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The Lipid Products of Phosphoinositide 3-Kinase Increase Cell Motility through Protein Kinase C*

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Phosphoinositide 3-kinase has been implicated as an activator of cell motility in a variety of recent studies, yet the role of its lipid product, phosphatidylinositol 1,4,5-trisphosphate (PtdIns-3,4,5-P3), has yet to be elucidated. In this study, three independent preparations of PtdIns-3,4,5-P3 were found to increase the motility of NIH 3T3 cells when examined utilizing a microchemotaxis chamber. Dipalmitoyl 1-α-phosphatidyl-L-phosphatidylinositol 3,4,5-trisphosphate (Di-C16-PtdIns-3,4,5-P3) also produced actin reorganization and membrane ruffling. Cells pretreated with 12-O-tetradecanoylphorbol-13-acetate to cause down-regulation of protein kinase C (PKC) exhibited complete inhibition of cell motility induced by Di-C16-PtdIns-3,4,5-P3. These results are consistent with previous observations that PtdIns-3,4,5-P3 activates Ca2+-independent PKC isoforms in vitro and in vivo and provide the first demonstration of an in vivo role for the lipid products of the phosphoinositide 3-kinase. PtdIns-3,4,5-P3 appears to directly initiate cellular motility via activation of a PKC family member.

Initiation of cellular motility has been demonstrated with multiple growth factors, including platelet-derived growth factor (PDGF)1 (1), hepatocyte growth factor (HGF) (2), and insulin (3). The mechanisms whereby cells undergo chemotaxis (directional cell movement) and chemokinesis (random cell movement) are complex, requiring dissolution of cell-cell contacts (such as tight junctions in epithelial cells) and cell-surface contacts, formation of lamellipodia, actin filament severing and nucleation, and finally contraction of the actin filament network leading to movement of the cell body (4). An understanding of the signaling pathways required to orchestrate these cellular events should provide critical new insights into numerous biological events such as cell migration and organization during organ development and wound healing, tumor cell metastasis, and progression of arterial atherosclerotic plaques.

Mutations in the PDGF receptor that eliminate binding of phosphoinositide 3-kinase PI 3-kinase-impair PDGF-dependent chemotaxis (1, 5, 6), and selective activation of the PI 3-kinase is sufficient to initiate motility (7). The lipid products of PI 3-kinase, PtdIns-3,4-P2, and PtdIns-3,4,5-P3 are elevated acutely in response to PDGF (8) and are thought to act as second messengers (9–11). Although the in vivo function of these lipids has not been demonstrated, they activate calcium-independent protein kinase C family members in a stereospecific manner (12–16). Thus, we investigated the possibility that PtdIns-3,4,5-P3 stimulates cell motility via activation of a PKC family member.

MATERIALS AND METHODS
Cell Culture and Reagents—The majority of experiments were performed with NIH 3T3 fibroblasts, using PDGF as the positive control. Selected experiments were repeated with mMCD-3 cells, a murine renal tubular epithelial cell line that expresses the c-PDGFR receptor and displays striking chemotaxis to a gradient of HGF (17–19). All cells were cultured in Dulbecco’s modified Eagle’s medium with 5% fetal calf serum using standard techniques. PDGF (Upstate Biotechnology, Inc., Lake Placid, NY) and HGF (Institute of Immunology, Tokyo, Japan) were used in concentrations of 10 and 40 ng/ml, respectively, based on previous dose response curves for maximal chemotaxis (19).

PtdIns-3,4,5-P3 was obtained from Upstate Biologicals, and phosphatidylserine (PtdSer) was from Avanti Polar Lipids. Diacylglycerol (DAG) and horseradish anti-mouse conjugate were purchased from Boehringer Mannheim. 12-O-Tetradecanoylphorbol-13-acetate (TPA) was obtained from Life Technologies, Inc., and wortmannin was from Sigma. Calphostin C was obtained from Calbiochem, and P81 phosphocellulose paper was purchased from Whatman. Thin layer chromatography plates (Silica Gel 60) were obtained from EM Separations.

Preparation of PtdIns-3,4,5-P3 from PtdIns-3,4,5-P2—Phosphoinositide 3-kinase was purified from rat liver cytosol as described previously (20) and used immediately for the preparation of PtdIns-3,4,5-P3. Lipid substrates were prepared by drying under a stream of nitrogen. PtdSer (10 mg/ml) was added to the PtdIns-3,4,5-P3 (2 mg/ml) as a carrier, and the mixture was sonicated in 10 mM Hepes, pH 7.0, 1 mM EDTA for 10 min using a bath sonicator. This mixture was then incubated with phosphoinositide 3-kinase at 37 °C in the presence of 50 μM [γ-32P]ATP (3000 Ci/mmol), 5 mM MgCl2, 50 mM Hepes, pH 7.5, for 60 min. The reaction (200 μl) was stopped by the addition of 65 μl of 5 N HCl, and lipids were extracted in 400 μl of CHCl3/MeOH (6:4). Lipids were dried and stored at −70 °C until needed (21). PtdIns-3,4,5-P3 was quantified by thin layer chromatography (napropanol, 2 M acetic acid extract (65:35) and radiation detection on a Bio-Rad molecular imager. Based on the specific activity of the [γ-32P]ATP, 20% of the PtdIns-4,5-P3 was converted to PtdIns-3,4,5-P3.

Preparation of Synthetic PtdIns-3,4,5-P3—Dipalmitoyl 1-α-phosphatidyl-L-phosphatidylinositol 3,4,5-trisphosphate (Di-C16-PtdIns-3,4,5-P3) (21) and dioleoyl-1-α-phosphatidyl-L-phosphatidylinositol 3,4,5-trisphosphate were synthesized as described previously (13, 22).

Chemotaxis Assay—Chemotaxis was evaluated using a modified Boyden chamber assay with a 48-well microchemotaxis chamber as described previously (Neuro Probe Inc., Cabin John, MD) (19, 23). Lipids...
FIG. 1. **PtdIns-3,4,5-P3 enhances motility of NIH 3T3 fibroblasts (A and B) and IMCD epithelial cells (C).** Cell motility was evaluated using a modified Boyden chamber assay with a 48-well microchemotaxis chamber. A, the lower section of the Boyden chamber was filled with media alone or media containing PDGF, PtdSer/PtdIns-4,5-P2 lipid substrate, or enzymatically generated PtdIns-3,4,5-P3. B and C, either chemically

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were dried in a stream of nitrogen and then sonicated for 5 min in serum-free media. The lower section of the Boyden chamber was filled with media alone or media containing either PDGF (10 ng/ml) or PtdSer/PtdIns-4,5-P$_2$ (100 µM/25 µM) or PtdSer/PtdIns-4,5-P$_2$/PtdIns-3,4,5-P$_3$ (100 µM/25 µM/5 µM). A polycarbonate filter (Nuclepore Corp., Pleasanton, CA) coated with rat tail collagen type I (Collaborative Biomedical, Bedford, MA) was placed over the lower compartment, and 1.5 x 10$^5$ cells were added to the upper compartment. In some experiments, wortmannin (Sigma) was added. In some experiments, 10 nM wortmannin (wort) was added. The number of chambers assayed for each condition is indicated by n. *, p < 0.001 compared with PtdIns-4,5-P$_2$ control.

Enzymatically Generated and Synthetic PtdIns-3,4,5-P$_3$ Initiate Cell Motility and Ruffling—Since exogenously added lipid indicated. In some experiments, 10 nM wortmannin (wort) was added. The number of chambers assayed for each condition is indicated by n. *, p < 0.001 compared with PtdIns-4,5-P$_2$ control.
m was chosen for further experiments, since this was the lowest dose that consistently resulted in a chemotactic response.

The polymerization of cytoplasmic actin that follows receptor stimulation and leads to membrane ruffling and lamellipodia formation is felt to be downstream of the PI 3-kinase (6, 25). To test this hypothesis, we evaluated actin filament reorganization and membrane ruffling following the addition of Di-C16-PtdIns-3,4,5-P3 (Fig. 3). The synthetic form of PtdIns-3,4,5-P3 stimulated membrane ruffling in 3T3 fibroblasts to the same extent as PDGF. PtdIns-4,5-P2 had no effect on quiescent cells.

Exogenously Added PtdIns-3,4,5-P3 Does Not Activate Endogenous PI 3-Kinase and Is Not Inhibited by Wortmannin—It was conceivable that a contaminant or a breakdown product of PtdIns-3,4,5-P3 might initiate the observed effects via activation of a cell surface receptor (as has been shown for lysophosphatidic acid). Although this seemed unlikely, since PtdIns-3,4,5-P3 made by three different procedures stimulated cell motility and comparable concentrations of PtdIns-4,5-P2 and/or PtdSer failed to stimulate cell motility, we searched for evidence that exogenously added PtdIns-3,4,5-P3 might act via cell surface receptor activation by examining intracellular production of PtdIns-3,4-P2 and PtdIns-3,4,5-P3 in 32PO4-labeled NIH 3T3 cells following the addition of extracellular Di-C16-PtdIns-3,4,5-P3. This approach was chosen because the receptors known to initiate chemotaxis (PDGF receptor, insulin receptor, c-met receptor, lysophosphatidic acid receptor) have also been found to activate the PI 3-kinase (8, 26–28). While stimulation with PDGF produced a dramatic rise in intracellular [32P]PtdIns-3,4-P2 and [32P]PtdIns-3,4,5-P3 (2.3- and 17-fold), no increase in either of these lipids was seen in cells treated with 5 μM Di-C16-PtdIns-3,4,5-P3.

![Fig. 3. Membrane ruffling by cells exposed to Di-C16-PtdIns-3,4,5-P3 mimics the response seen with PDGF. The top panels show that quiescent cells and cells exposed to 5 μM PtdIns-4,5-P2 (PIP2) are no different, whereas significant membrane ruffling can be seen in the cells exposed to either 40 ng/ml PDGF or 5 μM PtdIns-3,4,5-P3 (PIP3) at 10 (middle panels) and 30 min (bottom panels).](image-url)

![Fig. 4. TPA down-regulates PKCe in 3T3 cells. NIH 3T3 cells were treated for 12 h with either vehicle control (−) or 300 nM TPA (+) followed by SDS-polyacrylamide gel electrophoresis and immunoblotting with an antibody specific for PKCe. Densitometric analysis of the blot revealed 35.9 ± 4.6 densitometric units for control PKCe versus 8.2 ± 0.9 for cells pretreated with TPA (experiment performed in triplicate; p = 0.001).](image-url)
The PI 3-kinase inhibitor wortmannin binds irreversibly to the catalytic subunit of the enzyme and prevents production of the D3 phosphorylated lipid products of the enzyme. 10 nM wortmannin, the lowest dose that produces reliable inhibition of the PI3-kinase in vivo in 3T3 fibroblasts and mIMCD-3 cells (19), caused a 60% inhibition of PDGF- and HGF-dependent cell motility but had no effect on Di-C16-PtdIns-3,4,5-P3-stimulated cell movement in 3T3 cells or in IMCD cells (Fig. 1, B and C). These results demonstrate that wortmannin at a dose that inhibits PDGF receptor-mediated activation of the PI 3-kinase does not prevent PtdIns-3,4,5-P3-initiated motility, further supporting the hypothesis that these lipids are inserting into the membrane and directly initiating downstream signaling events.

100 nM wortmannin, a concentration where effects on several other kinases have been observed, caused essentially complete inhibition of both PDGF- and Di-C16-PtdIns-3,4,5-P3-stimulated cell motility (data not shown). In light of the observation by Kundra et al. (1) that selective activation of phospholipase Cγ by the PDGF receptor resulted in a substantial chemotactic response, even in the absence of PI 3-kinase activation, this result suggests that other targets of wortmannin that are likely to be inhibited at the higher concentration, such as myosin light chain kinase (29) or PtdIns 4-kinase (30), may be critical for cell motility as well.

Inhibition of PKC Prevents PtdIns-3,4,5-P3-mediated Cell Motility—It was previously shown that activation of PKC by DAG or TPA can stimulate chemotaxis (31–33). Therefore, we examined the role of activation of PKC in PtdIns-3,4,5-P3-mediated chemotaxis. 100 μM DAG produced a consistent increase in motility of NIH 3T3 cells (control, 1.3 ± 0.2 cells/mm²; DAG, 116.7 ± 13.4, p < 0.001). Of note, 5 μM DAG fails to induce chemotaxis, while 5 μM Di-C16-PtdIns-3,4,5-P3 does, indicating that the PtdIns-3,4,5-P3 effect is not due to hydrolysis to DAG. When DAG and Di-C16-PtdIns-3,4,5-P3 were both present in the bottom well, the chemotactic rate was similar to that seen with DAG alone (Di-C16-PtdIns-3,4,5-P3, 55.5 ± 4.5 cells/mm²; DAG, 116.7 ± 13.4; Di-C16-PtdIns-3,4,5-P3 with DAG, 127.3 ± 19.5, n = 12), suggesting that these two stimuli were acting via the same signaling pathway.

To examine this possibility, the TPA-activable PKC family members were down-regulated by overnight preincubation of NIH 3T3 cells with 300 nM TPA. Under these conditions, there was a 78% decline in the concentration of PKCe by Western analysis (Fig. 4), an effect comparable with that seen in human dermal fibroblasts (34). PKCe was chosen because it shows the greatest activation to PtdIns-3,4,5-P3 in vitro and in vivo. The migratory response to PtdIns-3,4,5-P3 was completely eliminated in NIH 3T3 cells pretreated with TPA (Fig. 5A), while PDGF-mediated cell movement was inhibited by 70%, a finding similar to that seen with exposure to 10 nM wortmannin. In addition, the specific PKC inhibitor calphostin C was tested (35, 36). NIH 3T3 cells exposed to 100 nM calphostin C (IC50 = 75–100 nM) for 30 min demonstrated a 90% reduction in PtdIns-3,4,5-P3-mediated cell motility (Fig. 5B). These results suggest that activation of the PI 3-kinase mediates cell motility via the local generation of PtdIns-3,4,5-P3 and subsequent activation of PKC.

DISCUSSION

The PI 3-kinase has been clearly implicated in cell motility by several laboratories (1, 6, 7, 19), yet the actual mechanism of...
this effect is poorly understood. The p85 subunit of the PI 3-kinase has a BCR homology domain, which is capable of binding GTP-Rac (37, 38) and may therefore act to recruit activated Rac or associated family members to the membrane where these signaling proteins have been shown to initiate motility (39, 40). In addition, PtdIns-3,4,5-P$_3$ has been shown to be capable of activating the actin-severing protein, gelsolin (28). The present results demonstrate that PtdIns-3,4,5-P$_3$, the lipid product of the PI 3-kinase, is capable of directly initiating cell motility and that this effect is mediated by activation of PKC. The greatest effect on cell motility occurred as directional movement toward a gradient of PtdIns-3,4,5-P$_3$ (i.e. chemotaxis), while non directional movement increased as well.

The model we propose to explain the ability of exogenously added PtdIns-3,4,5-P$_3$ to stimulate cell motility requires that some fraction of the lipid fuse with the plasma membrane and arrive at the inner leaflet over the 4-h assay period. There it can act similarly to endogenous PtdIns-3,4,5-P$_3$. This might occur by different mechanisms for the different PtdIns-3,4,5-P$_3$ isoforms. The enzymatically produced PtdIns-3,4,5-P$_3$, sonicated in the presence of excess PtdSer, is expected to be distributed in both the inner and outer leaflets of the newly formed vesicle. Fusion of these vesicles with the plasma membrane would presumably result in PtdIns-3,4,5-P$_3$ in both leaflets. The Di-C$_{16}$PtdIns-3,4,5-P$_3$ forms micelles when sonicated and is likely to cause local detergent-like effects when fusing with the plasma membrane, resulting in distribution of this lipid on both leaflets of the plasma membrane as well.

Recently, PKCz as well as the atypical PKCz isofrom have been found to be activated downstream of the PI 3-kinase in PDGF- and epidermal growth factor-stimulated cells (15, 16). In addition, several researchers have demonstrated that PtdIns-3,4,5-P$_3$ can directly activate calcium-insensitive PKC family members in vitro (12–14). Our results demonstrate that activation of the phorbol-sensitive PKC family members enhances cell motility in a fashion similar to that seen with PtdIns-3,4,5-P$_3$, while both down-regulation of PKC by over-night treatment with TPA and inhibition of PKC with calphostin C completely blocked the PtdIns-3,4,5-P$_3$ response. The present data cannot distinguish which PKC family member or members are directly involved, although in vitro data suggest that the PKCz is strongly up-regulated by these lipids. The observation that high concentrations of PtdIns-4,5-P$_2$ caused a modest increase in cell motility (Figs. IA and 2) is consistent with this hypothesis, since this polyphosphoinositide has also been found to weakly activate PKC in vitro (13).

In addition to PKC activation, there are several other targets for the lipid products of the PI 3-kinase. pp70$^{56k}$ was the first target shown to be downstream of PI 3-kinase (42), and recently, Akt (41, 43) and Rac (38) have also been implicated. The latter is of particular interest, since microinjection of constitutively active forms of rac leads to membrane ruffling (39). The recent availability of synthetic forms of these phosphoinositides should help identify their targets and determine the pathways that lead to the motile response.

Previous results from our laboratory and others have shown that activation of the PI 3-kinase is essential for PDGF and HGF-dependent cell movement. The present experiments demonstrate that the lipid products of the PI 3-kinase act directly as second messengers in cell motility and provide the first indication that PKC family members are required for the motility effects of this lipid in vitro.

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