Protein Kinase A Regulates 3-Phosphatidylinositol Dynamics during Platelet-derived Growth Factor-induced Membrane Ruffling and Chemotaxis

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Spatial regulation of the cAMP-dependent protein kinase (PKA) is required for chemotaxis in fibroblasts; however, the mechanism(s) by which PKA regulates the cell migration machinery remain largely unknown. Here we report that one function of PKA during platelet-derived growth factor (PDGF)-induced chemotaxis was to promote membrane ruffling by regulating phosphatidylinositol 3,4,5-trisphosphate (PIP3) dynamics. Inhibition of PKA activity dramatically altered membrane dynamics and attenuated formation of peripheral membrane ruffles in response to PDGF. PKA inhibition also significantly decreased the number and size of PIP3-rich membrane ruffles in response to uniform stimulation and to gradients of PDGF. This ruffling defect was quantified using a newly developed method, based on computer vision edge-detection algorithms. PKA inhibition caused a marked attenuation in the bulk accumulation of PIP3 following PDGF stimulation, without effects on PI3-kinase (PI3K) activity. The deficits in PIP3 dynamics correlated with a significant inhibition of growth factor-induced membrane recruitment of endogenous Akt and Rac activation in PKA-inhibited cells. Simultaneous inhibition of PKA and Rac had an additive inhibitory effect on growth factor-induced ruffling dynamics. Conversely, the expression of a constitutively active Rac allele was able to rescue the defect in membrane ruffling and restore the localization of a fluorescent PIP3 marker to membrane ruffles in PKA-inhibited cells, even in the absence of PI3K activity. These data demonstrate that, like Rac, PKA contributes to PIP3 and membrane dynamics independently of direct regulation of PI3K activity and suggest that modulation of PIP3/3-phosphatidylinositol (3-PI) lipids represents a major target for PKA in the regulation of PDGF-induced chemotactic events.

The ability of cells to undergo directional migration in response to environmental cues is required during embryonic development and is important during many physiological and pathological processes, including immune surveillance, wound healing, angiogenesis, inflammation, tumor growth, and metastasis. Cell migration in vivo is a complex process, and although some commonalities exist, the specific molecular mechanisms that govern the process are often unique products of the particular cell type (e.g. fibroblast versus neutrophil), stimulus (e.g. peptide growth factor versus chemokine), and cellular environment. The wide variety of initiating events, acting through distinct panels of intricate signaling networks, ultimately converge to effect common hallmarks of cell migration as follows: the spatial coordination of cell adhesion to the extracellular matrix; cytoskeletal assembly and disassembly; and membrane protrusion and retraction (1–3). One consequence of these interactions is dynamic cytoskeletal reorganization involving the polymerization of branched actin filaments against the leading edge cell membrane. It is through these events that the force to push the membrane forward is generated, and protrusive structures known as lamellipodia are formed (2). The small Rho GTPase Rac is a principal protein involved in regulating the formation of lamellipodia and membrane ruffles during cell migration. Rac is localized to the leading edge and thereby promotes spatially restricted activation of the actin polymerization machinery. Growth factor-induced Rac activity is associated with the formation of two distinct types of actin-rich membrane ruffles (4, 5). Ruffles that develop on the dorsal surface of the cell are thought to represent active sites of cytoskeletal reorganization important for invasion, migration, and macropinocytosis (5–9). Membrane ruffles that form at the cell periphery are believed to result from failed attachment of newly formed lamellipodia to the extracellular matrix and also serve as sites of active cytoskeletal reorganization (10).

Rac activation and membrane ruffling are common consequences of chemotactic stimulation in many cells, including the induction of cell migration of fibroblasts by peptide growth factors via receptor tyrosine kinases. The current model for growth factor-induced Rac activity involves the production of 3-phosphatidylinositides (3-PI), specifically phosphatidylinositol-3-phosphate (PIP3), phosphatidylinositol-3,4-phosphate (PIP2), and phosphatidylinositol-3,4,5-trisphosphate (PIP3), which recruit and activate the Rho GTPase Rac (11). The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3, Movies 1–10, and RuffleQuant.txt.

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3 The abbreviations used are: 3-PI, 3-phosphatidylinositol; AKAP, A-kinase anchoring proteins; FN, fibronectin; HDF, human dermal fibroblasts; H89, N-[2-(p-bromocinnamylamino)ethyl]-5-isouquinolinesulfonamide-2HCl; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol 3-kinase; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PKA, protein kinase A; PKI, PKA inhibitor; mPKI, myristoylated PKA inhibitor; REF52, rat embryo fibroblasts; DMEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin; PBS, phosphate-buffered saline; GFP, green fluorescent protein; EGFP, enhanced GFP; GEF, guanine nucleotide exchange factor; PH, pleckstrin homology.
PKA Regulates PIP₃ Dynamics

Sitol 3,4,5-trisphosphate (PIP₃), through activation of PI3K (11–14). The production of PIP₃ results in the recruitment of guanine nucleotide exchange factors (GEFs) containing pleckstrin homology (PH) domains to the cell membrane, subsequent exchange of GDP for GTP on Rac and activation of Rac effectors (15). PIP₃ is known to regulate many important aspects of cell migration, including the formation of lamellipodia, peripheral membrane dynamics (16–20), and cell polarity (21, 22).

As efficient chemotaxis requires distinct spatial regulation of cytoskeletal and membrane events, it then follows that the molecular pathways governing these processes also exhibit distinct distribution in subcellular space. Among several, the cyclic AMP-dependent protein kinase (PKA) is important for directional migration (23–25). PKA is activated in response to growth factors (26, 27), and this activity is required for growth factor-mediated cell migration, in part through the regulation of Rac activity. PKA appears to sustain Rac activity through both the enhancement of GEFs and inhibition of GTPase-activating protein function (24). Importantly, this requirement seems to involve distinct localized pools of PKA, as PKA is enriched within pseudopodia, and disruption of this localization prevents migration (24). PKA is spatially controlled within cells by AKAPs, which are scaffold proteins that promote the assembly of specialized multivalent signal transduction complexes through interactions with various protein kinases, phosphatases, and in some cases cell structural proteins (28).

Given that there are numerous intracellular substrates for PKA, including targets in the plasma membrane, nucleus, cytoplasm, mitochondria, and cytoskeleton (29, 30), anchoring of PKA to particular cellular subdomains provides an important additional facet of PKA regulation. The significance of PKA anchoring during directional migration has also been reported recently during α4-integrin-mediated motility where α4 appears to serve as a novel AKAP (31). Although an increasing body of evidence supports a role for PKA during directional cell migration, the precise mechanism(s) by which PKA exerts its effects on the migration machinery remain to be elucidated.

Previous studies that demonstrated a requirement for PKA during cell migration measured the overall ability of the cells to migrate in long term assays (wound healing; modified Boyden chamber) and were not amenable to the visualization of discrete cellular events that occur upon stimulation with growth factor (23–25). To gain a more detailed understanding of how PKA contributes to cell migration, the morphological features of cells following acute stimulation with PDGF in the presence and absence of PKA inhibition were examined. The results described here compliment and advance earlier studies by demonstrating that one function of PKA during chemotaxis is to promote Rac-mediated membrane ruffling through the modulation of 3-PI dynamics.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Other Reagents**—Primary antibodies were obtained commercially from Cell Signaling Technologies (total and pS473 Akt) and BD Biosciences (Rac1). Horseradish peroxidase-conjugated secondary antibodies were from Calbiochem, and Alexa Fluor-conjugated phalloidin was from Invitrogen. Platelet-derived growth factor type BB (PDGF-BB) was from Upstate Biotechnology, Inc. Fibronecin (FN) was from BD Biosciences. KT5720 and wortmannin were from Calbiochem; H89 and myristoylated PKA inhibitor (mPKI) were from Biomol. A myristoylated, scrambled peptide (GRTRGNRAI, single letter amino acid code) was synthesized by AnaSpec and served as a control for the mPKI peptide. Use of this reagent, rather than the typically used solvent (H₂O) control, was found to be especially important for biochemical analysis of PIP₃ levels, as phospholipids were more efficiently extracted from cells treated with either myristoylated peptide than from solvent-treated control cells (data not shown). The PIP₃ mass strip kit was from Echelon Biosciences (Salt Lake City, UT). Other chemicals were obtained from Sigma.

**Cell Culture and Transfection**—Swiss 3T3 cells, REF52 rat embryo fibroblasts, and human dermal fibroblasts were grown in Dulbecco’s modified Eagle’s medium (DMEM) plus 10% fetal bovine serum. For biochemical analysis NIH 3T3 (ATCC) cells were cultured in DMEM plus 10% bovine calf serum, grown to subconfluence (~85–90%), and then serum-starved overnight for 16–18 h. For transfection experiments, REF52 cells or human dermal fibroblasts were plated at 70% confluency onto 6-well plates (Corning Glass), and plasmid DNA was transfected with FuGENE 6 reagent (Roche Applied Science) as per the manufacturer’s instructions. Twenty four hours after transfection, cells were serum-starved overnight and then re-plated onto FN-coated (10 μg/ml) surfaces (coverslips or Delta-T dishes (Bioptechs, Butler, PA)) and assayed at the times indicated in the figure legends. Transfection efficiency, as determined by visualization of GFP-labeled cells after 24 h, was typically 40–70%, depending upon the cell type.

**DNA Constructs**—The plasmid encoding the Akt-PH-EGFP fusion protein was a generous gift from Dr. Y. Tsien (University of California, San Diego) and used to replace the EGFP coding sequence of pEGFP-PKI to create pmCherry-PKI. The plasmids expressing constitutively active (Q61L) and dominant-negative (T17N) Rac1 were obtained from Addgene (University of California, San Diego) and were used to replace the EGFP coding sequence of pEGFP-PKI to create pmCherry-PKI. The plasmids expressing constitutively active (Q61L) and dominant-negative (T17N) Rac1 were obtained from Addgene (Cambridge, MA).

**Immunofluorescence and Scanning Electron Microscopy**—Serum-starved cells were detached and replated onto FN-coated coverslips and allowed to adhere for 2–3 h at 37 °C. Cells were then either pretreated with DSMO solvent control or the PKA inhibitors H89 or KT5720 for 30 min after which PDGF-BB was added for the indicated times. Samples were then fixed and processed for either immunofluorescence or scanning electron microscopy. For visualization of actin, cells were fixed in 3.7% formaldehyde for 15 min, permeabilized in phosphate-buffered saline (PBS), 0.5% Triton X-100 for 10 min, blocked in PBS, 0.5% Triton X-100, 1% BSA for 30 min, and then stained with Alexa 488-phalloidin (1:100) for 30 min. Epifluorescence images were captured through ×40 or ×60 PlanApo objectives on a Nikon Eclipse TE-2000E inverted microscope using appropriate fluorophore-specific filters (Chroma Technology Corp., Rockingham, VT) and a CoolSnap HQ camera (Photometrics, Tucson, AZ) controlled by MetaVue (Molecular Devices) or...
Elements (Nikon) software. For scanning electron microscopy experiments, after the indicated treatments, fibroblasts were fixed with 2% glutaraldehyde/PBS and post-fixed in 1% osmium tetroxide in 0.05 M cacodylate buffer, pH 7.2. Samples were immersed in 1% tannic acid in cacodylate buffer and 0.5% uranyl acetate in distilled H₂O each for 1 h at room temperature. Samples were dehydrated in ethanol, critical point dried with liquid CO₂, mounted, and dried overnight. Specimens were sputter-coated with gold/palladium and imaged on a JSM-6060 scanning electron microscope (JEOL Inc., Peabody, MA).

Live Cell Imaging—Live cell imaging was performed on the Nikon TE-2000E inverted epifluorescence microscope described above, equipped with a dish heater (Delta T4; Bioptechs) to maintain cells at 35–37 °C. Cells were plated at a density of 3 × 10⁴ cells/cm² onto FN-coated Delta-T4 dishes and allowed to adhere for 2–3 h. Prior to imaging, the medium was replaced with fresh DMEM + 1% BSA or phenol red-free Iscove’s media (Invitrogen). Kymographs of phase-contrast movies were generated along 1–10 pixel wide boxed regions using the Multiple Kymograph plugin (by J. Rietdorf and A. Seitz) for ImageJ software (rsb.info.nih.gov, by W. Rasband). For imaging GFP fluorescence, cells were first transfected with various plasmids (as indicated in the figure legends) and then replated onto FN-coated Delta-T4 dishes. Cells with normal, comparable morphologies and moderate expression of fluorescent indicators were chosen for further analyses. Local chemotactic gradients were formed near target cells by placing a glass micropipette (1 mm inner tip diameter) coupled to an air pressure manifold (PLI-100; Medical System Corporation, Miami, FL) and filled with PDGF-BB (5–50 ng/ml) ~ 25 μm away from the cell. The gradient was initiated by increasing the holding pressure to 3 p.s.i. The linearity of the resulting gradients was routinely confirmed by measuring the graded fluorescence intensity of Alexa 594-conjugated dextran (M₅ 5000) added to the growth factor solution.

Image Analysis and Ruffle Quantification—An ImageJ macro was written to perform edge detection based on a modification of the Marr-Hildreth algorithm, in which a Difference of Gaussian filter function is used as a simple but faithful approximation of the Laplacian of Gaussian function commonly used in computer vision edge detection (32). The macro improves on similar previous approaches (33) in its simplicity, its ability to automatically process time-lapse image stacks, and the ability to exclude select regions of interest from the analysis (e.g. a neighboring cell that cannot be entirely cropped from the image). From each original grayscale fluorescence image in a time course stack, the macro generates two Gaussian blurred images at radius = 1.28 and 0.80. The 1.61 ratio of the two values was chosen to maintain the closest approximation of the Difference of Gaussian function to the true Laplacian of Gaussian function (32, 34), whereas the absolute values were determined empirically to maximize discrimination between ruffle and non-ruffle features. The images with the smaller blur radius are subtracted from those with the larger radius, and the resulting difference images are thresholded based on the mean image intensity of the entire stack. The macro then calls the ImageJ Analyze Particles function to select and analyze features from the thresholded difference image that are above a certain minimum pixel area and below a certain circularity (determined empirically to eliminate noise). Finally, the macro returns tabulated reports containing a frame-by-frame account of the number of objects per frame, as well as their total, average, and fractional area, and an account of the area of every object analyzed in the entire stack. In this study, the data from the macro are reported as the per-frame averages of the number of ruffle features (upon growth factor addition) and the total ruffle area.

The functionality of the macro is robust, as it faithfully recapitulates data on single images generated using an earlier approach (33) and readily analyzed and quantified ruffling responses in movies downloaded randomly from open-access sources (e.g. laboratory, journal, and consortium websites). The supplemental data contains a demonstration of the various processing steps of the macro (supplemental Movie 3) as well as the macro itself (RuffleQuant.txt), as a downloadable text file. For quantification of polarized responses to PDGF gradients, cells were bisected into gradient proximal and distal halves, and the proximal half was analyzed as described above. No ruffling was observed in the distal portion of the cells (Fig. 4, A and B; supplemental Movies 5 and 6).

Assessment of PIP₃ Levels—NIH 3T3 cells were serum-starved overnight and then treated with a myristoylated peptide control or mPKI for 30 min after which some cells were stimulated with PDGF-BB for 1 or 5 min. Extraction of PI(3,4,5)P₃ from cells was carried out following the supplier’s protocol (Echelon Biosciences). Briefly, cells were collected in cold 0.5 M trichloroacetic acid and washed with 5% trichloroacetic acid, 1 mM EDTA. After extraction of neutral lipids with MeOH: CHCl₃ (2:1), acidic lipids were extracted with CHCl₃, MeOH, 12 M HCl (40:80:1) and vacuum-dried (SpeedVac, Savant). Samples were dissolved in CHCl₃, spotted onto nitrocellulose membranes containing pre-spotted PIP₃ standards (PIP Strips; Echelon Bioscience), and processed by serial incubation in blocking solution (3% fatty acid-free BSA in PBS), PIP₃ detector, Secondary Detector solution, and detected by chemiluminescence.

PI3 Kinase Activity Assays—NIH 3T3 cells were serum-starved overnight and then treated with 20 μM of either control peptide or mPKI for 30 min after which some cells were stimulated with 20 ng/ml PDGF-BB for 3 min. Assessment of PI3K activity was performed on anti-phosphotyrosine immunoprecipitates (4G10; Millipore) as described previously (35).

Subcellular Fractionation—Serum-starved NIH 3T3 cells were pretreated for 30 min with either a control myristoylated peptide or mPKI and then stimulated with PDGF-BB for 10 min. Cells were then chilled on ice, rinsed in ice-cold PBS, and harvested in ice-cold hypotonic lysis buffer (10 mM Tris, pH 7.4, 1.5 mM MgCl₂, 5 mM KCl, 1 mM dithiothreitol, 0.2 mM sodium orthovanadate, leupeptin (10 μg/ml), 4-(2-aminoethyl)benzene-sulfonyl fluoride (400 μM), NaF (1 mM), pepstatin 1 μg/ml, aprotinin 1 μg/ml) and incubated on ice for 10 min. Cells were then passed 20 times through a 27-gauge needle and centrifuged at 700 × g for 10 min to pellet nuclei and intact cells. Supernatants were spun at 100,000 × g for 30 min at 4 °C. The membrane pellet was washed once in hypotonic lysis buffer, resuspended in hypotonic lysis buffer supplemented with 1% Nonidet P-40, and incubated on ice for 10 min to solubilize...
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FIGURE 1. PDGF-induced membrane ruffling requires PKA. A Swiss 3T3 fibroblasts were preincubated with either solvent control (DMSO), KT5720 (10 μM), or H89 (25 μM) and then left unstimulated, as controls (CTL), or stimulated with PDGF (10 ng/ml) (PDGF) for 15 min. Cells were then fixed and stained with Alexa 488-phalloidin to visualize actin. Arrows indicate peripheral membrane ruffles, and asterisks indicate dorsal ruffles. B, HDFs (upper panels) and Swiss 3T3 cells (lower panels) were preincubated with DMSO or KT5720 (10 μM) and then stimulated with PDGF (10 ng/ml) for 15 min after which cells were processed for scanning electron microscopy as described under “Experimental Procedures.” Scale = 5 μm. C, REF52 cells transfected with empty mCherry vector (left panels) or mCherry-PKI vector (right panels) were re-plated onto FN-coated glass-bottom dishes, after which live-cell imaging using phase contrast microscopy was performed. Frames corresponding to different times (min) after addition of PDGF (10 ng/ml) are depicted. Upper left panels show transfected cells. Images were captured every 30 s over the course of 40 min. Arrows indicate membrane ruffles (see supplemental Movie 1). Scale = 10 μm. D, kymographic analysis was performed on the cells depicted in C. Black lines represent the addition of PDGF. The data presented in C and D are representative of at least 10 different cells for mCherry and 11 different cells for mCherry-PKI from four independent experiments. Number in each kymograph corresponds to the line drawn in C.

membrane-associated proteins. Lysates were again spun at 100,000 × g for 30 min at 4 °C, and the supernatant was subjected to SDS-PAGE and immunoblot analysis using antibodies against total and phosphoserine 473 Akt (Cell Signaling Technologies).

Rac GTPase Assay—NIH 3T3 cells were serum-starved overnight and then re-plated onto FN-coated (10 μg/ml) dishes at a density of ~2.0 × 10⁶ cells per 150-mm plate. After 2 h, cells were pretreated for 30 min with either myristoylated control peptide or mPKI. After stimulation with 20 ng/ml PDGF for the indicated times, cells were harvested, and cell lysates were subjected to a pulldown assay by using a glutathione S-transferase fusion with the p21-binding domain of p21-activated kinase as described previously (24).

Scratch Migration/Wound Healing Assay—Serum-starved REF52 cells expressing either mCherry or mCherry-PKI were re-plated onto FN-coated (10 μg/ml) coverslips at ~10⁵ cells/cm². After 2 h, monolayers were scratched with a single pass of a pipette tip, rinsed with pre-warmed media, and incubated in DMEM containing 1% BSA and 10 ng/ml PDGF-BB at 37 °C for 5 h. Cultures were then washed, fixed in 3.7% formaldehyde, and stained with Alexa 488-phalloidin to visualize actin.

RESULTS

PKA Promotes PDGF-induced Membrane Ruffle Dynamics—Although previous studies definitively demonstrated a role for PKA in overall cell migration, they did not provide any clues as to the morphological deficits elicited by inhibition of PKA. Thus, in an effort to further investigate the mechanism(s) by which PKA regulates cell migration, experiments were performed to examine the effects of inhibiting PKA on morphological and cytoskeletal events occurring at early times following growth factor stimulation. As expected, initial observation of the actin cytoskeleton in control Swiss 3T3 fibroblasts 15 min following stimulation of cells with PDGF revealed the presence of both peripheral (denoted by the arrows) and dorsal membrane ruffles (denoted by the asterisks, Fig. 1A). Cells that had been pretreated with two different pharmacological inhibitors of PKA (H89 and KT5720) prior to stimulation with PDGF displayed slight differences in the morphology of the actin cytoskeleton (Fig. 1A). In particular, it appeared as if inhibition of PKA attenuated the dissolution of actin stress fibers and the formation of actin-rich peripheral membrane ruffles following stimulation with PDGF (Fig. 1A, compare lower middle and right panels to lower left panel). Interestingly, there appeared to be minimal effect on dorsal ruffle formation (Fig. 1A, asterisks denotes dorsal ruffles). To observe the effects of PKA inhibition on the actin cytoskeleton in finer detail, scanning electron microscopy was performed. Both Swiss 3T3 and human dermal fibroblasts stimulated with PDGF displayed prominent membrane ruffles, apparent as folds of membrane protruding up and off the peripheral cell surface (Fig. 1B, left panels). Underneath these ruffles were what appeared to be nascent lamellipodial protrusions. In contrast, fibroblasts that had been pretreated with PKA inhibitor (KT5720) exhibited a flattened appearance with a thickened membrane and a complete lack of ruffles around the cell periphery (Fig. 1B, right panels). These findings suggest that perturbation of PKA activity with pharmacological inhibitors...
modulates the dynamics of peripheral membrane ruffles in fibroblasts.

To further investigate this possibility, control and PKA-inhibited cells were monitored in real time following stimulation with PDGF. The inhibitory spectra of both H89 and KT5720 overlap only on PKA at the concentrations used in this study; however, both agents have been reported to inhibit kinases other than PKA. Therefore, to more specifically explore the role of PKA in regulating membrane ruffling dynamics, we transiently transfected REF52 fibroblasts with either a control plasmid encoding the monomeric red fluorescent protein mCherry or a plasmid encoding PKI, a specific inhibitor of PKA kinase activity, fused to mCherry (mCherry-PKI). Phase contrast microscopy was performed on live cells following stimulation with PDGF. REF52 fibroblasts were used for these experiments as they are efficiently transfected and display cell morphologies very amenable to microscopic observation of ruffling dynamics. As expected, within minutes of PDGF addition, fibroblasts transfected with empty mCherry responded by exhibiting broad intervals of peripheral membrane protrusion punctuated occasionally by periods of retraction and the formation of phase-dense ruffles that moved centripetally toward the cell body (Fig. 1C, left panels; supplemental Movie 1A). In contrast, fibroblasts transfected with mCherry-PKI showed a dramatic alteration in the dynamics of membrane protrusion, retraction, and ruffling (Fig. 1C, right panels; supplemental Movie 1B). Specifically, although there was some initial membrane protrusion in cells expressing PKI, the peripheral cell membrane appeared to fail to ruffle back toward the cell center. Kymographic analysis of time-lapse images captured every 30 s emphasized the remarkable differences in PDGF-induced membrane dynamics observed in control versus PKA-inhibited cells. The active cycles of protrusion and retraction (apparent as inflection points in the kymographs) and membrane ruffling (seen as phase-dense spots that move inward over time) observed in control cells (Fig. 1D, left panels) were all significantly blunted by the expression of the PKA inhibitor (Fig. 1D, right panels). Similar results were obtained with pharmacological PKA inhibitors (H89, KT5720) and in several other fibroblast cell lines (supplemental Fig. 1).

**PKA Is Required for Ruffling during Wound Healing**—To assess the requirement of PKA for membrane ruffling in a more complex system, the effect of PKA inhibition on actin cytoskeletal dynamics was ascertained in a wound healing assay. Five h after the formation of a scratch on a monolayer of control mCherry-expressing cells, 58% of the transfected cells present at the edge of the wound displayed prominent actin-rich membrane ruffles (Fig. 2, A, upper panels, and B). A similar percentage of nontransfected cells present at the edge of the wound were found to be ruffling (data not shown). In contrast, only 11% of mCherry-PKI-expressing cells exhibited actin-rich ruffles (Fig. 2, A, lower panels, and B). Of note, the overall number of mCherry-PKI-expressing cells located at the edge of the wound appeared to be decreased compared with the control cells (data not shown), suggesting that PKA inhibition may result in diminished movement of cells toward the open wound. As membrane ruffling is an integral aspect of cell migration, the observed ruffling defect in wounded monolayers upon PKA inhibition is consistent with our previous finding that PKA is required for directional migration (24). Taken together, the data from Figs. 1 and 2 soundly demonstrate that PKA activity is required for the formation and dynamics of peripheral membrane ruffles in response to PDGF.

**PKA Modulates 3-PI Dynamics in Vivo**—3-PIs, principally PIP₃, are growth factor-regulated membrane lipid products of PI3K that are generated at the leading edge and in membrane ruffles of migrating cells. They play a pivotal role in several aspects of cell migration, including the regulation of Rho GTPases and the establishment and maintenance of cell polarity (36, 37). Because PKA appeared to be required for PDGF-induced peripheral membrane ruffling, we hypothesized that PKA might exert its effects on cell migration, at least in part, through modulation of 3-PI lipid dynamics. To test this idea, the effect of PKA on the dynamics and distribution of 3-PIs was investigated within PDGF-stimulated cells. One tool frequently used to assess the subcellular localization of 3-PI lipids is a fusion protein comprising EGFP and the PH domain of Akt (Akt-PH-EGFP) (38–40). This domain binds specifically to PIP₃ and phosphatidylinositol 3,4-bisphosphate and mediates the recruitment of Akt to the plasma membrane following growth factor-induced accumulation of 3-PIs (41). REF52 fibroblasts were transiently co-transfected with Akt-PH-EGFP and either empty mCherry or mCherry-PKI and then analyzed...
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FIGURE 3 PKA is required for membrane localization of Akt-PH-EGFP following PDGF stimulation. A, REF52 cells were transfected with Akt-PH-EGFP and either empty mCherry vector (left panels) or mCherry-PKI (right panels), and live-cell phase contrast and epifluorescence imaging were performed on unstimulated cells (Un) or cells stimulated with PDGF (10 ng/ml) (PDGF) (supplemental Movie 2). Arrows indicate peripheral membrane ruffles showing high Akt-PH-EGFP fluorescence. Images are representative of four independent experiments. Scales = 10 μm. Images were transformed in pseudocolor using the same spectrum scale. Insets confirm co-expression of the respective mCherry construct. B, average number of ruffles and average ruffle area per frame after PDGF stimulation was quantified using the ImageJ macro described under “Experimental Procedures.” The values shown represent the averages (± S.E.) obtained from 11 cells for mCherry and 10 cells for mCherry-PKI from four independent experiments. Significance of the results was analyzed using an unpaired t test (*, p < 0.0001).

PDGF-induced Polarization of PIP₃ Requires PKA Activity—PI3K and 3-PIs are concentrated at the leading edge of neutrophils, Dictyostelium discoideum, and fibroblasts polarized in response to chemotactic gradients (42−46). As localized PKA activity has been shown to be required for polarized migration in fibroblasts, we next wished to determine whether inactivation of PKA alters the spatial localization of PIP₃ in cells exposed to a chemotactic gradient of PDGF. To assess this, fibroblasts were transfected with plasmids expressing Akt-PH-EGFP and either mCherry alone or mCherry-PKI and then exposed to a PDGF gradient using a micropipette. Control fibroblasts responded to the PDGF gradient in a polarized manner as evidenced by robust membrane protrusion and dramatic ruffling of the cell in the direction of the pipette (Fig. 4, A and C, upper right panels; supplemental Movies 5A and 6A). This directional response was also reflected by polarized relocalization of the Akt-PH-EGFP signal into membrane ruffles at the leading edge (arrows, Fig. 4, A and C, lower right panels; supplemental Movies 5B and 6B). As observed earlier in response to global PDGF stimulation, cells in which PKA was inhibited pharmacologically (by H89) or genetically (by expression of mCherry-PKI) also displayed a lack of dynamic membrane ruffling when subjected to a PDGF gradient (Fig. 4, B and D, upper panels; supplemental Movies 5C and 6C, respectively). Although exposure to the PDGF gradient caused some mobilization of the Akt-PH-EGFP domain from the center of H89-treated and PKI-expressing cells, there was little-to-no accumulation of the reporter on the side of the cell closest to the pipette as compared with the controls (compare lower panels of Fig. 4, B and D, to lower panels of Fig. 4, A and C; and supplemental Movies 5D and 6D to 5B and 6B, respectively). Moreover, inhibition of PKA with H89 or by expression of mCherry-PKI resulted in a significant diminution in both the average number (81 and 52%, respectively) and size (89 and 45%, respectively) of PIP₃-enriched ruffles formed in response to PDGF (Fig. 4, E and F). Taken together, these data demonstrate that PKA is required for the polarized localization of 3-PI to the leading edge in response to chemotactic stimulation with PDGF.
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PDGF, with maximal levels observed after just after 1 min (Fig. 5B). However, although there was no apparent effect of PKA inhibition on PDGF-induced P13K activity (Fig. 5C), there was a dramatic attenuation of PDGF-induced PIP₃ accumulation in fibroblasts pretreated with mPKI (Fig. 5B). The extent of inhibition of PIP₃ at the peak time point was 72% (±12). These data show that PKA is required for PDGF-induced accumulation of PIP₃, but not for activation of P13K, and suggest that PIP₃-dependent cell migration events may be similarly compromised by inhibition of PKA.

One event downstream of growth factor-induced PIP₃ that is pivotal for leading edge membrane and cytoskeletal dynamics is the activation of Rac (11, 15, 50). This, together with our finding that PKA is required for PDGF-induced PIP₃, led us to postulate that inhibition of PKA would block growth factor-induced Rac activity. As predicted, the activation of Rac by acute stimula-

The lessened ability of the PH domain of Akt to translocate to the peripheral cell membrane under conditions of PKA inhibition was also observed, biochemically, for endogenous Akt. For these and other biochemical experiments, NIH 3T3 cells were used in favor of REF52 or HDFs because they gave morphological effects comparable with REF52 and HDFs, but they provided higher yields of material for analyses. Growth factor-induced accumulation of 3-PIs recruits Akt to the plasma membrane (41) that promotes the phosphorylation of Akt on Thr-308 and Ser-473 and activation of its kinase activity (47, 48). Moreover, a recent report demonstrates that PIP₃ is principally responsible for regulating membrane-associated Akt activity (49). In control cells, both phosphorylated and total Akt were recruited to the membrane following stimulation with PDGF (Fig. 5A). However, membrane fractions prepared from cells that had been pretreated with a cell-permeable peptide PKA inhibitor (mPKI) prior to stimulation with PDGF displayed very little phosphorylated or total Akt in the membrane-rich fractions (Fig. 5A).

PKA Is Required for PDGF-induced PIP₃ Synthesis and Rac Activation—The lack of membrane recruitment of both the Akt-PH-EGFP fusion protein and endogenous Akt following stimulation with PDGF in PKA-inhibited cells suggested that the amount of 3-PI products at the cell membrane following stimulation with PDGF might be decreased. To test this possibility, cellular levels of PIP₃ were assessed biochemically by immunoblotting purified membrane phospholipids with a PIP₃-specific antibody. As expected, PIP₃ levels rapidly increased following stimulation of control fibroblasts with stimulation with PDGF was significantly blunted by specific inhibition of PKA over a time course similar to that observed for the defect in membrane ruffling (Fig. 5D).

PKA Regulates PIP₃ Accumulation Downstream of P13K and Upstream of Rac—The data presented to this point demonstrate that in response to PDGF, PKA is upstream of both PIP₃ and Rac. However, the relationship between PIP₃ and Rac is complex. As stated earlier, PIP₃ is required for, and is therefore upstream of, growth factor-induced activation of Rac via recruitment of Dbl homology/PH-containing Rac GEFs (11, 12). Conversely, the ability of Rac to bind and activate PI3K in vitro (51, 52), plus a number of elegant in vivo and in silico studies examining Rac activation and cellular PIP₃ levels (53–57), support a positive feedback loop from Rac back to PIP₃. Thus, it is possible that the effect of PKA inhibition on overall PIP₃ accumulation (Fig. 5B) may be caused by inhibiting the PIP₃ synthesis required for (and therefore independent of) Rac activation or by inhibiting a step downstream of initial PIP₃ synthesis but upstream of Rac, which would inhibit both Rac activation and subsequent PIP₃ synthesis driven by positive feedback. To begin to address this issue, we measured the effects of either blocking or forcing activation of Rac (alone or in combination with inhibition of PKA) on PDGF-induced PIP₃ ruffling responses.

As demonstrated previously, inhibition of PKA significantly reduced both the number and area of PDGF-induced ruffles (Fig. 6, A and B; supplemental Movie 7). Expression of the dominant-negative T17N allele of Rac1 (RacN17) also reduced both the number and size of growth factor-induced PIP₃-rich ruffles.

FIGURE 4. PDGF-induced Akt-PH-EGFP polarization is mediated by PKA. HDF cells were treated with DMSO (A) or H89 (25 µM) (B) for 30 min, and REF52 cells were transfected with plasmids encoding Akt-PH-EGFP and either empty mCherry (C) or mCherry-PKI (D). The distribution of Akt-PH-EGFP was imaged by live-cell phase contrast and epifluorescence microscopy of unstimulated cells (Un) or cells exposed to a gradient of PDGF emanating from a micropipette (position indicated by the asterisks). The images depicting PDGF-stimulated cells were taken 12–17 min after the beginning of stimulation (supplemental Movies 5 and 6). Arrows indicate peripheral ruffles. Scale = 10 µm. The average number of ruffles (D) and ruffle area (E) per frame was computed for DMSO (n = 4) and H89-treated (n = 11) and mCherry (n = 8) and mCherry-PKI-expressing cells (n = 15) as described under "Experimental Procedures." The values shown represent the mean (± S.E.) from at least three independent experiments. Significance of the results was analyzed using an unpaired t test (*, p < 0.0001).
PKA Regulates PIP₃ Dynamics

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FIGURE 5. PKA is required for PDGF-induced PIP₃ accumulation and Rac activation. A, subconfluent NIH 3T3 cells were serum-starved and then pretreated with either myristoylated control peptide or mPKI. Unstimulated cells (Un) or cells stimulated with PDGF (20 ng/ml) for 10 min were harvested for biochemical fractionation as described below “Experimental Procedures.” Membrane fractions were subjected to SDS-PAGE and immunoblot analysis using antibodies directed against total or serine 473-phosphorylated Akt. The images shown represent original images juxtaposed in Adobe Photoshop. The data are representative of three independent experiments. B, subconfluent NIH 3T3 cells were serum-starved and then incubated with myristoylated control peptide or mPKI (20 μM) for 30 min. The cells were then treated with PDGF (20 ng/ml) for the indicated times after which they were harvested and assayed for PIP₃ levels. The graph illustrates the relative levels of PIP₃ induced after 1 min of PDGF stimulation for the indicated conditions. The values shown represent the mean (±S.E.) of five independent experiments. Significance of the results was analyzed using a paired t test (**, p < 0.005). C, subconfluent NIH 3T3 cells were serum-starved and then incubated with myristoylated control peptide, mPKI, or wortmannin (Wort), with (+) or without (−) PDGF stimulation and assayed for PIP₃K activity. The graph shows the relative amount of PDGF-induced PI3K activity in the mPKI-treated cells compared with the myristoylated control. The values correspond to the mean (±S.D.) from four independent experiments (for control and mPKI). D, cells were treated as in B and then assessed for Rac GTPase activity using a p21-binding domain-pulldown assay as described under “Experimental Procedures.” The graph depicts the relative amount of Rac activity induced upon stimulation with PDGF (20 ng/ml) normalized to the unstimulated control. The values shown represent the mean (±S.D.) from four independent experiments. Significance of the results was analyzed using a paired t test (**, p < 0.005, 5-min time point; *, p < 0.05, 15-min time point).

to an extent that was statistically the same as the effect seen with PKI (Fig. 6, A and B; supplemental Movie 7). To represent the combined effects of the inhibitors, alone or in combination, on membrane ruffling, a ruffling index was calculated as the product of average ruffle number and average ruffle area (Fig. 6C; supplemental Movie 7). Interestingly, simultaneous inhibition of PKA and Rac activity reduced the number of ruffles to a greater extent than either reagent alone (to almost nil) and also further reduced the size of the few ruffles that did form (Fig. 6, A–C; supplemental Movie 7). The dramatic effect of each inhibitor alone, combined with the limits inherent in the techniques used for quantification, preclude the ability to discern whether the combined effect of inhibiting both PKA and Rac is synergistic (suggesting they act at different points in the same pathway) or simply additive (suggesting a potentially common point of action). Despite these limitations, the data clearly show that the combined inhibition of PKA and Rac has a cumulative effect on PDGF-induced formation of PIP₃-rich ruffles.

In contrast to the inhibitory effects on PIP₃ dynamics elicited by expression of dominant-negative Rac, expression of constitutively active (Q61L) Rac rescued the defect in PDGF-induced membrane ruffling observed in PKA-inhibited cells. Transfection of cells with Q61L Rac and empty mCherry increased basal ruffling compared with control-transfected cells (supplemental Fig. 3). However, cells expressing Q61L Rac were still able to respond robustly to PDGF (Fig. 7B). Cells co-expressing mCherry-PKI and Q61L Rac (Fig. 7, A and B, right panels; supplemental Movie 8C) ruffled in response to PDGF just as efficiently as cells expressing Q61L Rac alone (Fig. 7, A and B, right panels; supplemental Movie 8A), as clearly evidenced in kymographs by a dramatic increase in the number of inflection points observed following the addition of PDGF (Fig. 7B). Cells co-expressing mCherry-PKI and Q61L Rac were still able to respond robustly to PDGF (Fig. 7B). Cells co-expressing mCherry-PKI and Q61L Rac (Fig. 7, A and B, right panels; supplemental Movie 8C) ruffled in response to PDGF just as efficiently as cells expressing Q61L Rac alone (Fig. 7, A and B, right panels; supplemental Movie 8A), as clearly evidenced in kymographs by a dramatic increase in the number of inflection points observed following the addition of PDGF (Fig. 7B). Cells co-expressing mCherry-PKI and Q61L Rac (Fig. 7, A and B, right panels; supplemental Movie 8C) ruffled in response to PDGF just as efficiently as cells expressing Q61L Rac alone (Fig. 7, A and B, right panels; supplemental Movie 8A), as clearly evidenced in kymographs by a dramatic increase in the number of inflection points observed following the addition of PDGF (Fig. 7B).
mediated regulation of PI3P and membrane ruffling at play activity, clearly suggest otherwise. Thus, to determine whether growth factor-induced ruffling and PI3P dynamics in Q61L
to restore the formation of PI3P-rich membrane ruffles in response to PDGF.

The positive feedback loop connecting Rac to PI3P is thought to involve a complex and unclear path that ultimately relies on PI3K activity (54–57). Thus, if the effect of PKA on membrane ruffling was attributable solely to an effect on Rac activation, and Rac is supposed to enhance PI3K activity, one might expect that inhibition of PKA would have an effect on PI3K activity (through inhibition of the positive feedback loop). However, the data in Fig. 5C, showing no effect of PKA inhibition on PI3K activity, clearly suggest otherwise. Thus, to determine whether there might be another PI3K-independent mechanism for Rac-mediated regulation of PI3P and membrane ruffling at play here, and whether that mechanism might be dependent on PKA, we investigated the effect of wortmannin on Q61L Rac-induced ruffling with or without co-expression of PKI. Remarkably, although inhibition of PI3K activity with wortmannin completely inhibited growth factor-induced morphological responses in untransfected and control-transfected cells (supplemental Movie 9), it did not inhibit basal (data not shown) or growth factor-induced ruffling and PI3P dynamics in Q61L Rac-expressing cells (Fig. 7, D and F; supplemental Movie 10). Indeed, a small increase was observed. This lack of inhibition was observed at wortmannin concentrations as high as 20 μM (data not shown). These data show that both Rac and PKA contribute to PI3P accumulation and dynamics through mechanisms that do not involve direct regulation of PI3K activity on the overall pool of available PI3P. Nonetheless, our data suggest that the accumulation of PI3P in ruffles does not require synthesis of new PI3P in situ but may rather be more a product of re-localization or transport of existing of PI3P. Furthermore, it appears that constitutive activation of Rac in PKA-deficient cells is sufficient to restore the formation of PI3P-rich membrane ruffles in response to PDGF, even in the absence of PI3K activity. This suggests that the events downstream of activated Rac that promote PI3P dynamics and ruffling do not seem to require PKA activity, which in turn places the role of PKA firmly upstream of Rac. These data, plus those showing that inhibition of PKA prevents accumulation of PI3P and recruitment of endogenous PH domains to the plasma membrane, establish that PKA exerts a critical function at a point downstream of PI3K but upstream of Rac in the formation of PI3P and regulation of PI3P-rich membrane ruffling in the chemotactic response to PDGF (Fig. 8).

DISCUSSION

We and others have previously reported an important role for PKA activity during cell migration (24, 31); however the mechanism(s) through which PKA regulates the cell migration machinery remained largely unknown. In this regard, the present findings advance our knowledge by demonstrating that PKA activity presides over PDGF-induced membrane ruffling and PI3P dynamics. Here we report that in response to PDGF, PKA is required for the following: 1) the formation of peripheral...
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membrane ruffles, 2) the bulk accumulation of PIP₃, 3) the proper localization and polarization of 3-PIs to the peripheral membrane ruffles, and 4) the activation of Rac, a downstream effector of PIP₃. Importantly, the defects observed in membrane ruffling and 3-PI localization in the PKA-inhibited cells were rescued upon expression of constitutively active Rac independent of PI3K activity. These results strongly support the idea that one function of PKA during growth factor-induced chemotaxis is to regulate Rac-dependent membrane ruffling events through the modulation of 3-PI lipid dynamics.

As discussed previously, growth factor stimulation of fibroblasts is but one example, albeit a canonical one, of chemotactic movement. PIP₃ and other 3-PI lipids have been implicated as direct mediators of polarized migration and cell speed in several cell types, including neutrophils, D. discoideum, and fibroblasts (60–62). Although the Akt-PH domain principally binds PIP₃, it can also recognize phosphatidylinositol 3,4-bisphosphate, which is also asymmetrically distributed in migrating cells. Thus, although our biochemical data clearly demonstrate the effect of PKA on PIP₃, it is possible that the effects of PKA might also extend to this increasingly important phospholipid as well.

This study demonstrates a role for PKA in modulating PIP₃ dynamics during growth factor signaling; however, the precise target for PKA in this regard remains to be identified. Our results suggest that this target is not PI3K, as inhibition of PKA had no discernible effect on PI3K activity. Therefore, the effect of PKA is likely to be mediated through an alternative pathway. An obvious possibility is that PKA may negatively regulate one or more PIP₃ lipid phosphatases, such as PTEN (phosphatase and tensin homolog) or SHIP2 (Src homology (SH2) domain-containing inositol phosphatase-2), and thus inhibition of PKA may allow phosphatase activity and subsequent breakdown of PIP₃. However, there are no existing reports connecting PKA to these (or other) lipid phosphatases, and neither PTEN nor SHIP2 contains strong consensus sites for direct phosphorylation by PKA. Furthermore, we were
that the situation in vivo is, not surprisingly, far more complex. Specifically, Rac-dependent PIP₃ synthesis within this feedback loop seems to require Rac-mediated actin polymerization (54–56), and thus it is unlikely to be driven by direct activation of PI3K by Rac. How Rac-driven actin dynamics promote PIP₃ synthesis is unclear, but proposed possibilities include regulation of lipid phosphatases, synthesis, or delivery of substrate to active PI3K, or an undefined scaffolding function that coordinates multiple factors and/or enzymatic steps (54–56). An intriguing hypothesis is that Rac does not activate PI3K but rather provides, in an actin-dependent manner, a steady supply of substrate to an already fully activated PI3K, adding fuel to the fire, so to speak. Indeed, recent work demonstrates that unlike overexpression of constitutively active Rac, synthetic activation of endogenous Rac does not promote the production of PIP₃ (54) and is insufficient to initiate the positive feedback loop to PI3K-driven PIP₃ synthesis, even though this mode of activation is sufficient to promote protrusion and membrane ruffling (53, 54). This is consistent with earlier work showing that induction of endogenous Rac activity was insufficient to promote PIP₃ synthesis but could support this function with coincident addition of growth factor (i.e., nerve growth factor) (53). In this context, it is important to recall our current data in which the high basal level of ruffling elicited by Q61L Rac was further enhanced by PDGF stimulation, suggesting that the signals emanating from activated receptor tyrosine kinases provide more to the processes of PIP₃ synthesis and membrane ruffling than what is mediated by Rac alone.

What aspect of our data, then, definitively argues against the effects of PKA on ruffling/PIP₃ being mediated solely by effects on Rac itself, and not one level upstream at PIP₃? One might contend that if PKA affected an event further upstream than Rac, it would be expected that inhibition of PKA might have a slightly greater effect on membrane ruffling than inhibition of Rac. However, our data suggest that inhibition of Rac was equally effective as inhibition of PKA at blocking the ruffling response. The strong effect of dominant-negative Rac is not surprising, in and of itself, as inhibition of Rac has often been shown to inhibit ruffling responses (53–55, 58), and Rac activity is required for the positive feedback loop to PI3K-driven PIP₃ synthesis (53, 55, 56). It must be pointed out, however, that the experiments in Fig. 6 measure the dynamics of PIP₃ in membrane ruffles but do not directly assess the actual cellular levels of PIP₃. To address this question properly through these means would require being able to reliably discern the difference between a complete lack of PIP₃ synthesis and the amount synthesized in the absence of positive feedback. This amount of PIP₃ is likely to be very slight and to require high sensitivity, as well as high temporal and spatial resolution to detect, and thus would be quite technically challenging. Importantly, although the amount of PDGF-stimulated PIP₃ in the absence of PKA activity does appear to be slightly above basal, unstimulated levels (Fig. 5B, lanes 4 and 5), this amount is apparently not sufficient to support the membrane recruitment of the PH domain of Akt (Figs. 3, 4, and 5A). Thus, it is to be expected that PH domain-containing GEFs would show a similar lack of membrane recruitment, which would therefore preclude Rac activation. These data would argue strongly for the locus of

**PKA Regulates PIP₃ Dynamics**

**FIGURE 8. A model depicting possible connections between PKA, PIP₃, and Rac in PDGF-induced membrane ruffling.** PDGF-induced accumulation of PIP₃ occurs in two phases. First, an initial receptor-proximal, PI3K-dependent phase generates an early and likely modest amount of PIP₃ that is Rac-independent, but that promotes Rac activation through recruitment of Rac GEFs containing PIP₃-binding PH domains. A later phase then results from a positive feedback loop in which Rac promotes PIP₃ accumulation through an as-yet undetermined mechanism. This may involve either direct activation of PI3K and/or a more circuitous mechanism requiring Rac-mediated actin polymerization (see text for details). This study shows that inhibition of PKA limits initial PIP₃ accumulation through an unclear mechanism that does not involve regulation of PI3K activity. This prevents PH-domain recruitment to membranes, Rac activation, and the feed-forward loop required for further, robust PIP₃ accumulation. These deficits conspire to inhibit growth-factor-induced membrane ruffling.
PKA Regulates PIP$_3$ Dynamics

effect of PKA to occur upstream of both Rac activation and growth factor-mediated PIP$_3$ accumulation. Based on the following observations that inhibition of PKA: 1) has no effect on PDGF-induced PI3K activity; 2) all but ablates the PDGF-induced accumulation of PIP$_3$ (a necessary, upstream component of Rac activation); 3) is sufficient to prevent membrane recruitment of PH domain containing proteins; and 4) blocks PDGF-induced activation of Rac, we submit that PKA exerts a critical function at a point downstream of PI3K but upstream of Rac in the formation of PIP$_3$ and regulation of PIP$_3$-rich membrane ruffling (Fig. 8).

Despite the prominence of both Rac and PIP$_3$ in most other chemotactic models, it is evident that the mechanisms that regulate and connect these factors may differ among them (53–55). Thus, it would be of significant interest and importance to ascertain whether the effects of PKA on lipid dynamics during cell motility observed here also occur in other cell types (e.g. epithelial/endothelial cells, neutrophils) and in response to other motogenic stimuli (e.g. chemokines, G-protein-coupled receptor agonists). In addition to these considerations, future experiments will focus on identification of the discrete targets for PKA that mediate its effects on PIP$_3$, as well as investigating how PKA anchoring influences lipid dynamics during migration. Identification of the AKAPs that target PKA to various cytoskeletal and membrane domains during chemotaxis, as well as the identification of PKA substrates in those regions, are important and ongoing efforts and will likely provide further insights as to how the activity of PKA can specifically modulate distinct aspects of the complex process of cell migration.

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Addendum—While this study was under revision, the Ginsberg Laboratory reported integrin-mediated activation of PKA at the leading edge of cells (Lim, C. J., Kain, K. H., Tkachenko, E., Goldfinger, L. E., Gutierrez, E., Allen, M. D., Groisman, A., Zhang, J., and Ginsberg, M. H. (2008) Mol. Biol. Cell 19, 4930–4941). This activation was not dependent on PI3K activity but, consistent with our results, was required for polarized accumulation of Akt-PH-EGFP to the leading edge.

REFERENCES


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