Novel role of cPLA$_2$ in membrane and actin dynamics

Maarten Moes · Johannes Boonstra · Elsa Regan-Klapisz

Abstract Actin-directed processes such as membrane ruffling and cell migration are regulated by specific signal transduction pathways that become activated by growth factor receptors. The same signaling pathways that lead to modifications in actin dynamics also activate cPLA$_2$. Moreover, arachidonic acid, the product of cPLA$_2$ activity, is involved in regulation of actin dynamics. Therefore, it was investigated whether cPLA$_2$ plays a role in actin dynamics, more specifically during growth factor-induced membrane ruffling and cell migration. Upon stimulation of ruffling and cell migration by growth factors, endogenous cPLA$_2$ and its active phosphorylated form were shown to relocate at protrusions of the cell membrane involved in actin and membrane dynamics. Inhibition of cPLA$_2$ activity with specific inhibitors blocked growth factor-induced membrane and actin dynamics, suggesting an important role for cPLA$_2$ in these processes.

Keywords cPLA$_2$ · Actin · Arachidonic acid · Membrane dynamics · Ruffles · Lamellae · Leading edge · Migration

Abbreviations
PLA$_2$ · Phospholipase A$_2$
cPLA$_2$ alpha · Cytosolic PLA$_2$ alpha
HUVECs · Human umbilical vein endothelial cells

Introduction

Actin plays an essential role in a wide variety of dynamic events in mammalian cells, amongst them cell motility, cytokinesis, and cell–cell and cell–matrix interactions [1–4]. Actin dynamics are determined by the activities of a large number of actin-binding proteins. Extracellular stimuli, like growth factors and extracellular matrix components, stimulate intracellular signal transduction cascades that activate actin-binding proteins resulting in changes in local actin dynamics. These changes result in morphological changes like the formation of membrane ruffles and leading edges. In addition to changed actin dynamics upon growth factor addition or cell spreading, many signal transduction components are bound to actin, indicating the mutual relationship [5–7].

An important molecule involved in the regulation of cell metabolism is the unsaturated fatty acid arachidonic acid, which serves as a precursor for leukotriene and prostaglandin synthesis. However, arachidonic acid release has also been related to the regulation of actin dynamics [8]. In HeLa cells, an increase in arachidonic acid production was shown after cell attachment. This results in both actin polymerization and actin bundling via two distinct pathways involving lipoxygenase and cyclooxygenase [9, 10]. These enzymes metabolize arachidonic acid, resulting in the release of lipid second messengers that activate actin-binding proteins and consequently cause a change in actin...
dynamics. Additional to its role in cell spreading and immobilization, arachidonic acid was also shown to stimulate cell motility via the production of prostaglandins by cyclooxygenase. Prostaglandins play a role in actin cytoskeletal regulation and migration in a variety of ways. For example, stimulation of cells by chemokines resulted in an increase of prostaglandins and consequently in an increase of actin polymerization [11]. Moreover, several forms of stimulated migration require arachidonic acid metabolites [12, 13]. In addition, dissolution of adhesion structures like podosomes is dependent on the production of prostaglandins [14]. The role of arachidonic acid-mediated signaling in the regulation of small GTPases that modulate actin dynamics is highly specific for phospholipids carrying arachidonic acid at the sn-2 position and is therefore considered as the rate-limiting enzyme in receptor-mediated arachidonic acid release. cPLA2 requires Ca2+ to translocate from the cytosol to the membrane through its CaLB (calcium-lipid binding) domain, although Ca2+ is not directly involved in catalysis [18]. The activation of cPLA2 results from a phosphorylation by MAP kinase (for review, see [18]). This MAP kinase on its turn is activated by the same signal transduction cascades (growth factors and extracellular matrix components) that lead to modifications in actin dynamics. Therefore, it is tempting to suggest that cPLA2 is one of the key enzymes involved in local actin dynamics that are involved in the formation of membrane ruffles, and in cell motility.

Therefore, we have studied the localization of cPLA2 in fibroblasts during stimulation with platelet-derived growth factor (PDGF). Stimulation with PDGF introduces circular dorsal ruffles and lamellipodia. Here, we show that endogenous cPLA2 and its active, phosphorylated form are shown to localize at leading edges in primary human umbilical vein endothelial cells (HUVECs) and migrating mouse fibroblasts. Interestingly, inhibition of cPLA2 activity with specific inhibitors blocked PDGF-induced membrane and actin dynamics, suggesting a role for cPLA2 in these processes.

Materials and methods

Cell culture

Human umbilical vein endothelial cells were isolated according to Jaffe [19] and used from passage 1 to 4. HUVECs were cultured onto fibronectin-coated surfaces and grown in endothelial basal medium (EBM-2) supplemented with 2% fetal bovine serum (FBS) (Gibco, Paisley, UK), endothelial cell growth supplements (EGM-2) (Cambrex, NJ, USA), and 5 mM t-glutamine (Invitrogen). C3H10T1/2 mouse fibroblasts were cultured in HEPES buffered Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) supplemented with 7.5% FBS and 5 mM t-glutamine. Cells were grown at 37°C (humidified atmosphere, 5% CO2).

HUVECs were seeded on glass coverslips at 10,000 cells/cm², coated with fibronectin and allowed to grow for 2 or more days to obtain sub-confluent monolayers for localization studies and confluent monolayers for the scratch assays.

Fibroblasts were plated on glass coverslips at 12,000 cells/cm² and allowed to grow for 24 h. For PDGF stimulation experiments, cells were subsequently growth factor-starved for 24 h at 37°C and stimulated with 20 ng/ml of PDGF-BB (Upstate, Hampshire, UK). Treatment with cPLA2 inhibitors Pyrrolidine-2 S and C (sPLA2), and the group of cytosolic PLA2s (cPLA2) including sPLA2 and C (sPLA2), the intracellular calcium independent PLA2s (iPLA2), and the group of cytosolic PLA2s (cPLA2) including cPLA2 [17]. Interestingly, cPLA2 is highly specific for phospholipids carrying arachidonic acid at the sn-2 position and is therefore considered as the rate-limiting enzyme in receptor-mediated arachidonic acid release. cPLA2 requires Ca2+ to translocate from the cytosol to the membrane through its CaLB (calcium-lipid binding) domain, although Ca2+ is not directly involved in catalysis [18]. The activation of cPLA2 results from a phosphorylation by MAP kinase (for review, see [18]). This MAP kinase on its turn is activated by the same signal transduction cascades (growth factors and extracellular matrix components) that lead to modifications in actin dynamics. Therefore, it is tempting to suggest that cPLA2 is one of the key enzymes involved in local actin dynamics that are involved in the formation of membrane ruffles, and in cell motility.

Therefore, we have studied the localization of cPLA2 in fibroblasts during stimulation with platelet-derived growth factor (PDGF). Stimulation with PDGF introduces circular dorsal ruffles and lamellipodia. Here, we show that endogenous cPLA2 and its active, phosphorylated form are localized at the cell membrane in response to PDGF stimulation. They localize specifically to areas at the cell membrane that are reshaped by actin during PDGF stimulation. Furthermore, endogenous cPLA2 and its active phosphorylated form are shown to localize at leading edges in primary human umbilical vein endothelial cells (HUVECs) and migrating mouse fibroblasts. Interestingly, inhibition of cPLA2 activity with specific inhibitors blocked PDGF-induced membrane and actin dynamics, suggesting a role for cPLA2 in these processes.
goat-Alexa488 (Molecular Probes) and washed six times PBS containing 0.2% gelatin. In some samples, this was followed by a DAPI staining for 10 min. Finally, cells were mounted in Mowiol-DABCO.

Transfections

Transfections with GFP-cPLA2α were performed using Lipofectin (Invitrogen) according to the manufacturer’s instructions. This construct, kindly provided by Dr. C. Lenoir (Inserm Unit 538, Paris, France), was checked by sequencing.

Scratch assay

HUVECs were seeded on fibronectin-coated glass coverslips at 10,000 cells/cm² (≈10–20% confluency) and allowed to grow for 7 days with regular change of medium. Cells were gently scratched from the substrate using a pipette tip. The remaining cells were washed with culture medium and allowed to reoccupy the wounded area for various time intervals. Mouse C3H10T1/2 fibroblasts were seeded at 20,000 cells/cm² and allowed to grow to confluency before performing the scratch assay as described above.

Acquisition of images

Samples were studied with a Leitz microscope (Orthoplan Flu 043944) equipped with Leitz oil objectives ×40 NA 1.3 and ×63 NA 1.4. Images were acquired using a Leica CCD camera (model DC350F; Leica Microsystems) using Leica Image Manager 50 software. Confocal pictures were obtained using a Zeiss CLSM (Pascal 510) equipped with Zeiss water objectives (×40 NA 1.3 and ×63 NA 1.4). Images were processed with Adobe Photoshop 8.0.

Results

cPLA2α is recruited to PDGF-induced dorsal membrane ruffles and lamellae in C3H10T1/2 fibroblasts

Mouse C3H10T1/2 fibroblasts provide an excellent model cell line to study actin dynamics. When these cells are serum-starved for 24 h, they are spread and exhibit a clear network of actin stress fibers as shown by staining of F-actin with phalloidin (Fig. 1b). Addition of PDGF-BB, (20 ng/ml for 15 min at 37°C), results in the partial disappearance of stress fibers, and the formation of large circular dorsal membrane ruffles, characterized by a ring of F-actin beneath the membrane (Fig. 1e). Successive optical sections obtained by confocal scanning light microscopy confirmed that these ruffles extend from the dorsal plasma membrane (Fig. 1e, h). As reported previously, ruffle formation is dependent upon actin polymerization and is highly transient [20]. Since actin polymerization is induced by growth factors [21] and is dependent on the production of arachidonic acid metabolites [8, 9], it is tempting to suggest that cPLA2α plays a role in actin dynamics, because cPLA2α is also activated by growth factors [22–24]. Therefore, the subcellular localization of endogenous cPLA2α was determined upon PDGF-induced changes in actin polymerization. Cells were labeled with an antibody specific for cPLA2α as characterized previously [25]. As shown in Fig. 1a, in serum-starved fibroblasts, cPLA2α is mainly detected in the perinuclear area and throughout the cytoplasm. Upon addition of PDGF-BB to the cells, cPLA2α is recruited to newly formed circular dorsal ruffles (Fig. 1d, g) as demonstrated by the colocalization of cPLA2α and PDGF-induced F-actin structures (Fig. 1f, i). This result indicates that upon growth factor stimulation, cPLA2α is rapidly and specifically recruited at areas of actin polymerization at the plasma membrane.

In addition to the formation of membrane ruffles, C3H10T1/2 cells also form lateral lamellae upon addition of PDGF (Fig. 1n). Whether the cells form lamellae or circular ruffles is determined by the PDGF concentration used. At concentrations >20 ng/ml, abundant circular ruffling takes place, whereas at concentrations in the range of 1–5 ng/ml, less circular dorsal ruffles occur and more lamellae are formed. The formation of lamellae is related to cell spreading and/or cell motility. As clearly shown in Fig. 1n, treatment of cells with 20 ng/ml PDGF for 10 min at 37°C results in the formation of lamellae characterized by a lateral band of newly formed F-actin (Fig. 1n) in which cPLA2α accumulates (Fig. 1m), while the perinuclear localization of cPLA2α, as observed in untreated cells (Fig. 1j), disappeared. Together these data clearly demonstrate that PDGF-induced actin remodeling is accompanied by a recruitment of cPLA2α to sites of new actin polymerization and membrane dynamics.

cPLA2α is localized at leading edges of migrating mouse fibroblasts

To determine whether the recruitment of cPLA2α at sites of newly formed F-actin represents a feature common to other cellular processes that depend on actin polymerization, the localization of cPLA2α was determined in the context of cell migration. We performed a commonly used wound-healing assay, also called scratch assay. In this assay, a confluent monolayer is gently scratched with a pipette tip to introduce a wound. Cells located at the border of the wound respond by polarizing towards the wound, emit protrusions, and start to migrate into the wounded area which is finally healed. Directly after scratching
monolayers of C3H10T1/2, cPLA2a was mainly cytoplasmic and perinuclear in cells lining the wound (Fig. 2b). Seven hours after the scratch, cells located at the border of the wound displayed protrusions of the plasma membrane in which cPLA2a was detected (Fig. 2e, f). This membrane localization was restricted to the leading edge of the
migrating cells. These edges are enriched in F-actin (Fig. 2d) and in ARP 3 indicating active actin remodeling (data not shown). At later time points, cPLA$_2$ was detected at leading edges of cells that had migrated into the wound. In conclusion, these data show that in migrating cells, endogenous cPLA$_2$ is recruited to the leading edges of migrating cells, i.e. at sites of active actin remodeling.

cPLA$_2$ is localized at leading edges of migrating primary human endothelial cells

To determine whether the recruitment of cPLA$_2$ at sites of actin polymerization as observed in fibroblasts is a conserved phenomenon, the relationship between cPLA$_2$ and F-actin was studied in primary endothelial cells of human origin, i.e. HUVECs. It has been demonstrated that cPLA$_2$ translocates to the Golgi complex in response to confluency in HUVECs [25, 26]. A scratch assay was performed on a confluent monolayer of HUVECs and the localization of cPLA$_2$ and F-actin was examined. Immediately after scratch, cPLA$_2$ was localized at the Golgi apparatus in all cells (lining the wound or not) (Fig. 2h). Four hours after the scratch, cells located at the border of the wound displayed protrusions of the plasma membrane in which cPLA$_2$ was detected and where cPLA$_2$ locally co-localized with F-actin (data not shown).
shown). Subsequently migrating cells reoccupy the wounded area. Twenty-two hours after scratch, migrating cells filled up the wounded area (Fig. 2j, k, l) and displayed cPLA$_2^a$ staining at the cell membrane (Fig. 2k) and leading edge (Fig. 2k insert), where it locally colocalizes with F-actin. In contrast to confluent cells (Fig. 2h), in the wounds area, cPLA$_2^a$ is localized primarily in the cytoplasm and also at the cell periphery (Fig. 2k). This latter localization may both be related to cell spreading and cell migration.

To determine whether the recruitment of cPLA$_2^a$ to leading edges of migrating HUVECs was not solely occurring in the context of the scratch assay, the localization of cPLA$_2^a$ was compared in non-confluent versus confluent cultures. In contrast to confluent cultures, in which HUVECs are growth-arrested by contact inhibition and are unable to migrate, cells in non-confluent cultures do proliferate and migrate. Both migration and cell proliferation are dependent on actin polymerization, and therefore non-confluent cells display numerous and extended membrane areas where actin polymerization takes place. In confluent HUVECs, cPLA$_2^a$ is located at the Golgi and does not co-localize with F-actin (Fig. 3c). In contrast, in non-confluent cells that display a migratory phenotype, cPLA$_2^a$ is present at the cell periphery in restricted membrane areas which correspond to sites of actin polymerization (Fig. 3b, d).

The goat anti-cPLA$_2^a$ antibody used in our study is specific for cPLA$_2^a$ (confirmed by western blotting of HUVEC and C3H10T1/2 lysates (data not shown and [26–28]). However, to further confirm the presence of cPLA$_2^a$ at the cell membrane, we examined the subcellular localization of GFP-tagged human cPLA$_2^a$ in transiently transfected HUVECs. In non-confluent cells, GFP-cPLA$_2^a$ was localized at the cell membrane, in addition to its cytoplasmic and perinuclear localization (Fig. 4d, f). This confirms our data on the localization of endogenous cPLA$_2^a$ at the cell membrane in leading edges. Additionally, GFP-cPLA$_2^a$ was detected at the Golgi apparatus of confluent HUVECs (Fig. 4a), showing that GFP-cPLA$_2^a$ exhibits the same translocation property as endogenous cPLA$_2^a$. Finally, GFP-cPLA$_2^a$ was recognized by the goat anti-cPLA$_2$ antibody (Fig. 4b, c).

Phosphorylated cPLA$_2^a$ is present in circular dorsal ruffles and leading edges

In view of our data demonstrating the recruitment of cPLA$_2^a$ at sites of actin remodeling and the possible involvement of the arachidonic acid pathway in actin remodeling, it is tempting to suggest that the enzymatic activity of cPLA$_2^a$ is important for this process. Since phosphorylation of cPLA$_2^a$ at serine residue 505 by mitogen-activated protein kinases (MAPK) is involved in cPLA$_2^a$ activation [29], we studied the localization of phosphorylated cPLA$_2^a$ using an antibody specific for Ser505-phosphorylated cPLA$_2^a$. In cultures of serum-starved mouse fibroblasts, phospho-S505-cPLA$_2^a$ was detected in the cytoplasm and in the nucleus of most cells (Fig. 5a). After stimulation with PDGF for 15 min, phospho-S505-cPLA$_2^a$ was found in circular dorsal ruffles (Fig. 5d). Additionally, phospho-S505-cPLA$_2^a$ was detected at leading edges of migrating mouse fibroblasts (Fig. 5g) and of migrating HUVECs (Fig. 5h). In conclusion, cPLA$_2^a$ and phospho-S505-cPLA$_2^a$ were found at protrusions of the cell membrane where actin remodeling takes place, indicating that cPLA$_2^a$ activity plays an active role in this process.
Inhibition of cPLA₂ enzymatic activity prevents the formation of PDGF-induced circular ruffles and lamellae.

To determine whether the enzymatic activity of cPLA₂ is required for actin remodeling, we used the cPLA₂ inhibitors Pyrrolidine-2 and Wyeth-1 characterized previously [30]. These non-structurally related inhibitors do not interfere with the enzymatic activity of calcium-independent PLA₂ (iPLA₂) or secreted PLA₂s (sPLA₂s) [30]. Serum-starved mouse fibroblasts were pre-treated with Pyrrolidine-2 or Wyeth-1 or mock-treated for 45 min. Pretreatment of cells with these inhibitors did not affect cellular morphology, nor did it affect the localization of cPLA₂ compared to mock-treated cells (Fig. 6a, g). Subsequently, cells were treated for 15 min with 20 ng/ml PDGF-BB, they were fixed and F-actin was stained with phalloidin in parallel to cPLA₂ immunodetection. Interestingly, when cells were pre-treated with either inhibitor, PDGF treatment did not induce circular dorsal ruffles nor lamellae (Fig. 6g–l), whereas in mock-treated cells, lamellae (data not shown) and dorsal ruffles containing cPLA₂ were induced by PDGF (Fig. 6d–f, arrowheads). These data show that inhibition of cPLA₂ enzymatic activity prevents PDGF-induced actin remodeling.

Discussion

Arachidonic acid release has been demonstrated to play a role in actin polymerization. Since activation of cPLA₂ results in the preferential release of arachidonic acid, it is tempting to suggest that cPLA₂ is a key enzyme involved in the regulation of local actin dynamics. Therefore, we have studied the localization of cPLA₂ in fibroblasts and HUVECs in relation to induced actin dynamics. Endogenous cPLA₂ was shown to localize at the plasma membrane in both migrating mouse fibroblasts and migrating HUVEC. Moreover, the phosphorylated form of cPLA₂ (on Ser505) was found at the plasma membrane in both cell types. Both cPLA₂ and phospho-S505-cPLA₂ localize at protrusions of the cell membrane that show active actin and membrane remodeling such as circular dorsal ruffles, lamellipodia, and leading edges. Interfering with the activity of cPLA₂ in mouse fibroblasts resulted in the blocking of PDGF-induced actin and membrane dynamics.

cPLA₂ is present at the cell membrane in a spatio-temporal manner. Upon PDGF stimulation, cPLA₂ docks at specific sites at the cell membrane, namely at lamellipodia and ruffles. Upon stimulation of migration, cPLA₂ docks at the leading edge of motile cells. Also here, the localization of cPLA₂ at the cell membrane is local and temporal. This indicates that its docking at the cell membrane is tightly regulated. It was suggested that cPLA₂ interacts with membranes via PtdIns(4,5)P₂. It was shown that PtdIns(4,5)P₂ stimulates cPLA₂ activity independent of calcium suggesting an interaction between PtdIns(4,5)P₂ and cPLA₂ allowing cPLA₂ to reach its substrate at membranes [31]. PtdIns(4,5)P₂ is primarily located in the cytoplasmic leaflet of the plasma membrane. Interestingly, it has been demonstrated that a variety of signal transduction proteins, including growth factor receptors, reside in areas of high actin polymerization,
such as membrane ruffles [7]. Receptor activation can increase or decrease local PtdIns(4,5)P$_2$ concentrations via the local activation of kinases and phosphatases like PI5K, phospholipase C, phospholipase D, and PI3K [32]. By doing so, receptor activation might also regulate the docking of cPLA$_2$ at the cell membrane upon growth factor stimulation. Interestingly PtdIns(4,5)P$_2$ was shown to be enriched locally at the leading edge [33] and phosphatidylinositol metabolism was shown to be involved in ruffle formation [34–36].

The local presence of cPLA$_2$ and its phosphorylated active form at sites of actin and membrane remodeling indicates a role for cPLA$_2$ in regulating membrane dynamics. This is also suggested by blocking of PDGF-induced actin
and membrane dynamics after exposure to the cPLA\textsubscript{2}\textalpha\ inhibitors Pyrrolidine-2 and Wyeth-1. Of note, these inhibitors also block the enzymatic activity of another cPLA\textsubscript{2} from group IV, namely cPLA\textsubscript{2}\textgamma\ [30]. This enzyme can release arachidonic acid from membrane phospholipids but, contrary to cPLA\textsubscript{2}\textalpha, it does not have specificity for arachidonic acid versus other fatty acids [30]. However, in C3H10T1/2, cPLA\textsubscript{2}\textgamma is not expressed, as confirmed by RT-PCR (our unpublished data). This observation strengthens our inhibitors-based results, reinforcing the idea that cPLA\textsubscript{2}\textalpha plays a major role in regulating actin dynamics.

The local presence of the active form of cPLA\textsubscript{2}\textalpha might indicate a local production of arachidonic acid by cPLA\textsubscript{2}\textalpha. Subsequently, arachidonic acid might affect locally the behavior of actin via arachidonic metabolites. Arachidonic acid release has been previously related to the behavior of actin. During cell spreading, actin is changing the cytoskeleton and thereby changing the cell morphology. In HeLa cells, an increase in arachidonic acid production was shown after cell attachment. This results in both actin polymerization and actin bundling via two distinct pathways involving lipoxygenase and cyclooxygenase [9, 10].
Lipoxygenase metabolizes arachidonic acid and via a cascade of lipid second messengers protein kinase C epsilon (PKCε) is subsequently activated which triggers actin polymerization leading to cell spreading. Cyclooxygenase generates prostaglandins resulting in the activation of cyclic AMP-dependent protein kinase A (PKA) that induces actin bundling. Moreover, PKA was shown to play a role in the actin cytoskeleton regulation in a variety of ways (for review, see [37]).

So, an increase of arachidonic acid causes an increase in actin polymerization and bundling during cell spreading by activating actin-binding proteins like PKA and PKCε. Furthermore, arachidonic acid and some of its metabolites can activate signaling pathways leading to the activation of small GTPases such as Rac1 and Rho [15, 16], ultimately resulting in actin remodeling. It has long been known that arachidonic acid and some of its metabolites can bind directly to and regulate the activity of specific GTPase-activating proteins (GAPs), which are regulators of small GTPases [38, 39]. Altogether, it is tempting to suggest that the local production of arachidonic acid and some of its metabolites could trigger signaling pathways resulting in the local remodeling of actin.

In addition, cPLA2α is able to change the local characteristics of the plasma membrane by interacting with membrane phospholipids. cPLA2α-mediated hydrolysis of cellular membranes results in the production of lysophospholipids within the lipid bilayer and the lysophospholipids thus produced change the plasma membrane microviscosity. A change in the plasma membrane microviscosity by lysophospholipids was shown to affect cell migration [40]. Plasma membrane microviscosity has an important role on actin dynamics and thereby regulates cell motility [40, 41]. It was suggested that an increase in the plasma membrane microviscosity in lamellipodia might result in a local increase of actin-directed movement [41]. Our results show active cPLA2α at sites of active actin remodeling, and cPLA2α might here locally direct the behavior of actin by changing the plasma membrane microviscosity.

Altogether, our data show that cPLA2α is specifically recruited at sites of actin and membrane remodeling in different cellular processes. Since this phenomenon occurs in mouse fibroblasts and in primary human endothelial cells, it is likely to represent a general property of cPLA2α. However, further research is required to fully elucidate the role of cPLA2α in the above-mentioned processes.

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