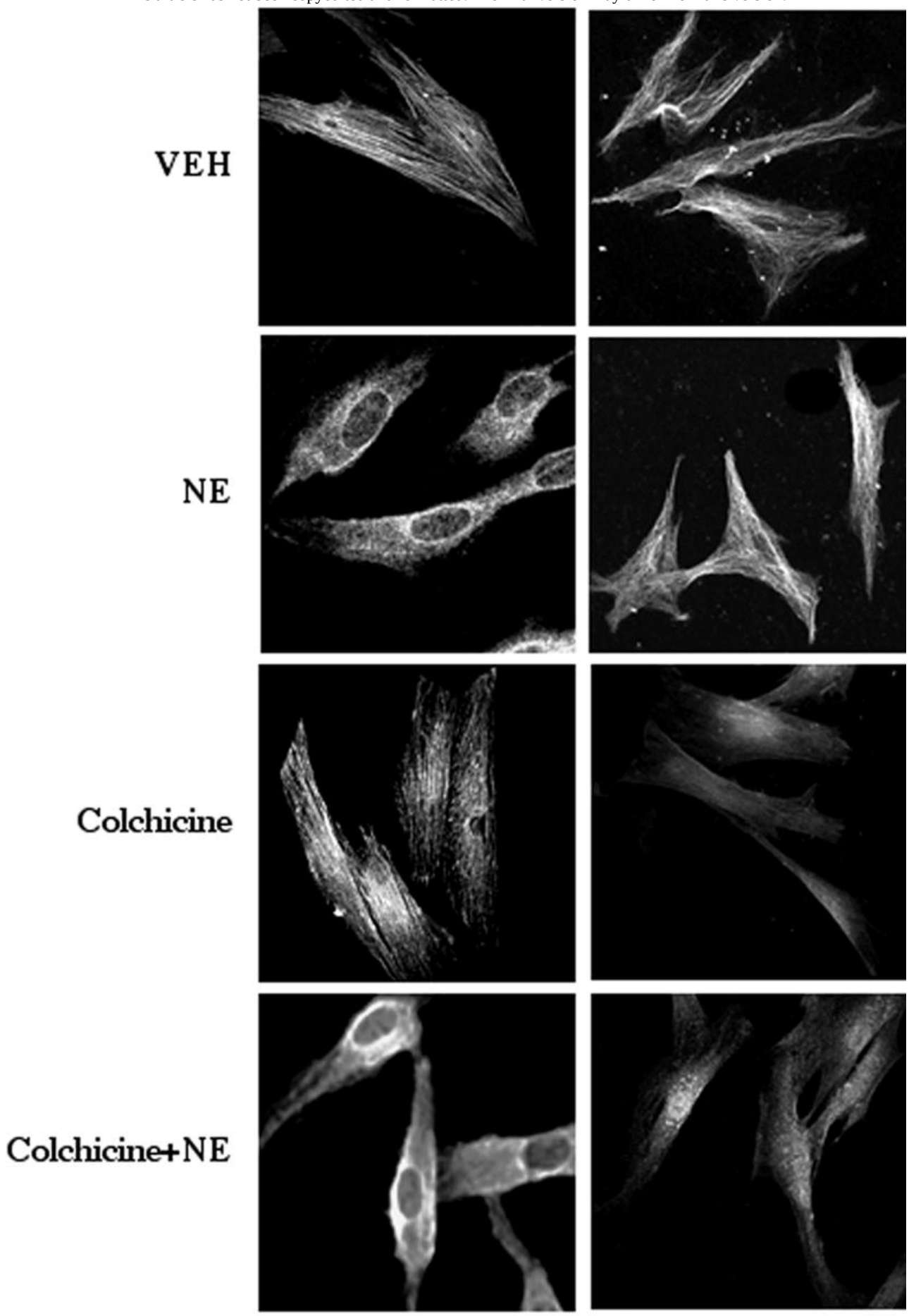


Figure 2

JPET Fast Forward. Published on February 10, 2005 as DOI: 10.1124/jpet.104.081992
This article has not been copyedited and formatted. The final version may differ from this version.

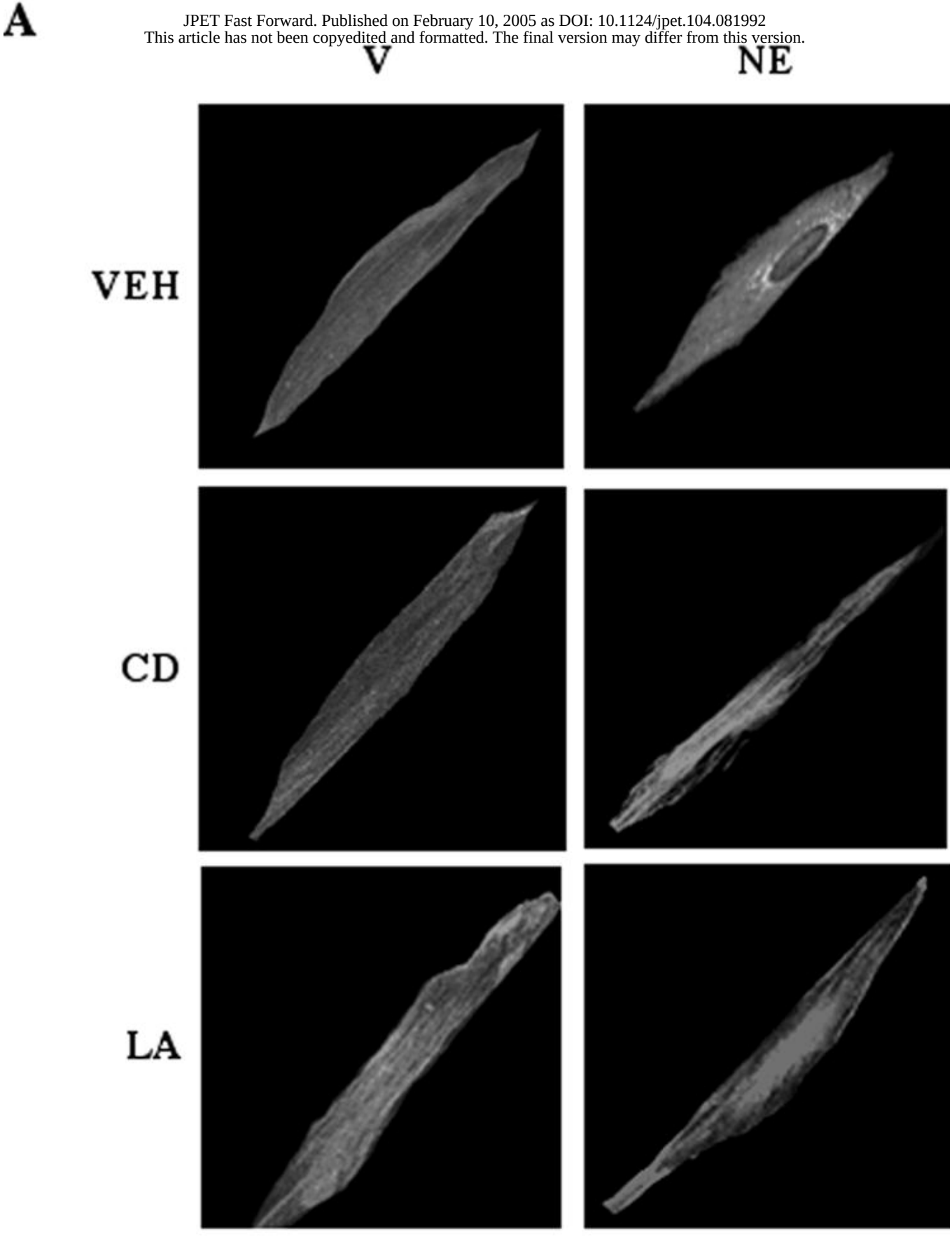


Figure 3 A

JPET Fast Forward. Published on February 10, 2005 as DOI: 10.1124/jpet.104.081992
This article has not been copyedited and formatted. The final version may differ from this version.

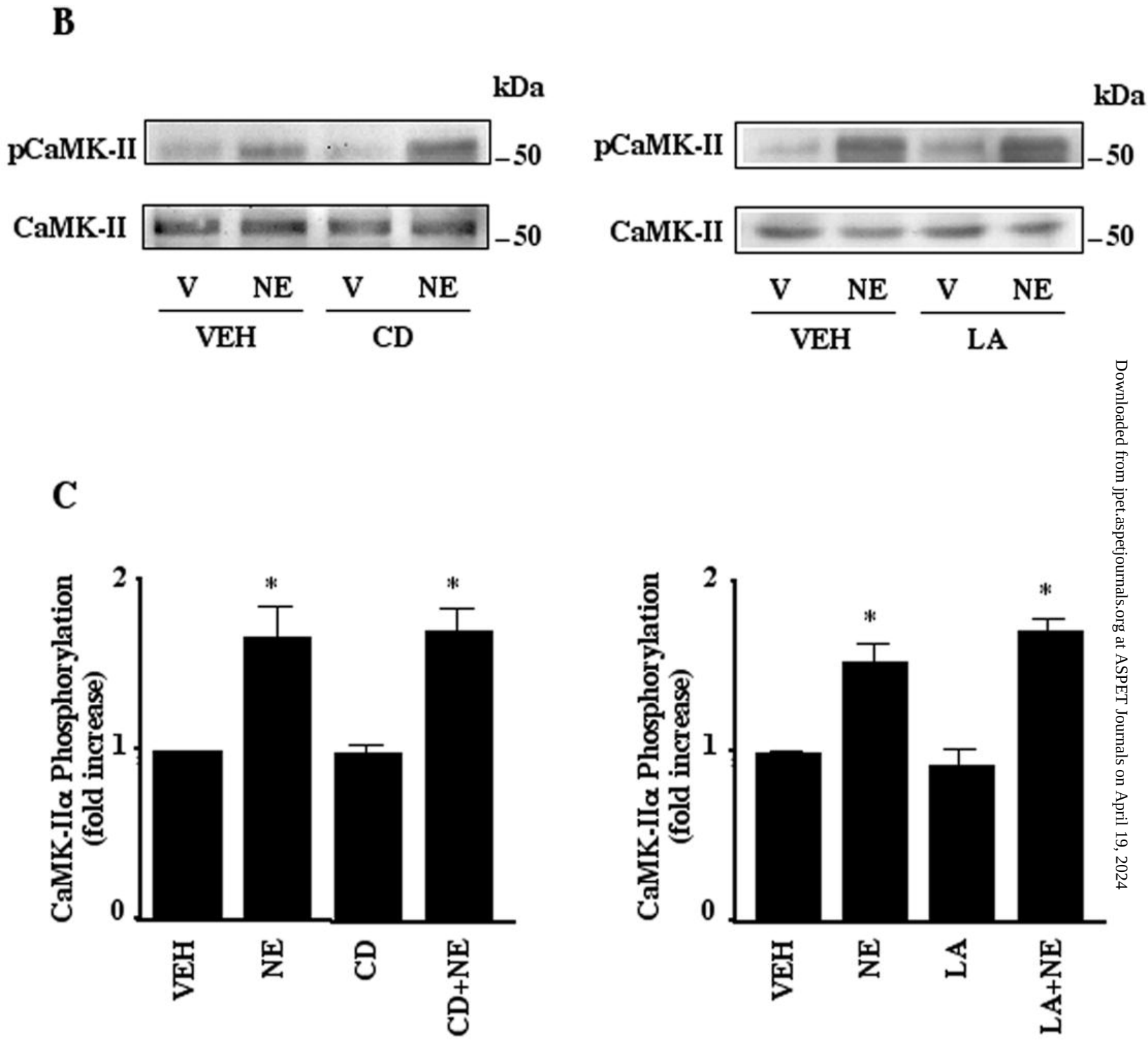


Figure 3 B & C

JPET Fast Forward. Published on February 10, 2005 as DOI: 10.1124/jpet.104.081992
This article has not been copyedited and formatted. The final version may differ from this version.

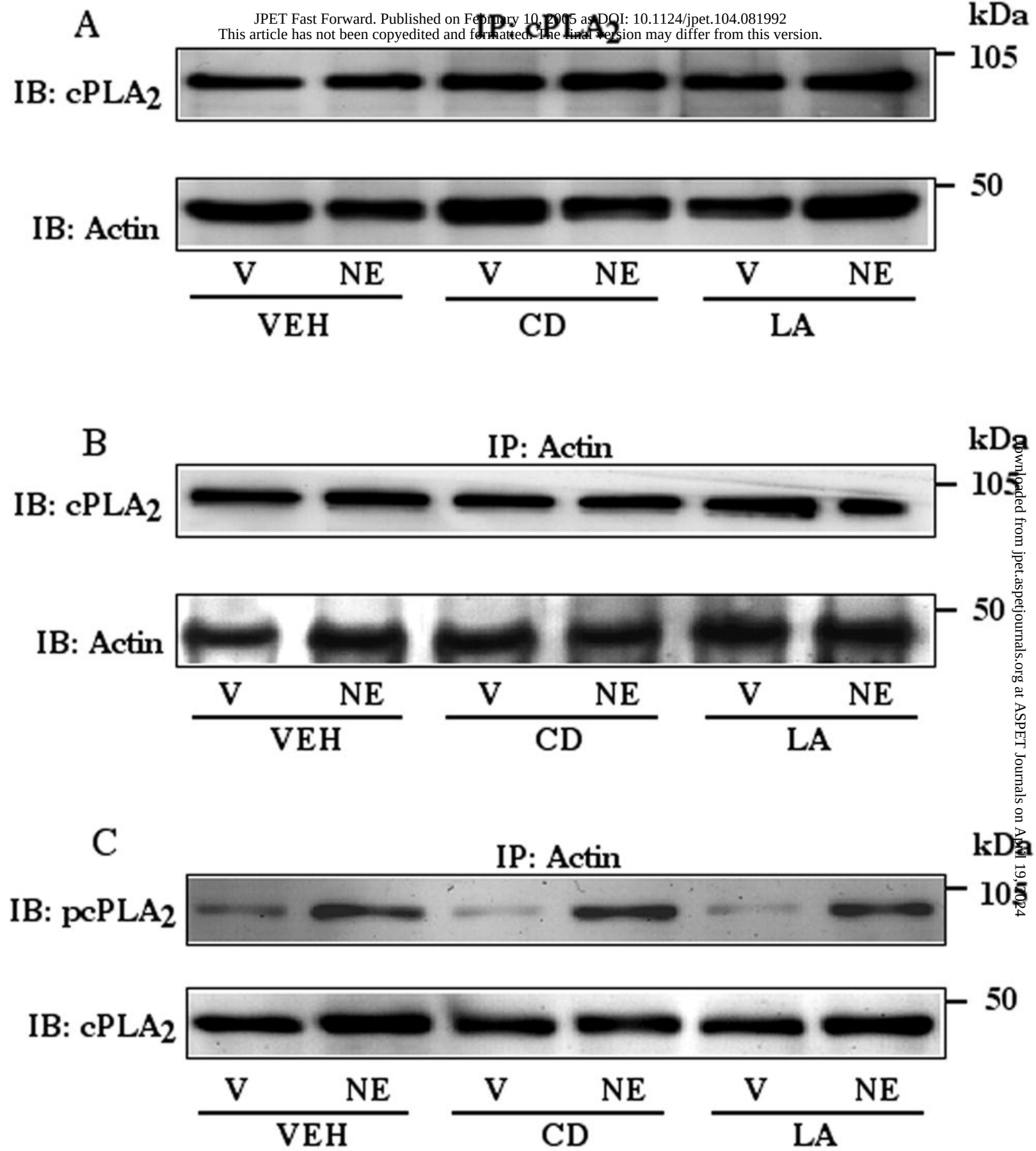
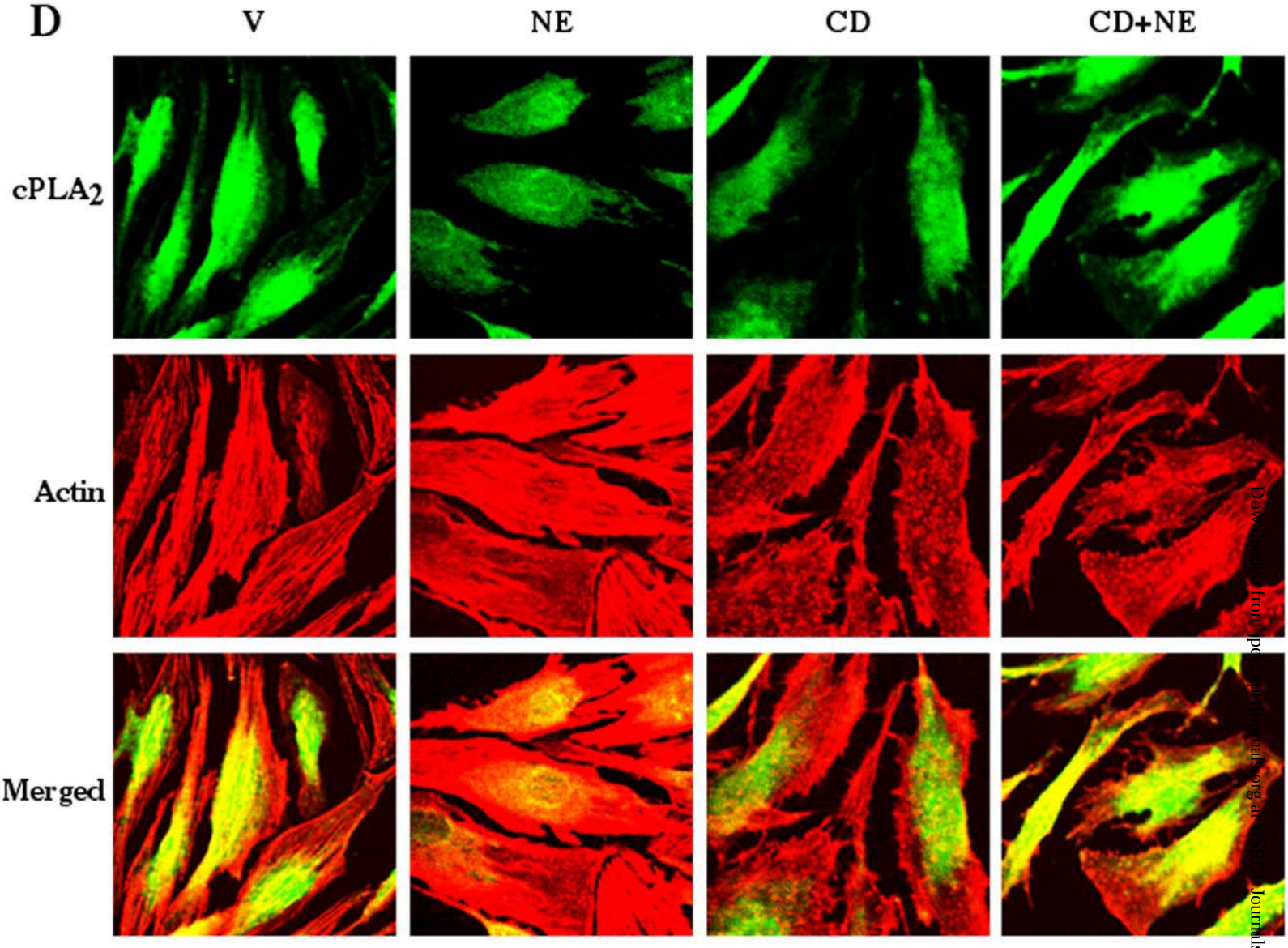


Figure 4 A-C

JPET Fast Forward. Published on February 10, 2005 as DOI: 10.1124/jpet.104.081992
This article has not been copyedited and formatted. The final version may differ from this version.



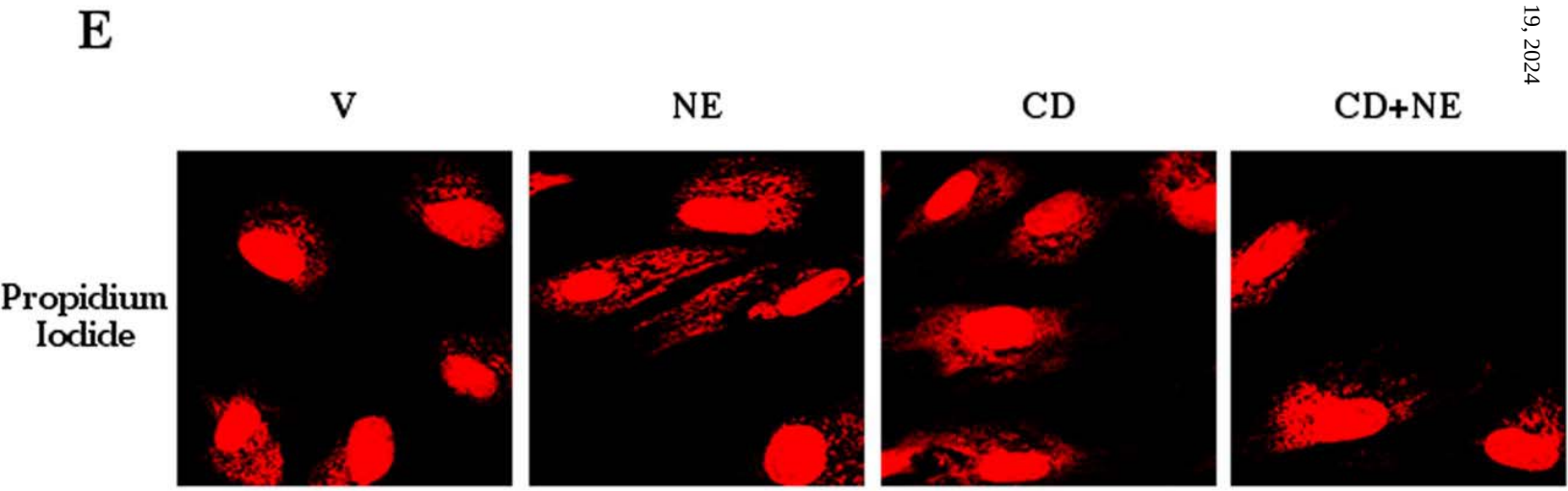


Figure 4 D-E

JPET Fast Forward. Published on February 10, 2005 as DOI: 10.1124/jpet.104.081992
This article has not been copyedited and formatted. The final version may differ from this version.

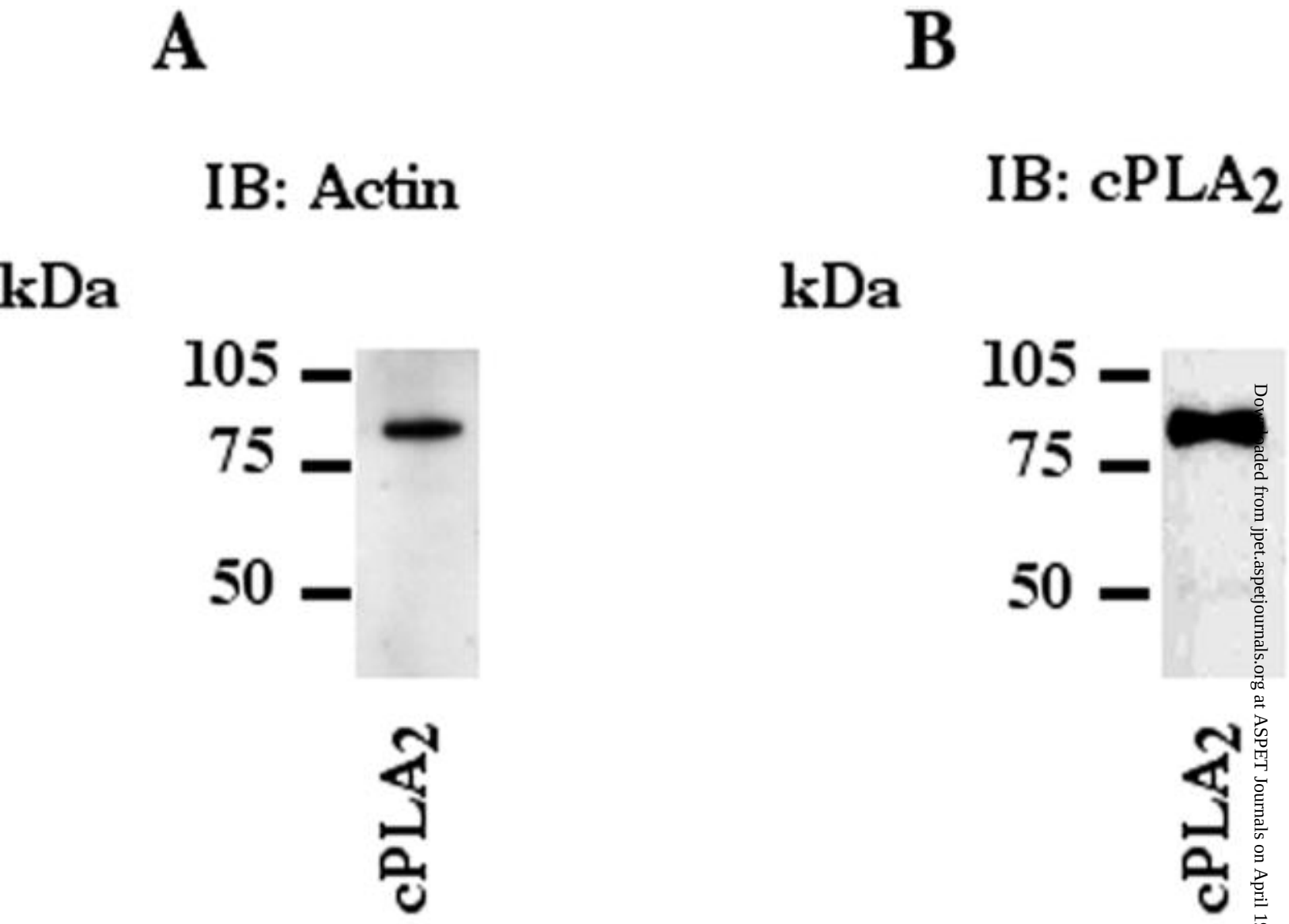


Figure 5

JPET Fast Forward. Published on February 10, 2005 as DOI: 10.1124/jpet.104.081992
This article has not been copyedited and formatted. The final version may differ from this version.

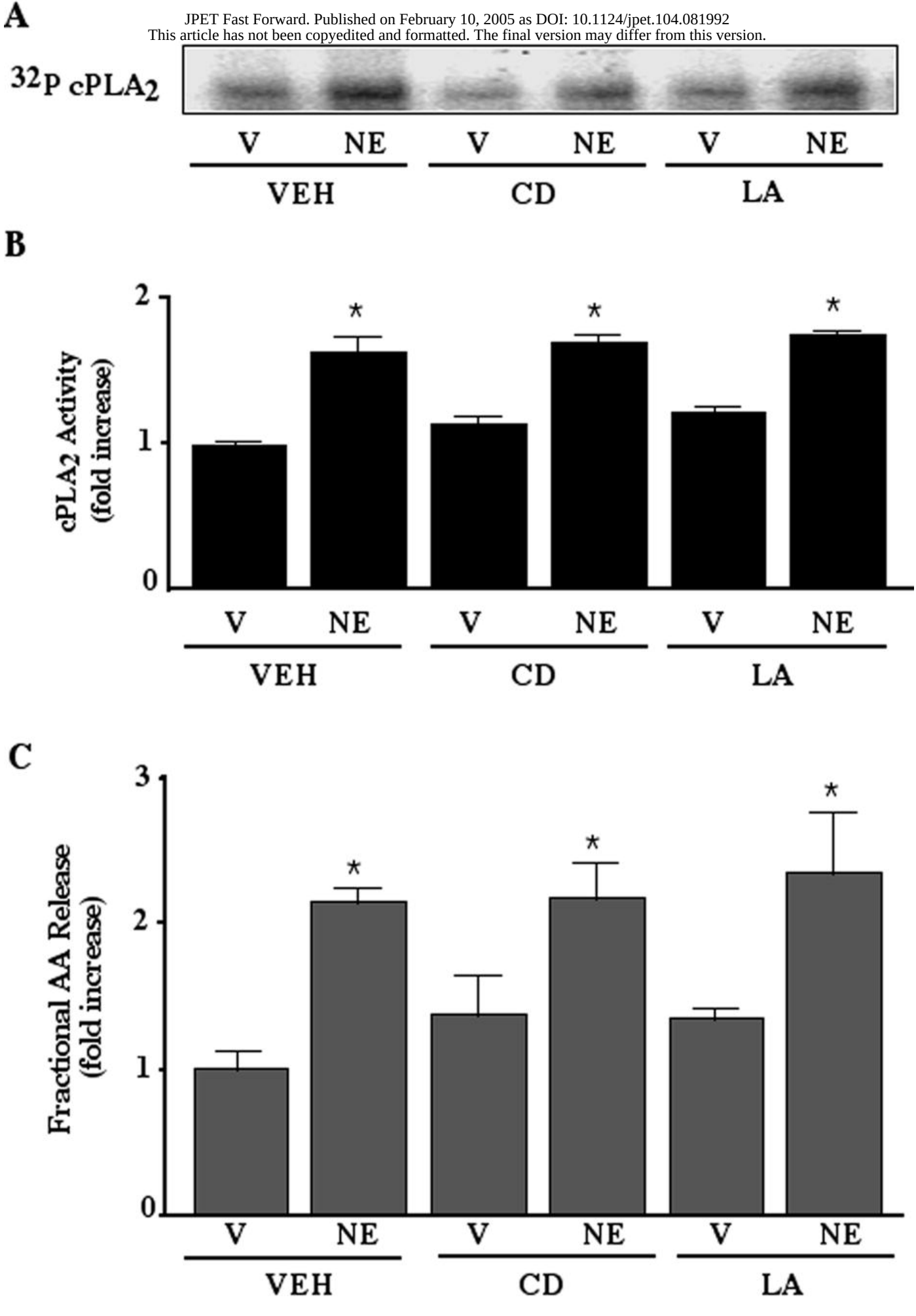


Figure 6

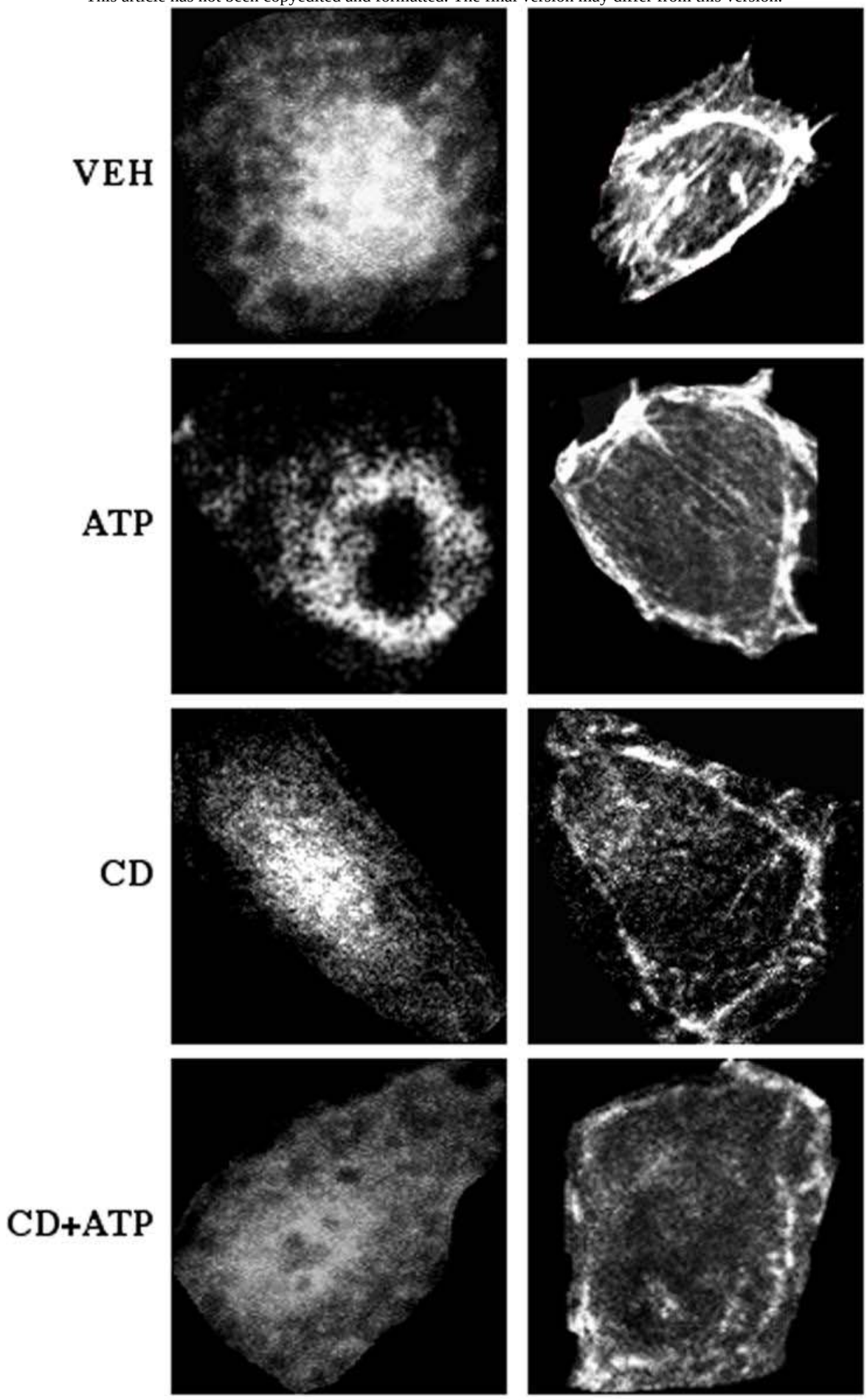


Figure 7