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α_1-Adrenergic Receptor-Induced Cytoskeletal Organization and Cell Motility in CCL39 Fibroblasts Requires Phospholipase D1

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ABSTRACT

The role of phospholipase D (PLD) in cytoskeletal reorganization, ERK activation, and migration is well established. Both isoforms of PLD (PLD1 and PLD2) can independently activate stress fiber formation and increase ERK phosphorylation. However, the isoform's specificity, upstream activators, and downstream targets of PLD that coordinate this process are less well understood. This study explores the role of α_1-adrenergic receptor stimulation and its effect on PLD activity. We demonstrate that PLD1 activators, RhoA, and PKCα are critical for stress fiber formation and ERK activation, and enhance the production of phosphatidic acid (PA) upon phenylephrine addition. Ectopic expression of dominant negative PLD1 and not PLD2 blocks ERK activation, inhibits stress fiber formation, and reduces cell motility in CCL39 fibroblasts. Furthermore, we demonstrate the mechanism for PLD1 activation of ERK involves Ras. This work indicates that PLD1 plays a novel role mediating growth factor and cell motility events in α_1-adrenergic receptor-activated cells. J. Cell. Biochem. 112: 3025–3034, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: PHOSPHOLIPASE D; PLD1; PLD2; STRESS FIBER; CCL39 FIBROBLAST; CELL MOTILITY; RAS; PROTEIN KINASE C ALPHA; ALPHA ONE ADRENERGIC RECEPTOR; PHENYLEPHRINE; RHOA; MAPK; ERK; RAF

Directed cell migration is critical in the process of wound repair and development, but when disregulated, may lead to invasion and migration. Directed cell migration is regulated by a variety of extracellular cues whose mechanism includes growth factor and small G protein signaling pathways. A key factor influencing cell motility is the reorganization of the cytoskeletal scaffolding including the formation of actin stress fiber. However, the mechanism by which signaling pathways regulate cytoskeletal reorganization and cell migration remains less certain. Using the α_1-adrenergic receptor agonist phenylephrine (PE), we have found evidence that supports a role for α_1-adrenergic receptor and the ERK-growth factor pathway in the regulation of stress fiber formation and directed cell motility in fibroblast cells [Sang et al., 2007; Taves et al., 2008].

Actin stress fibers are bundles of actin filaments that provide contractile force and structure for actively moving cells. The precise role of stress fibers in cellular migration is not universal. For many cell types, the appearance of stress fibers is associated with reduced mobility and increased focal adhesions. There are numerous reports showing that increases in the level of stress fibers correlate to a decreased cell motility and enhanced cell spreading and fixing to the substratum [see Pellegrin and Mellor, 2007 for reviews]. In contrast to these observations, there is a growing number of examples where stress fiber formation is associated with and supports cell motility. Stress fiber assembly at the leading edge of a migrating cell produces structural support for filopodial protrusions and provides a footing for the cell as the fibers connect the leading edge of the cell to focal adhesions [Raftopoulou and Hall, 2004]. Directed cell migration in keratinocyte cells show a direct relationship between cell motility and the formation of stress fibers, while inhibition of the fibers reduce the migration speed and directionality [Jackson et al., 2011]. In another study, transforming growth factor β increased cell motility in wound assays and enhanced the formation of stress fibers in a manner which affected directionality [Morin et al., 2011]. Thus, the view of stress fibers reducing cell motility may not be completely correct and demonstrates the need to understand the regulation of these cellular functions.

For a number of years, phospholipase D (PLD), a lipase that catalyzes the hydrolysis of phosphatidylcholine to phosphatidic acid (PA) has been implicated in the activation of growth factor signaling

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and cell migration events [Lacal, 1997; Rizzo et al., 1999, 2000; Andrensen et al., 2002; Rizzo and Romero, 2002; Rudge and Wakelam, 2009]. Exogenously added PA (generated enzymatically or using cell permeable PA) leads to substantial formation of stress fibers, activates growth factor pathways, increases transformation in soft agar assays, and tumor formation in athymic mice [Buchanan et al., 2005]. Two mammalian isozymes of PLD (PLD1 and PLD2) have been implicated in these processes. In myoblasts, RhoA regulation of PLD1 promoted actin organization [Komati et al., 2005]. Induction of PLD1 activity enhanced the tumor formation and transformation of colorectal cells and increased anchorage-independent growth in fibroblasts [Min et al., 2001]. Yet PLD2 has also been found to be critical to cell motility events. An increase in PA level generated by PLD2 is responsible for cell migration [Gorshkova et al., 2008]. Knockdown of PLD2 blocks growth factor signaling in ovarian cancer cells [Snider et al., 2010], while overexpression of a kinase deficient PLD2 inhibited proliferation in EL4 lymphoma cells [Knoepp et al., 2008]. Thus, while there is some evidence for both isozymes being involved in the regulation of cell growth events, a precise role for PLD1 in signaling cell growth and motility biology remains somewhat ambiguous.

Our previous work has demonstrated that the α_1-adrenergic receptor activates growth factor pathways, stress fiber formation and cell motility in a PLD-dependent manner [Sang et al., 2007; Taves et al., 2008]. Therefore, in the present study we hypothesize that a specific PLD isoform mediates α_1-adrenergic receptor signaling in Chinese hamster lung fibroblasts (CCL39 fibroblasts). Our results support this hypothesis, as we find that PE stimulation enhances directed cell migration and stress fiber formation and that RhoA and PKCα are required for PE-induced generation of PA and the activation of growth factor pathway signaling. Furthermore, we show that PLD1 mediates α_1-adrenergic receptor activation of ERK. Finally, we provide evidence of the mechanism by which PLD activates ERK. Thus, our data identifies a new role for PLD1 in ERK pathway signaling.

MATERIALS AND METHODS

CELL CULTURE AND MATERIALS

CCL39 Chinese hamster lung fibroblasts (American Type Culture Collection, Manassas, VA) were cultured in high glucose DMEM plus 10% fetal bovine serum with 100 U/ml penicillin, 100 μg/ml streptomycin. Cells were cultured onto 35-mm tissue culture dishes and allowed to grow to approximately 70–80% confluence. Cells were growth-arrested for 12 h with high glucose DMEM containing 0.5% serum with antibiotics. One hour prior to agonist treatment, the cells were rinsed with PBS and incubated in serum-free high glucose DMEM without antibiotics. Cells were then treated with specific agonists, inhibitors, or activators as indicated. CCL39 cells were transiently transfected upon reaching 60–70% confluence with Fugene-6 (Roche Biochemical) using serum- and antibiotic-free OptiMEM 1 media (Invitrogen, Carlsbad, CA). Cells were then allowed to incubate overnight at 37°C in a 5% CO_2 incubator. The next morning, 1.5 ml of high glucose DMEM with 0.5% heat-inactivated FBS was added to each dish and the cells returned to the incubator for 24 h. Following the incubation, media were removed and cells incubated with high glucose DMEM with 0.5% FBS. Cells were used for experiments 24 h following transfection.

Dominant negative constructs of phospholipase D1 (DN-PLD1), phospholipase D2 (DN-PLD2), and dominant negative RhoA (DN-RhoA) were kind gifts from Dr. Michael Frohman, Stony Brook University [Sung et al., 1997]. Dominant Negative Ras (DN-Ras) was a gift from Dr. Natalie Ahn, Department of Chemistry and Biochemistry, University of Colorado. Plasmids containing EGFP-RhoA and EGFP-PKCα were purchased from Clonetech Laboratories, Inc. (Mountain View, CA). Dulbecco's modified Eagle's medium with high glucose, 5-*N*(ethyl)-isoproplyamiloride (EIPA), FITC-phalloidin, and PE were purchased from Sigma Chemical Co. (St. Louis, MO). Short chain (dihexanoyl)-phosphatidic acid (sc-PA) was from Avanti Polar Lipids (Alabaster, AL). ERK, PKCα primary antibodies, secondary HRP-linked antibodies and chemiluminescence detection reagent were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). P-ERK antibody was from Cell Signaling Technologies (Beverly, MA). [^{3}H] myristic acid was from Amersham Biosciences (Piscataway, NJ).

WOUND HEALING ASSAY

For ligand-induced wound healing assays, CCL39 cells were seeded into culture dishes and allowed to grow to confluence in high glucose DMEM containing 10% serum and antibiotics. A wound was created using a sterile pipet. Residual cells were removed by washing with PBS. Media and, as indicated, agonist or inhibitors, were replaced at 12 h. Migration was measured in the presence of the indicated agents in high glucose DMEM using grids as a reference mark. Micrographs were taken with an Olympus IX 70 equipped with an Orca CCD camera in phase contrast mode at 100× magnification. The media were replaced following measurement. For analysis, each wound was measured at five locations each at three individual fields.

STRESS FIBER FORMATION DETERMINATION

Treated and control cells grown on glass coverslips were fixed with 3% paraformaldehyde at 4°C for 30 min and permeabilized with 0.4% Triton X-100 for 10 min actin stress fibers were stained with 0.5 μg/ml fluorescein isothiocyanate (FITC)–phalloidin for 60 min. The cells were treated with prolong anti-fade prior to mounting the glass slides and analyzed on an inverted Olympus IX70 microscope in fluorescence mode at 100× magnification. Cells displaying significant and strong stress fibers (well formed and prominent fibers which are organized and stretched through the majority of the cell) and weak stress fibers (thin less chorded stress fibers which extend through most of the cell) were counted in five random fields for each slide. Percent of cells displaying stress fibers was determined as the total number of cells with stress fibers versus the total number of cells observed in the observed fields.

PLD ASSAY

In vivo PLD activity assay was performed using the transphosphatidylation assay with metabolically labeled cells). [^{3}H] myristic acid (2 μCi/ml [9,10-^{3}H] myristic acid, American Radiolabelled Chemicals, Inc.) was added to the cell culture media for 16–18 h prior to agonist stimulation. One hour prior to treatment, unincorporated [^{3}H] myristic acid was removed by washing with PBS and the cells

were incubated in serum-free high-glucose DMEM medium. For the final 15 min of preincubation, 0.3% 1-butanol was included. The cells were harvested following treatment with or without agonists in the presence of 0.3% 1-butanol. Lipids were extracted using an acidified chloroform methanol extraction and resolved by thin-layer chromatography. Standards for PA and phosphatidylbutanol (PtdBut) were visualized with iodine and bands co-migrating with the authentic standards were scraped into scintillation vials. PLD activity was determined as the incorporation of radioactivity into PtdBut and expressed as a percentage of total lipid recovered.

PKC TRANSLOCATION

Following agonist treatment for 15 min, cells were washed twice with ice-cold phosphate-buffered saline and then scraped in 500 μl of homogenization buffer (20 mM Tris–HCl, pH 7.5, 10 mM EGTA, pH 7.4, 2 mM EDTA, pH 7.4, 250 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, and 0.2% (w/v) leupeptin). The cells were then sonicated twice for 10 s each and centrifuged in a TL-100.3 rotor at 40,000 rpm for 40 min at 4°C. The supernatant (cytosol) was removed and solubilized with an equal volume of 2× sample buffer. The membrane pellet was resuspended in an equal volume of homogenization buffer and mixed 1:1 with 2× sample buffer. All fractions were boiled for 5 min and analyzed by Western blot analysis.

RhoA AND RAS ACTIVATION ASSAY

G-Protein activation was determined by a pull-down assay using either GST-Raf1 Ras-binding domain or GST-Rhotekin (CellBio Labs, San Diego, CA). CCL39 cells were cultured in cell culture dishes and starved in serum-free medium for 12 h and then exposed to agonist and/or inhibitors as indicated, quickly washed with ice-cold Tris-buffered saline, and lysed in 500 μl of lysis buffer containing 20 mM Tris–HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 50 mMβ-glycerophosphate, 10 mM NaF, 1 mMNa_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, and 2 μg/ml leupeptin. Cell lysates were immediately centrifuged at 8,000 rpm at 4°C for 5 min and 50 μg of lysates were incubated with 30 μg GST-fusion protein beads for 1 h at 4°C. The beads were washed with wash buffer, and bound small G-protein was eluted by boiling each sample in Laemmli sample buffer. Eluted samples from the beads and total cell lysate were then electrophoresed on 14% SDS–polyacrylamide gel electrophoresis gels, transferred to nitrocellulose, and analyzed by Western blotting using a monoclonal anti-Ras or RhoA antibody.

RESULTS

The involvement of the α_1-adrenergic receptor in directed cell migration and invasion was shown to require both ERK and the formation of PA [Sang et al., 2007; Taves et al., 2008]. To examine this effect in actively migrating cells, studies were performed in CCL39 fibroblasts treated with PE. Because our earlier work showed that addition of PE to CCL39 cells increased the number of non-confluent cells forming stress fibers and enhanced the rate of CCL39 invasion [Taves et al., 2008], we first investigated the organization of actin cytoskeleton at the leading edge of a wound. At the edge of the wound in either control or PE stimulated cells, all cells showed some level actin stress fibers (Fig. 1A). The stress fibers in non-stimulated cells were randomly oriented at the wound edge with little cell movement into the wound. This was in contrast to PE stimulated cells, where cells migrating into the wound edge had a strong stress fiber formation oriented into the migratory direction (Fig. 1A). The fold increase in cells expressing strong stress fibers for PE-treated cells at the edge of the wound (4.38 ± 0.18 SEM) as compared to control cells displaying strong stress fibers was very highly significant ($P < 0.0001$) (Fig. 1C). This is further illustrated when examining the intensity and thickness of the stress fibers seen in cells that were actively migrating within the wound where PE stimulated cells display much more prominent and strong stress fibers than in the non-stimulated control cells (Fig. 1B,C). To quantify stress fiber response, cells at the wound edge or within the wound were scored as containing strong (thick distinct fibers reaching through most of the cell), weak (thin and shortened stress

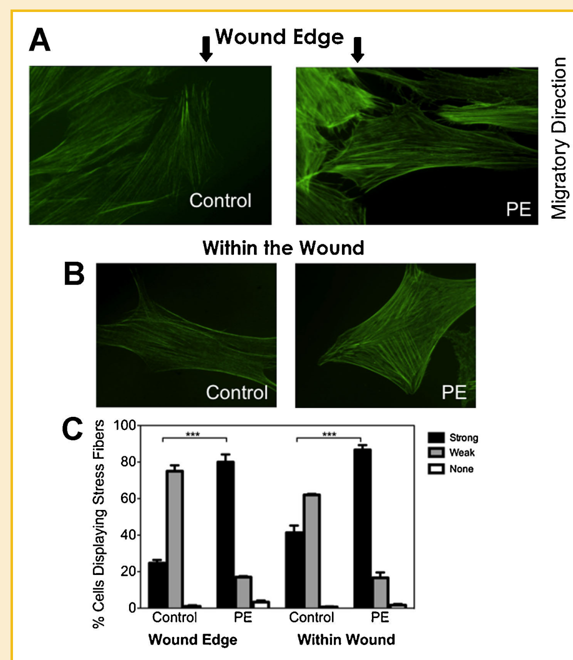

Fig. 1. α_1-Adrenergic receptor stimulation regulates stress fiber formation in directionally motile cells. A confluent monolayer of CCL39 cells were grown in culture dishes and incubated in serum-free medium for 1 h prior to wounding. Cells were treated with either 50 μM PE or vehicle for 24 h with a change of media containing vehicle or agonist at 12 h. Cells were fixed and stained for stress fiber formation using FITC-labeled phalloidin. Representative fluorescence micrograph of CCL39 cells. A: At the edge of the wounded monolayer and (B) of cells migrating into the vacated wound. (C) Quantification of stress fiber formation at the wound's edge and those cells within the wound. Cells with thick organized stress fibers stretching throughout the cell were counted as strong stress fibers while those cells with thin-cabled bundles extending through most of the cell were counted as cells displaying weak stress fibers. Five fields were counted for each sample. Values represent averages of three independent assays, and error bars show SEM values ($P < 0.001$). Fold increase in the number of cells with strong stress fibers was determined using a one-tailed unpaired t-test with a 95% confidence interval (***).

fibers) or no stress fibers. As expected, strong stress fiber formation was observed in cells treated with PE compared to control cells (3.90 ± 0.78 SEM; $P < 0.001$) using a one tailed, unpaired t-test with a 95% confidence interval (Fig. 1C).

Cells displaying prominent stress fibers were then assayed in non-confluent conditions to determine the impact of various agonists and inhibitors (Fig. 2). In agreement with previous studies, stress fiber formation was dependent on RhoA, PLD, and PKC (Fig. 2A). Expression of dominant negative RhoA abrogated the ability of PE to stimulate the formation of stress fibers. As seen in other work, pretreatment with a primary alcohol that blocks the formation of PLD-generated PA significantly inhibited PE-mediated stress fiber formation. Because PKCα activation has been linked to formation of actin reorganization, we also examined the effect of treating CCL39 cells with either PMA or inhibiting PKCα prior to PE stimulation. The addition of PMA significantly activated the formation of stress fibers while pretreating cells with the PKCα inhibitor, Ro-31-8220, significantly ($P < 0.001$) blocked the formation of PE-mediated stress fibers (Fig. 2B). The percent of cells displaying stress fiber in RhoA, PLD, and PKC inhibited cells with PE was 4.5- to 5-fold lower than control cells, suggesting a basal control of actin polymerization. However, the inability of PE to induce stress fiber formation in any of these cells indicates an involvement of the adrenergic signaling pathway or that there is a basal level of signaling in resting cells that is lost when these signaling intermediates are inhibited. These results suggest that PE-mediated stress fiber formation in actively migrating cells is directionally coordinated and indicate that actin polymerization requires a signaling pathway involving RhoA, PKCα, and PLD activity.

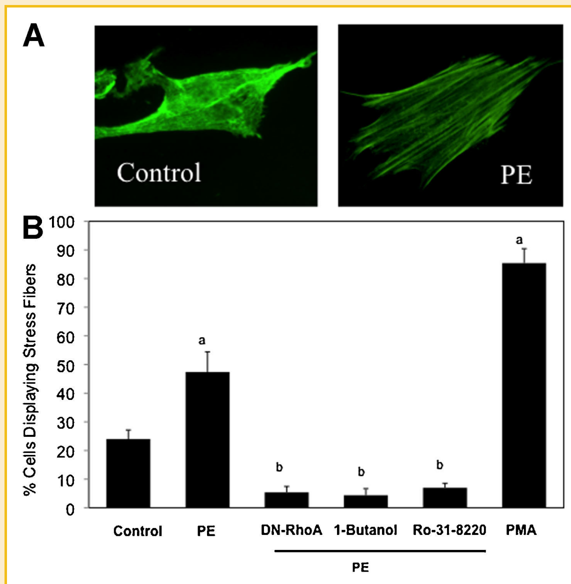

Fig. 2. α_1-Adrenergic receptor-regulated stress fiber formation requires RhoA, PLD, and PKC activity. Cells were grown to 20–30% confluency on glass coverslips and cultured 12–16 h in media containing 0.5% serum. The cells were then deprived of serum for 1 h in high-glucose Dulbecco's modified Eagle's medium media. Fifty micrometers PE was added and cells were incubated at room temperature for 30 min and stained for actin polymerization. Where indicated cells were incubated with either 50 μm PE or 100 nm phorbal-12-myristate-13-acetate (PMA), for 30 min 1 μM Ro-31-8220 or 0.5% 1-butanol was used as indicated to pretreat cells 15 min prior to agonist addition. Cells were transfected with DN-RhoA 24 h prior to determination of actin polymerization. A: Representative micrographs were obtained by using an Olympus IX70 fluorescence microscope. B: Five random fields were counted for each coverslip. The average percent of cells displaying strong stress fiber formation was determined for each field in each experiment. All data are presented as the mean ± SEM. Significance of the difference between means was determined by non-paired, one-way Anova ($P > 0.001$). Data indicated with "a" were significantly different from the control cells, while those marked with "b" were significantly different from the PE agonist-treated cells. Data represent four separate experiments.

To study which isoform of PLD is activated by the α_1-adrenergic receptor, we next investigated the level of PLD activity with its upstream regulators and dominant negative PLD expression. As expected, activation of PKC via phorbal ester addition resulted in high levels of PLD activation as assayed by phosphatidylbutanol (PtdBut) formation (Fig. 3A). Consistent with these results, 1 μM addition of the PKCα inhibitor, Ro-31-8220 abrogated the ability of PE to stimulate phosphatidylbutanol (PtdBut) formation to near basal level. Expression of dominant negative RhoA (DN-RhoA) also significantly blocked the ability of PE to increase PtdBut production (Fig. 3A). Specific analysis of PLD isoforms activated by PE was determined in CCL39 cells transiently transfected with dominant negative PLD (DN-PLD1 or DN-PLD2). DN-PLD is a catalytically inactive mutant that dimerizes with endogenous PLD rendering the native lipase inactive and has been used in a number of cell lines to block isoforms specific PLD activity [Sung et al., 1997; Kam and Exton, 2002; Knoepp et al., 2008]. Cells expressing flag tagged, DN-PLD1 or DN-PLD2 (as determined by Western blot, data not shown) were treated with 50 μM PE for 15 min before analysis for PLD activity. Expression of DN-PLD1 significantly blocked PE-mediated PLD activity (0.5-fold less than PE-treated cells $P < 0.001$) whereas expression of DN-PLD2 expression did not significantly alter the ability of the cells to respond to PE (Fig. 3B). While DN-PLD1 did not fully block the PE-mediated PLD activity, it should be noted that transfection efficiencies of CCL39 fibroblasts using GFP as a reporter typically approach 80%. Thus, the remaining α_1-adrenergic receptor-mediated increase in PLD activity transfected with DN-PLD1 is likely due to non-expressing cells. Taken together, these results suggest that PLD1 but not PLD2 is activated by α_1-adrenergic receptor in CCL39 fibroblasts.

These results implicate PKCα and RhoA in the activation of PLD, which in turn, may activate ERK for stress fiber formation and cell migration. As signaling of α_1-adrenergic receptor in non-cardiac tissue is not well described, we focused on the involvement of PKCα and RhoA in PE-induced ERK activation. Immunoanalysis demonstrated that endogenous PKCα is expressed in CCL39 cells (Fig. 4A). Transient transfection of EGFP-PKCα was monitored by immunofluorescence microscopy, and EGFP-PKCα was capable of intracellular movement to the cell membrane upon treatment with the agonists PE or PMA (Fig. 4B). To further corroborate the activation of PKCα in fibroblasts, the translocation of PKC from cytosol to membranes was determined. Addition of PE or PMA to cultured cells resulted in a pronounced relocation of PKCα from the cytosol to the plasma membrane fraction of the cell (Fig. 4C). In addition, parallel studies were performed to show the involvement of RhoA in

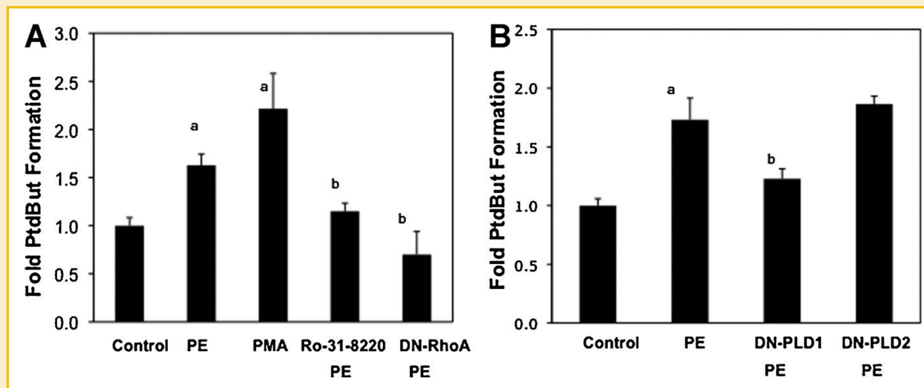

Fig. 3. PtdButanol accumulation is regulated by RhoA, PKCα, and dominant negative PLD1 expression. Quiescent CCL39 cells were incubated overnight with [H3] myristic acid in media containing 0.5% serum, washed with PBS, incubated for 1 h with serum-free Dulbecco's modified Eagle's medium high-glucose media before treatment. Where indicated, the cells were transfected with various constructs for 24 h prior to labeling. PLD assays were terminated with addition of methanol and lipids extracted. Incorporation of radioactivity into the PLD product phosphatidylbutanol (PtdBut) was measured after TLC. Cells were treated with either 50 μm PE, or 100 nm PMA for 30 min. All data are presented as the mean ± SEM ($n \geq 4$) from a representative experiment. Statistical analysis was performed by using non-paired, one-way Anova ($P > 0.001$). Data indicated with "a" were significantly different from the control cells, while those marked with "b" were significantly different from the PE agonist-treated cells.

PE-mediated signaling. Addition of PE or activation of PKC by PMA induced the recruitment of EGFP-RhoA to the membrane. Inhibition of PKC by R0-31-8220 blocked the ability of PE to induce EGFP-RhoA translocation (Fig. 5A). Interestingly, PMA also induced EGFP-RhoA to the membrane indicating that PKC could impact PLD by direct and indirect signaling (Fig. 5A). Additional insight can be obtained by pursuing additional studies using a range of PKC specific inhibitors with both the pull-down and EGFP-RhoA assay approaches. Having established that α_1-adrenergic receptor agonist in CCL39 fibroblasts activates both PKCα and RhoA, the interdependence of both signaling proteins on ERK activation was next investigated. Phorbol ester produced a robust and expected activation of ERK, while 30-min treatment of the PKCα inhibitor Ro-31-8220 resulted in a loss of PE-induced ERK activation (Fig. 4D). Somewhat surprisingly, expression of DN-RhoA reduced the pERK levels after PE stimulation (Fig. 5B). These data imply a signaling relationship between PKCα and RhoA in ERK activation.

To further establish the identity of which PLD isoform is responsible for stress fiber formation and regulation of cell motility in α_1-adrenergic-stimulated fibroblasts, cells were transiently transfected with either DN-PLD1 or DN-PLD2 and assayed for stress fiber formation. Inhibition of PLD1 activity blocked the formation of stress fibers (Fig. 6A,B) and decreased both the distance PE-stimulated cells migrate and the number of cells moving into the vacated space as compared to control conditions (Fig. 6C). Initial work with DN-PLD1 and DN-PLD2 alone showed no significant change (data not shown). Expression of DN-PLD2 did not inhibit the ability of PE to induce actin reorganization (and even slightly increased the numbers of cells displaying stress fibers) but often altered the morphology of the cell to a more rounded appearance (Fig. 6A—lower right panel). The shortened stress fibers were reminiscent of dorsal stress fibers, with one end of the fiber fixed at focal adhesions [Pellegrin and Mellor, 2007]. A second but not further examined effect of DN-PLD2 expression was a slight increase in the motility of un-stimulated cells over control cells (Fig. 6). When taken together with earlier data presented here, this work establishes the role of PLD1 and not PLD2 in regulating cell motility events in α_1-adrenergic receptor-stimulated CCL39 fibroblasts.

While the independent roles of PLD and ERK have been investigated in a number of cell types and with a range of agonists, the mechanism of PLD activation within the ERK signaling pathway has only been described in a few systems. As the product of PLD, PA can bind and activate Raf independent of Ras [Rizzo et al., 2000; Cockcroft, 2001; Huang and Frohman, 2007; Rudge and Wakelam, 2009], we hypothesized that Ras may be dispensable for ERK activation. We observed that expression of DN-Ras was, surprisingly, sufficient to block PE induced ERK activation (Fig. 7A). To confirm that PE-activated Ras and that PLD activity is responsible for the activation, a Ras pull-down activation assay was performed (Fig. 7B). When cells were treated with the α_1-adrenergic agonist, the level of active Ras increased, while addition of soluble, cell-permeable phosphatidic acid (sc-PA) also led to an increase in active Ras (Fig. 7B). We next investigated the identity of the PLD isoform responsible for PE-mediated Ras activation. Ras activity was measured in CCL39 cells treated with the PLD inhibitor, 1-butanol (which inhibits the formation of PA) or in cells transfected with dominant negative PLD1 and PLD2. Reduction of PLD-generated PA by 1-butanol and expression of DN-PLD1 both attenuated PE-induced activation of Ras, whereas expression of DN-PLD2 did not have a significant effect on Ras activation by PE (Fig. 7C). These results demonstrate that PLD1 regulates PE-induced Ras activation in CCL39 fibroblasts.

DISCUSSION

There is considerable evidence for a significant role of PLD activity in cytoskeletal organization, cell migration, and invasion. However, the questions of which PLD isoform is involved and the mechanism by which PLD regulates these processes remain unanswered. Our previous studies have demonstrated PLD activity is crucial in ERK activation, stress fiber formation and cell motility in CCL39

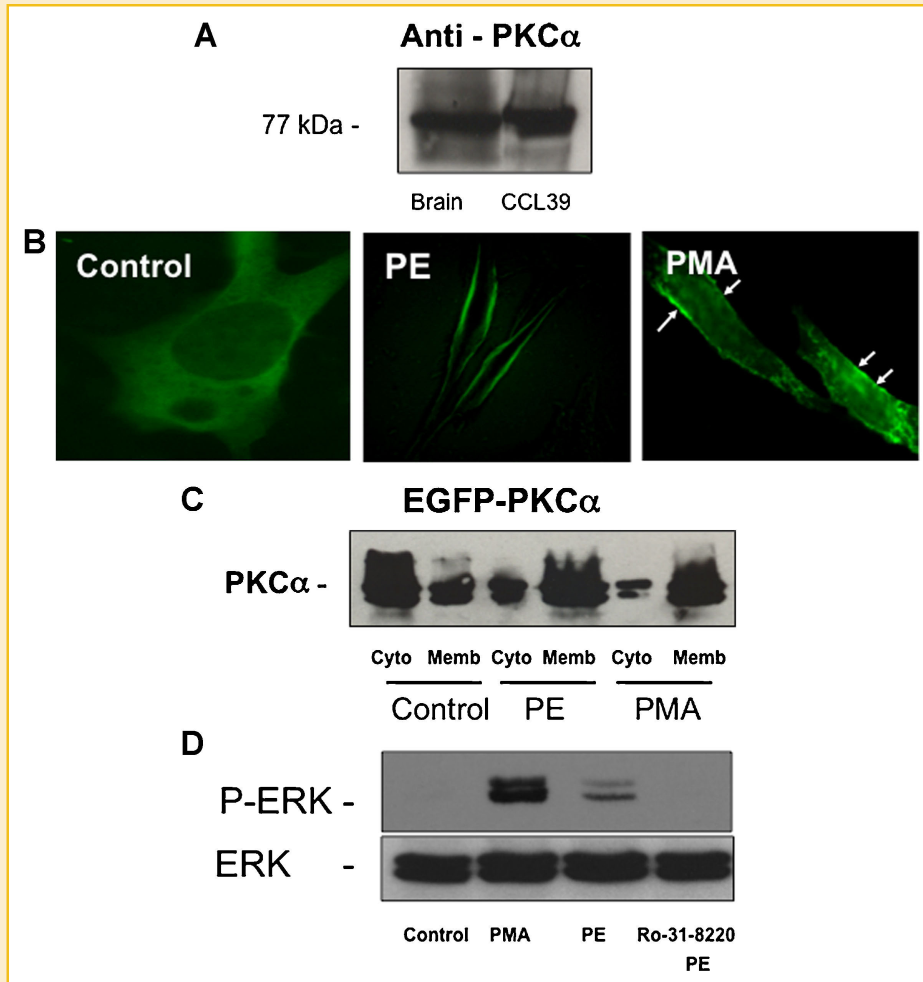

Fig. 4. Effect of PE stimulation on PKCα and ERK activation. A: Protein kinase Cα expression in CCL39 fibroblasts was determined by Western blot of 15 μg of lysate. Rat brain lysate (15 μg) was used as a blotting control. B: CCL39 cells transfected with GFP-PKCα made quiescent in serum-deprived cells were treated with either 50 μm PE, or 100 nm PMA for 15 min. After treatment, cells were fixed and analyzed by fluorescence microscopy. C: Cells were treated with PE or PMA for 15 min, lysed and membrane fraction separated from soluble, cytosolic fraction by centrifugation. Equal mass (20 μg of total protein) of each fraction was subjected to Western blot analysis to determine PKCα translocation. D: ERK activation was determined in quiescent, serum-deprived cells incubated with the indicated agonist or for 15 min. Following treatment, cells were rinsed with PBS and lysed in Laemmli sample buffer. A total of 20 μg of each sample was resolved on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and the phosphorylation state of ERK was determined by immunoanalysis. Data are representative of four separate experiments.

fibroblasts [Sang et al., 2007; Taves et al., 2008]. In this study we found that PE-activated signaling pathways upstream of PLD1 was associated with stress fiber formation and ERK activation. Dominant negative PLD1, and not PLD2, blocked PE-mediated PLD activity, cytoskeletal reorganization, and directed cell migration. The activation of ERK was Ras dependent requiring PLD1 activity. The results from this study reveal the influence of PLD1 as an essential intermediate in the growth factor pathway regulation and stress fiber formation in response to α_1-adreneric receptor activation.

Agonist-induced activation of PLD activity is found in most mammalian cells. Nearly all animal cells express both PLD1 and PLD2 isoforms [Exton, 2002; Foster and Xu, 2003; Huang and Frohman, 2007; Mansfeld and Ulbrich-Hofmann, 2009]. In earlier work we found that α_1-adreneric receptor activation of CCL39 fibroblasts resulted in the generation of PA and the regulation of a number of signaling intermediates involved with PLD activity including ERK, PLCβ_1, and PLCβ_3 [Provost et al., 2005]. While these previous studies did not identify the PLD isoform associated with α_1-adreneric receptor activation, Gαq activation of PLCβ_1 is enhanced by PA-generated via PLD1 [Litosch, 2009]. This observation indicated a potential role for PLD1 membrane signaling to ERK and cell motility in our studies. PLD2 is often found involved in membrane signaling [Vorland et al., 2008; Rudge and Wakelam, 2009] and there is growing evidence that PLD1 can be found at the plasma membrane and is involved in GPCR and growth factor signaling [Han et al., 2007; Zhao et al., 2007; Vorland et al., 2008]. As both RhoA and PKCα are stimulated by PE, and are regulators of PLD1, and because PLD activity is critical for PE-stimulation of ERK and stress fiber formation in CCL39 cells [Sang et al., 2007], we next tested the specific requirement for both RhoA and PKC in the activation of ERK and PLD activity. The assessment of PLD1

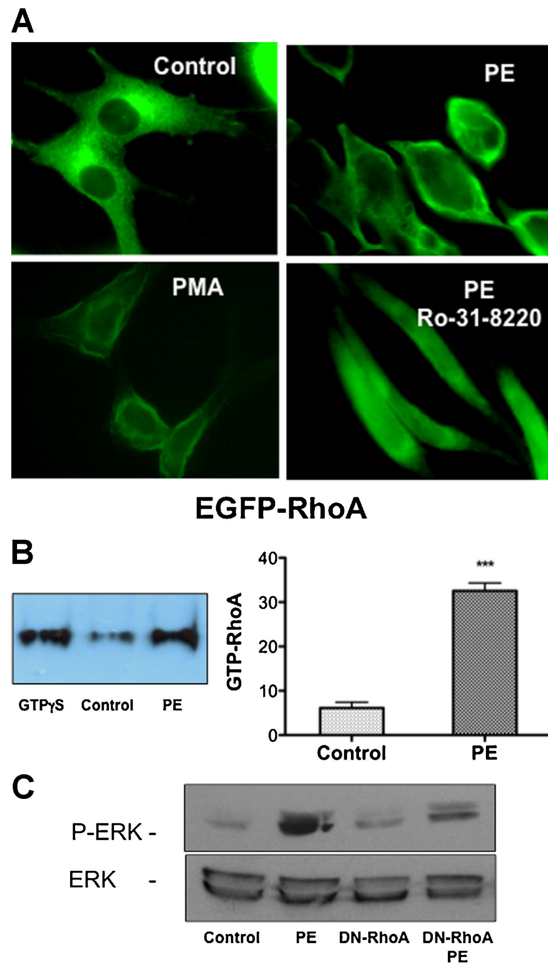

Fig. 5. RhoA activation by α_1-adrenergic receptor stimulation is required for phosphorylation of ERK. A: EGFP-RhoA was transfected in CCL39 cells and, as indicated, incubated with 1 μM Ro-31-8220 15 min prior to stimulation. Cells were then treated with 50 μM PE or 100 nm PMA for 15 min, fixed and analyzed by fluorescence microscopy. B: ERK activation in control (mock transfected) or DN-RhoA transfected cells was determined following PE stimulation. Cell lysates from control and treated cells were separated by SDS-PAGE, probed with anti-phosphoERK and total ERK antibodies. Representative fluorescence micrographs and Western blots are from three independent experiments.

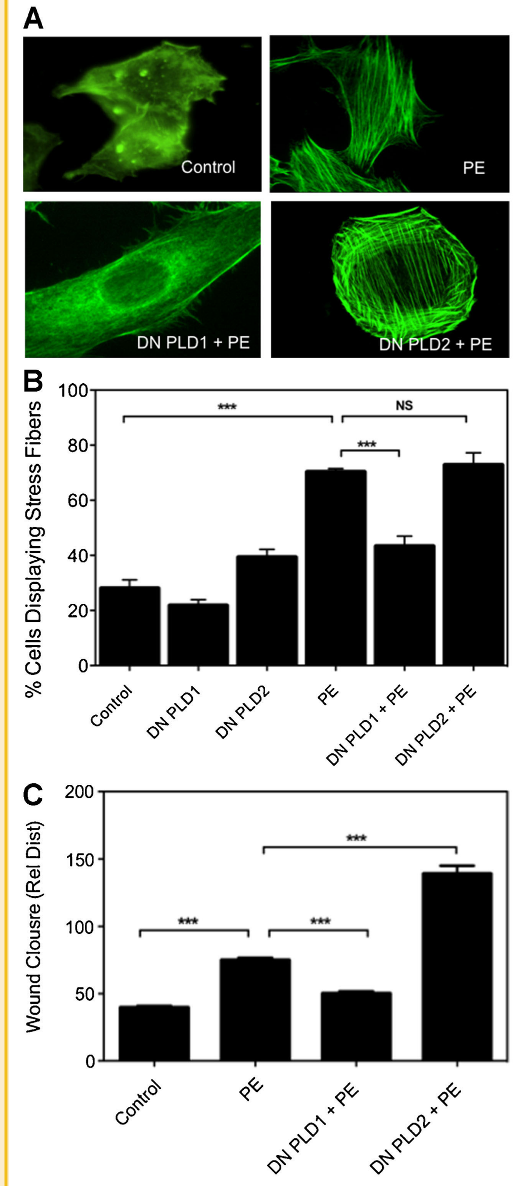

Fig. 6. Expression of dominate negative PLD isoforms on stress fiber formation and cell motility. A: CCL39 fibroblasts grown to 20–30% confluency on glass coverslips were transfected with DN-PLD1 or DN-PLD2 before being made quiescent in medium containing 0.5% serum. Cells were incubated with 50 μm PE for 30 min and stained for actin polymerization. Representative images were obtained by using an Olympus IX70 fluorescence microscope. B: Five random fields were counted for each coverslip. The average percent of cells displaying strong stress fiber formation was determined for each field in each experiment. C: Forty-eight hours post-transfection, a wound was created in a confluent monolayer of CCL39 fibroblasts by scraping away half of the cells. Cell debris was removed by rinsing and the cells were incubated for 24 h without (control) or with 50-μm PE. Images were taken at the initiation of the experiment ($t = 0$) and after 24 h ($t = 24$). Movement of cells into the scarred region was quantified by measuring the migration distance at five locations per wound and is displayed graphically. All data are presented as the mean ± SEM. Significance of the difference between means was determined by non-paired, one-way Anova ($P > 0.001$). NS = not statistically significant at a 99% confidence interval. Data represent three separate experiments.

activation was performed using two complementary approaches. The first was to provide evidence suggesting the involvement of well-known PLD1 regulators, RhoA and PKC. The potential impact of PKC activation of RhoA as observed in Figure 5A suggests that PKC could activate both RhoA [likely by phosphorylation of P115RhoGEF, Peng et al., 2011] and by direct interaction with PLD1. Both signaling components are activated by addition of PE and their activity was dependent on and required for PLD activity and the ERK phosphorylation. These results highlight the dependence of PLD1 activation in ERK and stress fiber formation. The second approach to identify which PLD isoform is involved in PE signaling utilized dominant negative PLD1 and PLD2 expression. In the present study, dominant negative PLD isoforms were effectively utilized to block

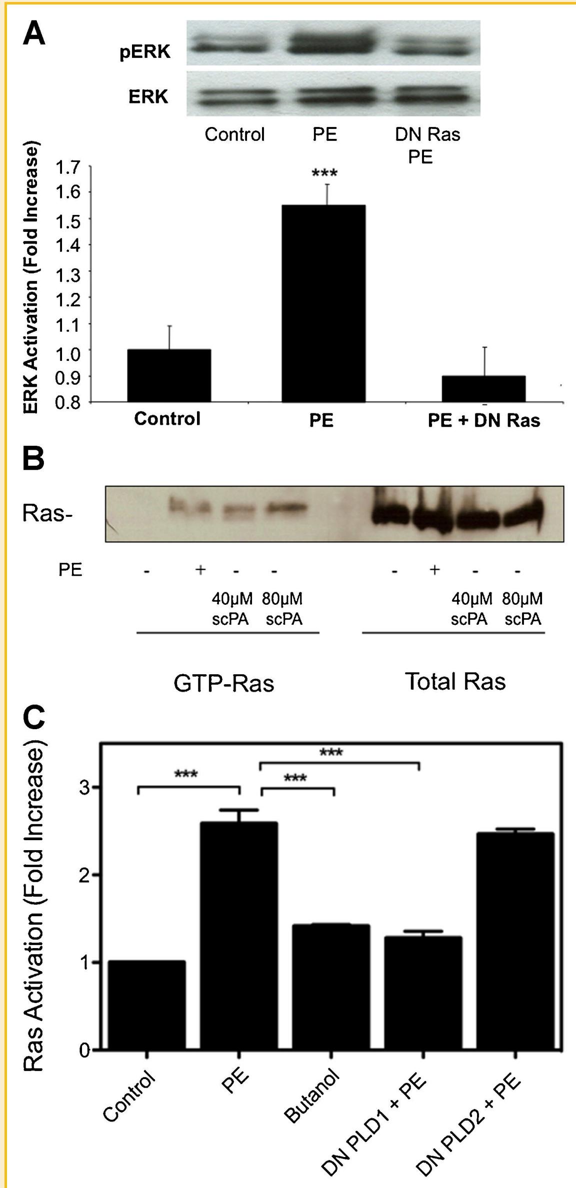

Fig. 7. PE-mediated PLD signaling to ERK involves Ras activation. A: Cell lysates were prepared in serum-starved control and DN-Ras transfected cells after 50 μM PE stimulation for 15 min where indicated. PhosphoERK was determined by immunoblotting, the blot stripped and analyzed for ERK levels using anti-ERK antibodies. ERK activation is expressed as the relative intensities of pERK/total ERK ratio quantitated by densitometry. Data are expressed as fold increase over basal control and are means ± SEM measured from four independent experiments. Statistical analysis was performed by using non-paired, one-way Anova. ***Significant difference from control ($P > 0.001$). B: Serum-starved cells were incubated with 40 or 80 μm of the short chain-lipid permeable dihexanoyl phosphatidic acid (sc-PA) or 50 μM PE for 15 min Ras activation was estimated with the pull-down assay. The total Ras in cell lysates is also shown. Results shown are representative of three separate experiments. C: PE (50 μM) induced Ras activation in 0.5% butanol, DN-PLD1 or DN-PLD2-treated cells were measured using the Ras pull down assay. The degree of Ras activation was determined using densitometric analysis of the ratio of total Ras and Ras-GTP. Data are mean ± SEM from four separate experiments. Significance of the difference between means as shown by the connecting bars above each bar graph was determined by non-paired, one-way Anova ($P > 0.001$).

specific PLD isoform activity in this work (Fig. 3B), which reduced PE-mediated induction of stress fiber formation (Fig. 6B) and cell motility (Fig. 6C). Dominant negative PLD constructs have been used by others to identify PLD1 and PLD2 signaling [Vitale et al., 2001; Zhao et al., 2007; Garrido et al., 2009; Snider et al., 2010]. While utilizing RNA-interfering technology would be a useful tool to examine PLD in this work, the sequence for PLD2 has not been published for the Cricetulus griseus genome. Most work with siRNA with PLD has been performed with human cell lines and there the difference in base pair sequences between human and other species (>90% between human and hamster PLD1) would make it difficult to generate functional PLD knockdowns of both isotypes. Collectively these data suggest that PLD1 activity is required for the α_1-adreneric receptor stimulation of cell motility events.

Stress fibers are found along the base of the cell fixed at each end to focal adhesions and in migrating cells are typically oriented parallel to the direction of motility [Zimerman et al., 2004; Pellegrin and Mellor, 2007]. This relationship between cell migration into wound areas and stress fiber formation is in agreement with the changes seen in stimulated epithelial cells [Morin et al., 2011] and in Hep3B cells where upstream regulation of stress fiber formation is concomitant with increases in cell motility [Leung et al., 2010]. We observed that, compared to non-stimulated control cells, cells treated with α_1-adreneric receptor agonist actively moved into a wound and displayed a higher level of cytoskeletal organization (Fig. 1A). These cells also migrated further into the wound (Fig. 5C) than control, non-stimulated cells. While it is not clear from these studies whether these stress fibers stimulated by PE in a PLD-dependent manner are truly ventral or a combination of dorsal stress fibers cross-linked to traverse arcing actin fibers, the dually fixed, cell-length stress fibers can provide contractile force for migrating cells [Hotulainen and Lappalainen, 2006]. Evidence supports that both PLD1 and PLD2 play a significant role in cell motility. Both PLD1 and PLD2 knockdown cells display poor cell spreading and stress fiber formation [Chae et al., 2008]. However, loss of PLD1 expression did not inhibit the serum-induced formation of stress fibers in CHO cells, indicating a role for PLD2 in this phenomenon [Su et al., 2006]. We find here that inhibition of PLD1 and not PLD2 blocked nearly all PE-induced stress fiber formation and significantly decreased the distance these cells migrated and the total number of cells able to migrate into vacated space in response to PE stimulation (Fig. 5C). The necessity of PLD1 in cell motility presented here is in agreement with the PLD1-dependent neurite outgrowth in growth factor-stimulated H19-7 cells [Oh et al., 2007], in L6 myogenic cells where PLD1 localizes to actin structures and is responsible for the stress fiber formation [Komati et al., 2005], and in HEK-293 cells stimulated with carbachol where PLD1 activity, under the control of cofillin, was found to be directly responsible for the formation of stress fibers [Han et al., 2007].

The activation of growth factor pathway signaling by PLD generated PA has been identified in a number of studies [Zhang and Du, 2009]. While there are several putative PA binding proteins, the binding and activation of Raf by PA is demonstrated to be the primary means by which PLD activates growth factor pathways [Daub et al., 1997; Della Rocca et al., 1999; Rizzo et al., 1999, 2000; Kranenburg and Moolenaar, 2001]. Thus, it was surprising to find

that dominant negative Ras inhibited ERK activation (Fig. 7A). This finding was supported by evidence that the addition of exogenously added cell-permeable PA increased the level of GTP-Ras (Fig. 7B). Our earlier work showed that short chain PA-activated ERK and stress fiber formation. Similar activation of Ras by PLD2, but not PLD1, was identified in activated lymphocytes [Mor et al., 2007]. The mechanism for an upstream-Raf activation of ERK was identified as the Ras activator, SOS [Zhao et al., 2007]. Moreover, it was found that the PH domain of SOS was able to directly bind to PA and recruit SOS to the membrane, leading to Ras activation and demonstrating that PLD2 was responsible for the PA-mediated activation of SOS and Ras. While the work performed in activated lymphocytes certainly supports our findings, the requirement of PLD1 and not PLD2 isoform activity for Ras activation (Fig. 7C) potentially points to a larger involvement of both isoforms of PLD in Ras activation.

PLD activity is elevated in a number of cancers and is implicated in motility regulation of neutrophil and fibroblasts [Foster and Xu, 2003; Huang and Frohman, 2007; Rudge and Wakelam, 2009]. However, the isoform of PLD involved in these cell functions varies, is not canonical, and is somewhat conflicting. An increase in PLD1 activity is critical for Ras transformation of tumor cells in soft agar [Buchanan et al., 2005], ERK activation is enhanced by an increased translocation of Raf to membranes upon PLD-generated PA [Lacal, 1997; Rizzo et al., 1999, 2000; Andrensen et al., 2002; Rizzo and Romero, 2002; Rudge and Wakelam, 2009], and lysophospatidic acid activation of PLD1 increases ERK phosphorylation [Hong et al., 2001]. Yet PLD1 knockdown increased the number of focal adhesions, induced the formation of stress fibers reaching throughout the cell, and decreased cell mobility [Kim et al., 2006]. PLD2, but not PLD1, is critical for enhanced motility and tumor formation in a number of cell types. Reduction of PLD2 expression suppressed PA levels, causing an increase in cell spreading and a concomitant decrease in cellular motility [Du and Frohman, 2009]. Thus, there are conflicting accounts of which PLD isoform is involved in cytoskeletal organization and cell motility.

Here, we provide additional evidence identifying PLD as a critical player in α_1-adrenergic receptor stimulation of stress fiber formation and increased directional migration. This work also demonstrates the involvement of both RhoA and PKCα in the α_1-adrenergic receptor activation of PLD1 leading to an enhanced organization and motility of CCL39 fibroblasts (Fig. 8). In addition, our findings identify a novel role for PLD1 where PLD1-generated PA is critical for activation of growth factor pathways upstream of Raf. This indicates a provocative model by which a cell specific PLD isoform can be involved in the regulation of cell motility where both Raf and Ras are involved in cell growth factor pathways.

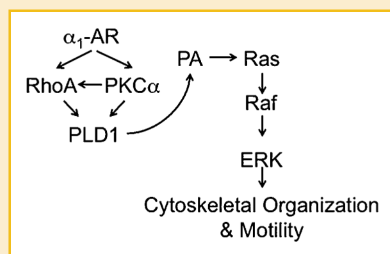

Fig. 8. Model of PLD1 signal-regulated cell motility and stress fiber formation by α_1-adrenergic receptor activation in CCL39 cells. This model demonstrates the putative signaling pathway for α_1-adrenergic receptor signaling to induce motility events in CCL39 fibroblasts. GPCR activation of the receptor activates both upstream regulators of PLD1 activity. PLD1 catalyzes the formation of PA, which leads to the activation of Ras, required for ERK activation. The stimulation of the Ras–Raf–ERK pathway leads to stress fiber formation and cell motility in α_1-adrenergic stimulated cells.

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