Intact Actin Filaments Are Required for Cytosolic Phospholipase A\textsubscript{2} Translocation but Not for Its Activation by Norepinephrine in Vascular Smooth Muscle Cells\textsuperscript{S}

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ABSTRACT

Cytosolic phospholipase A\textsubscript{2} (cPLA\textsubscript{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\textsubscript{2} translocation to the nuclear envelope is mediated via its phosphorylation by calcium/calmodulin-dependent kinase-II in rabbit vascular smooth muscle cells (VSMCs). 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\textsubscript{2} translocation to the nuclear envelope and its activation by NE in rabbit VSMCs. NE (10 \textmu M) caused cPLA\textsubscript{2} translocation to the nuclear envelope as determined by immunofluorescence. Cytochalasin D (CD; 0.5 \textmu M) and latrunculin A (LA; 0.5 \textmu M) that disrupted actin filaments, blocked cPLA\textsubscript{2} translocation elicited by NE. On the other hand, disruption of microtubule filaments by 10 \textmu M colchicine did not block NE-induced cPLA\textsubscript{2} translocation to the nuclear envelope. CD and LA did not inhibit NE-induced increase in cytosolic calcium and cPLA\textsubscript{2} activity, determined from the hydrolysis of L-1-[\textsuperscript{14}C]arachidonoyl phosphatidylcholine and release of AA. Immunoprecipitation studies showed an association of actin with cPLA\textsubscript{2}, which was not altered by CD or LA. Far-Western analysis showed that cPLA\textsubscript{2} interacts directly with actin. Our data suggest that NE-induced cPLA\textsubscript{2} translocation to the nuclear envelope requires an intact actin but not microtubule filaments and that cPLA\textsubscript{2} phosphorylation and activation and AA release are independent of its translocation to the nuclear envelope in rabbit VSMCs.

Phospholipase A\textsubscript{2} (PLA\textsubscript{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 hydroxyeicosatetraenoic and trienoic acids (Capdevila et al., 1982; Serhan, 1997; Funk, 2001). Cytosolic PLA\textsubscript{2} (cPLA\textsubscript{2}), the group IVA of the PLA\textsubscript{2} enzymes, is an 85-kDa protein with a preference for AA over the other fatty acids and is activated by submicromolar calcium (Ca\textsuperscript{2+}) and by phosphorylation (Lin et al., 1993; Kudo and Murakami, 2002). Structural analysis of cPLA\textsubscript{2} has shown the presence of an N-terminal Ca\textsuperscript{2+}-phospholipid binding domain (C2 domain) and a C-terminal catalytic domain (Dessen, 2000). cPLA\textsubscript{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\textsubscript{2} has been shown to be phosphorylated on Ser\textsuperscript{505} by the mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase (ERK)1/2, and p38 MAPK (Lin et al., 1993; Kramer et al., 1996; Borsch-Haubold et al., 1998; Gijon et al., 2000); on Ser\textsuperscript{515} by calcium/calmodulin-dependent protein kinase-II (CaMK-II; Muthalif et al., 2001); and on Ser\textsuperscript{727} by MAPK-interacting kinase I (Hefner et al., 2000).

ABBRiVIATIONS: PLA\textsubscript{2}, phospholipase A\textsubscript{2}; AA, arachidonic acid; cPLA\textsubscript{2}, cytosolic phospholipase A\textsubscript{2}; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; CaMK-II, calcium/calmodulin-dependent protein kinase-II; NE, norepinephrine; ER, endoplasmic reticulum; MDCK, Madin-Darby canine kidney; ECL, enhanced chemiluminescence; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminoophenyl)butadiene; VSMC, vascular smooth muscle cell; CD, cytochalasin D; LA, latrunculin A; KN-93, 2-[[2-(2-hydroxyethyl)-N-(4-methoxybenzenesulfonfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; TBST, Tris-buffered saline/Tween 20.

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Several agonists that increase cellular levels of Ca\(^{2+}\), including norepinephrine (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 Carvalho 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 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 Madin-Darby canine kidney (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) 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; Valderama 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; Valderama et al., 2001). Similarly, inhibitors of microtubule filaments nocodazole or colchicine 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 VSMCs. 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 VSMCs.
Phosphorylation of cPLA2. Phosphorylation of cPLA2 in response to NE was measured as described previously (Lin et al., 1993). Briefly, growth-arrested VSMCs were labeled with 300 μCi/ml [32P]orthophosphoric acid for 4 h in plain M-199 medium. The medium was removed, and the cells were washed with plain M-199 medium and treated with 0.5 μM CD, 0.5 μM LA, or their vehicle for 30 min followed by stimulation with 10 μM NE or its vehicle for 10 min. The cells were lysed in lysis buffer, and cPLA2 was immunoprecipitated using anti-cPLA2 monoclonal antibodies. [32P]-Labeled cPLA2 immunoprecipitate was subjected to 10% SDS-PAGE. The gel was dried, and the radioactivity was detected by autoradiography.

**Immunoprecipitation.** Cells were grown on 100-mm tissue culture dishes to subconfluence and arrested for growth for 48 h. Immunoprecipitation was performed as described previously (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 10 μM NE for 10 min. 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). cPLA2 and actin were immunoprecipitated using anti-cPLA2 or anti-actin goat polyclonal antibodies, respectively. cPLA2 and actin immunoprecipitates were subjected to 10% SDS-PAGE followed by immunoblotting.

**Immunoblotting.** To determine CaMK-II and cPLA2 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 20 (TBST buffer) for 2 h, the membrane was incubated overnight with anti-phospho-CaMK-IIα goat polyclonal or anti-phospho-cPLA2 (Ser655) rabbit polyclonal antibodies at 1:1000 dilution in TBST buffer containing 5% BSA followed by incubation with anti-goat IgG horseradish peroxidase antibody (1:20,000 dilution in TBST buffer) and anti-rabbit IgG horseradish peroxidase antibody (1:1000 dilution in TBST buffer) for 1 h at 25°C, respectively. The immunoreactive protein was detected using the ECL Plus system. CaMK-IIα and cPLA2 protein levels were detected using anti-CaMK-IIα goat polyclonal and anti-cPLA2 mouse monoclonal antibodies (Santa Cruz Biotechnology, Inc.), respectively.

For coinmunoprecipitation studies, the immunoprecipitates obtained with anti-cPLA2 and anti-actin antibodies were subjected to SDS-PAGE analysis followed by immunoblotting using both anti-cPLA2 and anti-actin mouse monoclonal antibodies (Santa Cruz Biotechnology, Inc.).

**Far-Western Analysis.** To determine whether cPLA2 binds directly to actin, we performed Far-Western analysis. Briefly, pure recombinant cPLA2 was subjected to SDS-PAGE and transferred to a nitrocellulose membrane, which was blocked with 5% (w/v) BSA in TBST for 2 h at room temperature or overnight at 4°C. The nitrocellulose membrane, with immobilized proteins, was 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 VSMCs were treated with CD or LA (0.5 μM each) for 30 min. The cells were washed with Hank’s balanced salt solution three times and trypsinized. Two milliliters 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. Then, 40 μl of trypan blue dye (1:1; v/v) was added to the 40-μl pellet and left at room temperature for 5 min. Ten microliters of the re suspended cells was 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 nonviable cells over total cells was used as an index of viability.

**Measurement of [3H] AA Release from VSMCs.** The release of AA and its tritiated products from VSMCs was measured as described previously (Muthalif et al., 1996). Briefly, the cells labeled overnight with [3H]AA were washed with Hanks’ balanced salt solution 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 [3H]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. [3H] 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 CaMK-IIα, cPLA2, catalytic activity, and AA release were expressed as mean ± S.E.M. Data were analyzed by one-way analysis of variance; 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 cPLA2 to the Nuclear Envelope Is Blocked by Actin Disruption.** Previous studies from our laboratory have shown that cPLA2 is translocated from the cytoplasm to the nuclear envelope in response to NE (Fatima et al., 2003). CD (0.5 μM), a fungal toxin that 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 disassembly, caused disruption of actin filaments in the VSMCs (Fig. 1B) and prevented NE-induced translocation of cPLA2 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 VSMCs were viable as indicated by trypan blue exclusion test.

**Disruption of Actin Filaments Does Not Block NE-Induced cPLA2 Translocation by Inhibiting Calcium Influx.** Ca2+ is required for cPLA2 activation and translocation from the cytoplasm to the nuclear envelope in response to NE in VSMCs (Muthalif et al., 1996). To determine whether inhibitors of actin polymerization block NE-induced cPLA2 translocation to the nuclear envelope by blocking Ca2+ influx, the effect of CD and LA on cytosolic Ca2+ in VSMCs was examined. Exposure of VSMCs to NE twice at 10-min intervals increased the level of cytosolic Ca2+; 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 Ca2+ between the first and second exposure to NE obtained during the infusion of these agents was not different from the corresponding ratio obtained during infusion of their vehicle (Supplemental Data Fig. 1). Therefore, blockade of NE-induced cPLA2 translocation to the nuclear envelope is not due to decreased Ca2+ influx, but rather by the disruption of actin filaments per se.

**NE-Induced Translocation of cPLA2 Is Not Blocked by Disruption of Microtubule Filaments.** To determine whether microtubule filaments are also required for NE-
induced cPLA₂ 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 cPLA₂ translocation to the nuclear envelope in response to NE in VSMCs (Fig. 2). Therefore, translocation of cPLA₂ 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 Ca²⁺ that binds to calmodulin and activates CaMK-II (Muthalif et al., 1996). Activated CaMK-II phosphorylates cPLA₂ 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 VSMCs (Muthalif et al., 1996). 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 VSMCs. 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 Fig. 3C, whereas CaMK-II translocation, as determined by confocal microscopy (Fig. 3A), was blocked by CD and LA, indicating that intact actin filaments are required for CaMK-II as well as for cPLA₂ 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 cPLA₂ 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 Fig. 2). Although they inhibited NE-induced CaMK-II phosphorylation (Supplemental Data Fig. 2), which suggests that actin polymerization in VSMCs is not dependent on CaMK-II activity.

**cPLA₂ Is Associated with Actin Filaments.** To determine whether there is any association between cPLA₂ and actin filaments, cPLA₂ immunoprecipitates were probed with anti-actin antibodies and actin immunoprecipitates with anti-cPLA₂ antibodies. Our studies showed that cPLA₂ and actin coimmunoprecipitate, as well as disruption of actin filaments by CD and LA does not affect this association (Fig. 4, A and B). There was a possibility that actin disruption may alter the amounts of phosphorylated cPLA₂ bound to actin. To test this, we immunoprecipitated actin and determined the amount of Ser⁵⁰⁵-phosphorylated cPLA₂ bound to it (VEH). A, distribution of cPLA₂ (n = 5). B, density of cPLA₂ fluorescence around nuclear envelope (n = 5). C, distribution of actin filaments (n = 5) was visualized by confocal microscopy (40× magnification) in VSMCs treated with 10 μM NE or its vehicle (V) in the presence of CD, LA, or their VEH. *p value is significantly different from VEH.

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**Fig. 1.** cPLA₂ accumulation around the nuclear envelope in response to NE in VSMCs is lost when actin filaments are disrupted by inhibitors of actin filament polymerization. VSMCs were treated with inhibitors of actin polymerization, including 0.5 μM CD and 0.5 μM LA or their vehicle.
phosphorylation of cPLA2 at Ser515 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 cPLA2 antibody specific against this phosphorylation sites of cPLA2.

cPLA2 Binds Directly to Actin. Com-immunoprecipitation and colocalization studies demonstrated the association of cPLA2 with actin. To determine whether cPLA2 binds directly to actin, we performed Far-Western analysis with pure recombinant cPLA2. Our studies revealed that cPLA2 binds to actin (Fig. 5).

NE-Induced cPLA2 Phosphorylation and Activation and AA Release Were Not Altered by Actin Filament Disruption. To determine the consequence of actin filament disruption on cPLA2 phosphorylation and activity, cells labeled with or without 32P were treated with CD or LA, exposed to NE, and then lysed. The lysates were assayed for cPLA2 phosphorylation, measured by 32P incorporation (Fig. 6A); cPLA2 activity was determined from the hydrolysis of L-1-[14C]arachidonyl phosphatidylethanolamine (Fig. 6B); and fractional AA release was measured by labeling the cells with [3H]AA (Fig. 6C) as described under Materials and Methods. Neither NE-induced cPLA2 phosphorylation nor activation or AA release was altered by CD or LA. Therefore, it seems that intact actin filaments provide tracks for the translocation of cPLA2 to the nuclear envelope, but they are not involved in its phosphorylation or activation process in rabbit VSMCs.

Translocation of cPLA2 to the Nuclear Envelope by Angiotensin-II and Ionomycin is Also Blocked by Actin Disruption in Rabbit VSMCs. To determine whether disruption of actin filaments interferes with cPLA2 translocation elicited by stimuli other than NE, we examined the effect of CD on ionomycin- and angiotensin-II-induced cPLA2 translocation in rabbit VSMCs. Angiotensin-II (0.1 μM) and 1 μM ionomycin caused translocation of cPLA2 to the nuclear envelope, and this translocation was blocked by the disruption of actin filaments with 0.5 μM CD (Supplemental Data Fig. 3). These observations indicate that translocation of cPLA2 to the nuclear envelope by agents other than NE also requires intact actin filaments in rabbit VSMCs.

Translocation of cPLA2 to the Nuclear Envelope by ATP Is Blocked by Actin Disruption in MDCK Cells. Disruption of actin filaments blocked the translocation of cPLA2 to the nuclear envelope in rabbit VSMCs. To determine whether an intact actin is also essential for the translocation of cPLA2 in other cells, we examined the effect of CD on cPLA2 translocation in response to ATP in MDCK cells. In these cells where ATP is known to cause cPLA2 translocation to the nuclear envelope (Evans et al., 2002), disruption of actin filaments with 0.5 μM CD blocked 100 μM ATP-induced cPLA2 translocation to the nuclear envelope (Fig. 7). Therefore, it seems that intact actin filaments are also required for translocation of cPLA2 to the perinuclear region in cells other than rabbit VSMCs.

Discussion

This study demonstrates a novel role of actin filaments in NE-induced translocation of cPLA2 to the nuclear envelope in VSMCs. Moreover, it shows that phosphorylation and activation of cPLA2 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 VSMCs with CD or LA prevented cPLA2 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 cPLA2 to the nuclear envelope seems to be specific to this cytoskeletal structure, because disruption of microtubule filaments by colchicine in VSMCs did not prevent NE-induced translocation of cPLA2 to the nuclear envelope.

Ca2+ is essential for the binding of cPLA2 to phospholipid vesicles or membranes (Channon and Leslie, 1990; Wijker and Sundler, 1992; Nalefski et al., 1994). This enzyme contains an N-terminal C2 domain that binds Ca2+ and promotes attachment of cPLA2 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 VSMCs (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 VSMCs (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 seems to be unlikely because neither CD nor LA decreased NE-induced rise in cytosolic Ca$^{2+}$ in VSMCs. The effect of CD to inhibit Ca$^{2+}$ current in A7r5 cells and not to decrease cytosolic Ca$^{2+}$ levels in rabbit VSMCs 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 VSMCs (Fatima et al., 2003). Therefore, CD and LA could block cPLA$_2$ translocation by inhibiting CaMK-II activity in the VSMCs. 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 VSMCs. **Fig. 3.** Translocation of CaMK-II to the nuclear envelope in response to NE in VSMCs is lost when actin filaments are disrupted by inhibitors of actin filament polymerization. VSMCs were treated with 0.5 µM CD, 0.5 µM LA, or their vehicle (VEH) and exposed to 10 µM NE (10 min) or its vehicle (V). A, CaMK-II translocation to the nuclear envelope was visualized by confocal microscopy (40× magnification) (n = 5). B, CaMK-II phosphorylation was detected using anti-phospho-CaMK-II antibody (top), and the CaMK-II protein levels (bottom) were detected using anti-CaMK-II goat antibodies in cells treated with 10 µM NE or its V as described under Materials and Methods (n = 3). C, density of CaMK-II phosphorylation was quantified using NIH Image 1.63 program. *, value significantly different from VEH.
CaMK-II is a Ser/Thr kinase that phosphorylates cPLA₂ at Ser⁵¹⁵ (Muthalif et al., 2001). Previously, we have shown that CaMK-II also translocates to the nuclear envelope in response to NE in a Ca²⁺/H₁₁₀₀₁-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 cPLA₂ to the nuclear envelope. Since CD and LA did not alter phosphorylation of either CaMK-II or cPLA₂, it seems that these enzymes are phosphorylated/activated before translocation to the nuclear envelope. Since CaMK-II in some cells

![Image of immunoblotting experiments showing cPLA₂ and actin association](image-url)
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 cPLA2 to the nuclear envelope in response to NE in VSMCs. 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 VSMCs.

The role of actin in cPLA2 transport to the nuclear envelope in response to NE was also indicated from our coimmunoprecipitation experiments showing that actin is associated with cPLA2. Since anti-actin antibody does not differentiate between polymerized (F-actin) and depolymerized (monomeric G-actin) form of actin, we cannot differentiate which of these forms of actin binds to cPLA2. Our finding that CD or LA did not alter this association suggests that cPLA2 most likely binds to both forms of actin. Moreover, NE also failed to alter the association of cPLA2 and actin. Therefore, it seems that actin is not directly involved in the transport of cPLA2 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 cPLA2 on actin filaments remains to be determined. The requirement of intact actin filaments for cPLA2 translocation to the nuclear envelope in VSMCs was not unique to the action of NE because cPLA2 translocation elicited by angiotensin II and ionomycin was also
inhibited by disruption of actin filaments with CD. Moreover, it seems that intact actin is also required for cPLA₂ translocation in other cells as indicated by our demonstration that disruption actin filaments with CD prevented localization of cPLA₂ to the perinuclear region in response to ATP in MDCK cells.

cPLA₂ can also bind with other cellular proteins, including vimentin (Nakatani et al., 2000); calpain light chain; p11, a Ca²⁺ binding protein; annexin I; annexin V; and the nuclear protein PLIP, and most of these proteins inhibit the activity of cPLA₂ or AA release (for review, see Kudo and Murakami, 2002). The molecular mechanism of interaction of these proteins with cPLA₂ and their contribution to cPLA₂ translocation to the perinuclear region is not known. cPLA₂, which colocalizes with vimentin, an intermediate filament protein, in the perinuclear region of human embryonic kidney 293 cells in response to calcium ionophore has been shown to bind to C2 domain of cPLA₂ (Nakatani et al., 2002). In our study the Far-Western analysis showed that the full-length cPLA₂ binds directly to actin. It is possible that actin also binds to C2 domain of cPLA₂. However, studies with various cPLA₂ mutants, including cPLA₂ deleted of its C2 domain, would be required to address this issue.

The translocation of cPLA₂ 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 cPLA₂ releases AA for prostanoxin synthesis from the membrane phospholipids around the nuclear envelope and/or adjacent ER (Wood et al., 1993; Regier et al., 1995). However, in MDCK cells ATP and ionomycin caused phosphorylation, activation, and translocation of cPLA₂ to the nuclear envelope, but the inhibitor of MAPK kinase (MEK) U0126 reduced cPLA₂ activity, as measured by release of AA, without altering its phosphorylation or translocation (Evans et al., 2002). On the other hand, phosphorylation of cPLA₂ in VSMCs 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 VSMCs is mediated by AA metabolites generated subsequent to activation of cPLA₂ by CaMK-II (Muthalif et al., 1998) and the inhibition of cPLA₂ catalytic activity with methyl arachidonyl fluorophosphate attenuated ERK1/2 MAPK activity without blocking cPLA₂ translocation (Fatima et al., 2003), it seems that phosphorylation of cPLA₂ by ERK1/2 in VSMCs (Muthalif et al., 1998), like in MDCK cells (Evans et al., 2002), is not required for cPLA₂ translocation to nuclear envelope. The translocation of cPLA₂ to the nuclear envelope in response to NE is also independent of p38 MAPK in rabbit VSMCs (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 cPLA₂ 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 VSMCs. Our findings also raise an important question regarding the functional significance of cPLA₂ translocation to the nuclear envelope in VSMCs. It is possible that cPLA₂ 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 VSMCs. Whether the interruption of cPLA₂ translocation to the nuclear envelope by disruption of actin filaments affects 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 are required for cPLA₂ translocation to the nuclear envelope in response to NE in VSMCs. Moreover, phosphorylation and activation of cPLA₂ are not dependent upon its translocation to the nuclear envelope; activated cPLA₂ may release AA from the nuclear membrane as well as cell membrane in VSMCs in response to NE.

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References


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Figure 1: Disruption of actin filaments by CD or LA does not alter NE-induced rise in cytosolic calcium ([Ca^{2+}]_{i}). Rabbit VSMC were exposed to NE (10 µM) twice, first in the absence (first response) and then in the presence (second response) of CD (0.5 µM), LA, (0.5 µM) or their vehicle (VEH). (A) Cytosolic increase in Ca^{2+} was measured using fura-2 as described under Experimental Procedures (n=3). (B) Ratio of the increase in cytosolic [Ca^{2+}]_{i} of second/first exposure of NE in VSMC.
Figure 2: Inhibition of NE-induced activation of CaMK-II does not disrupt actin filaments. VSMC were treated with inhibitor of CaMK-II activity (KN-93, 10 μM, 30 min), or autacamtide inhibitory peptide (AIP; 50 μM, 30 min) or their vehicle (VEH) and then exposed to NE (10 μM, 10 min) or its vehicle (V). (A) Actin filaments remained intact in the presence of CaMK-II inhibitors (n=5). (B) KN-93 and AIP blocked NE-induced CaMK-II phosphorylation (upper panel). CaMK-II protein levels are shown in the lower panel (n=3).
Figure 3: Translocation of cPLA2 to the nuclear envelope in response to angiotensin-II (Ang) and ionomycin (ION) in VSMC is lost when actin filaments are disrupted with CD, an inhibitor of actin filament polymerization. VSMC were treated with CD (0.5 μM), or its vehicle (VEH) and exposed to Ang II (100 nM, 10 min), ION (1 μM) or their vehicle (V). cPLA2 translocation to the nuclear envelope and actin filament polymerization was visualized by confocal microscopy (40 X magnification) (n=5).