The signaling pathway that triggers morphological differentiation of PC12 cells is mediated by extracellular signal-regulated kinase (ERK), the classic mitogen-activated protein (MAP) kinase. However, mediators of the pathway downstream of ERK have not been identified. We show here that phospholipase D2 (PLD2), which generates the pleiotropic signaling lipid phosphatidic acid (PA), links ERK activation to neurite outgrowth in nerve growth factor (NGF)-stimulated PC12 cells. Increased expression of wild type PLD2 (WT-PLD2) dramatically elongated neurites induced by NGF stimulation or transient expression of the active form of MAP kinase-ERK kinase (MEK-CA). The response was activity-dependent, because it was inhibited by pharmacological suppression of the PLD-mediated PA production and by expression of a lipase-deficient PLD2 mutant. Furthermore, PLD2 was activated by MEK-CA, whereas NGF-stimulated PLD2 activation and hypertrophic neurite extension were blocked by an MEK-specific inhibitor. Taken together, these results provide evidence that PLD2 functions as a downstream signaling effector of ERK in the NGF signaling pathway, which leads to neurite outgrowth by PC12 cells.

Axonal outgrowth and guidance are critical events in the establishment of neuronal networks during brain development and are regulated by extracellular guidance cues such as chemoattractants and chemorepellents (1, 2). The attractant and repellent signals are sensed by growth cones at the tips of axons and on the other hand, Rac has been implicated in axonal outgrowth (12) via controlling the neuron-specific, cytoplasmic GTPases, Rho, Rac, and Cdc42, direct the axonal extension by reorganizing the growth cone actin cytoskeleton (3–6). RhoA regulates the repulsive signaling pathway through its target molecule Rho-associated kinase, termed ROCK (7–9), which in turn regulates myosin II and LIM kinase (8, 9). We and others have also recently reported that phosphatidylinositol 4-phosphate 5-kinase (PI4P 5-kinase), which phosphorylates phosphatidylinositol 4-phosphate at the D-5 position of the inositol ring to produce the versatile lipid signaling molecule phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2), functions as a downstream effector of Rho/ROCK in lysophosphatic acid-induced neurite retraction for mouse neuroblastoma N1E-115 cells (10, 11). On the other hand, Rac has been implicated in axonal outgrowth (12) via controlling the neuron-specific, cytoplasmic GTPases, Rho, Rac, and Cdc42, direct the axonal extension by reorganizing the growth cone actin cytoskeleton (3–6). RhoA regulates the repulsive signaling pathway through its target molecule Rho-associated kinase, termed ROCK (7–9), which in turn regulates myosin II and LIM kinase (8, 9). We and others have also recently reported that phosphatidylinositol 4-phosphate 5-kinase (PI4P 5-kinase), which phosphorylates phosphatidylinositol 4-phosphate at the D-5 position of the inositol ring to produce the versatile lipid signaling molecule phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2), functions as a downstream effector of Rho/ROCK in lysophosphatic acid-induced neurite retraction for mouse neuroblastoma N1E-115 cells (10, 11). On the other hand, Rac has been implicated in axonal outgrowth (12) via controlling the neuron-specific, cytoplasmic GTPases, Rho, Rac, and Cdc42, direct the axonal extension by reorganizing the growth cone actin cytoskeleton (3–6).

Another signaling pathway intermediate well known to be involved in regulating neurite outgrowth is the classic mitogen-activated protein (MAP) kinase, extracellular signal-regulated kinase (ERK). Neurite outgrowth from neurons and neuronal cell lines induced by stimulation of neuronal cell adhesion molecules, such as L1, or by neurotrophines, such as nerve growth factor (NGF), absolutely requires the activation of ERK (15, 16). The one or more downstream signaling pathways that couple ERK to neurite outgrowth, however, have not yet been clarified.

A candidate for signaling downstream of ERK would be the lipid-metabolizing enzyme, phospholipase D2 (PLD2). PLD catalyzes the hydrolysis of the major membrane phospholipid phosphatidylcholine to produce the pleiotropic signaling lipid messenger phosphatidic acid (PA). The mammalian PLD family consists of two related gene products, PLD1 and PLD2 (17, 18). PLD1 is directly regulated by classic protein kinase C and members of the ADP-ribosylation factor and Rho family small

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**Footnotes:**

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‡ The abbreviations used are: PI4P 5-kinase, phosphatidylinositol 4-phosphate 5-kinase; PLD, phospholipase D; PA, phosphatidic acid; PEt, phosphatidylethanol; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; MEK, MAP kinase-ERK kinase; GFP, green fluorescent protein; NGF, nerve growth factor; PBS, phosphate-buffered saline; BSA, bovine serum albumin; WT, wild type; LD, lipase-deficient; MEK-CA, active form of MEK; HA, hemagglutinin; HRP, horseradish peroxidase.
GTPases in conjunction with polyphosphoinositides, PI(4,5)P₂ and phosphatidylinositol 3,4,5-trisphosphate (17). This PLD isozyme appears to play substantial roles in a wide variety of cellular signaling pathways, membrane vesicle trafficking, and exocytosis (19–22). In contrast to PLD1, little is known regarding the physiological functions and regulatory mechanisms of PLD2, which is constitutively active in the presence of polyphosphoinositides and is fundamentally insensitive to PLD1 activators in vitro (18). We previously reported that PLD2, but not PLD1, translocates to actin-based membrane ruffles produced by epidermal growth factor stimulation of HeLa cells (23), indicating that PLD2, possibly through its product PA, is involved in membrane dynamics. Actin-based membrane dynamics in the growth cone is also a critical event for neurite outgrowth in response to chemoattractants (24). Taken together, these findings led us to speculate that PLD2 is involved in the mechanisms of neurite remodeling. This hypothesis is supported by the report that PLD2 mRNA levels increase strikingly in dentate granule cells in the pre-natal hippocampus and the granule cell layer in cerebellum at early post-natal life (25, 26), in coincidence with the period during which maximal numbers of neurons are differentiating and pathfinding (27). Moreover, Parmentier et al. (28) have reported that PLD2 activation through norepinephrine stimulation of rabbit vascular smooth muscle cells is dependent upon ERK activation. These reports prompted us to investigate whether PLD2 serves as a signal-transducing enzyme downstream of ERK in neurite outgrowth. Since PC12 cells have been well established as a model system in which ERK plays a crucial role in NGF-induced neurite outgrowth (29–31), we established PC12 cell clones that inducibly express wild type (WT) and lipase-deficient (LD) mutants of PLD1 and PLD2. Here we show that PLD2, but not PLD1, is involved in NGF-stimulated neurite outgrowth from PC12 cells. PLD2 activity is up-regulated by ERK upon stimulation of PC12 cells with NGF, and we show that this is functionally critical. These results provide evidence that PLD2 is a novel signaling molecule in the pathway that mediates signaling from ERK to neurite outgrowth in PC12 cells.

MATERIALS AND METHODS

Plasmids and Antibodies—cDNAs for WT-PLD1 and PLD2 and their LD mutants were constructed as described previously (32) and subcloned into pTRE vector (Clontech Laboratories, Palo Alto, CA) to establish PC12 cell clones in which these proteins can be inducibly expressed. To transiently express WT-PLD2 in PC12 cells, the cDNA for WT-PLD2 was subcloned into pCGN (pCGN-WT-PLD2) (32). The expression plasmid for a constitutively active form of MAP kinase-ERK kinase (MEK-CA), pSRs-HA-MEK-CA, was a generous gift of Dr. E. Nishida (Kyoto University, Japan). The expression plasmid for an green fluorescent protein (GFP), pEGFP-N3, was purchased from Clontech Laboratories.

A rabbit polyclonal anti-PLD2 D4 antibody that recognizes both PLD1 and PLD2 was raised against the bacterial recombinant peptide corresponding to amino acids 714–1074 of PLD1 and then affinity-purified. A rat monoclonal anti-mouse PLD2 411A antibody that specifically recognizes mouse PLD2 was generated by a rat medial iliac lymph node method (33). In brief, WKY/NCrj rats were immunized with 0.2 mg of a rat monoclonal anti-mouse PLD2 411A antibody that specifically recognizes mouse PLD2, and spleen cells were fused with SP2/O myelomas. The hybridoma clones that produce the anti-PLD2 antibody were selected by enzyme-linked immunosorbent assay and Western blotting. The following antibodies and probes were used: anti-ERK2 D2 and -phosphorylated ERK E-4 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); rat anti-PLD1 and -PLD2 antibodies (Roche Applied Science, Indianapolis, IN); anti-GFP antibody (MBL, Nagoya, Japan); horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (Amersham Biosciences, Arlington Heights, IL); HRP-conjugated anti-mouse IgG antibody (BD Transduction Laboratory, Lexington, KY); fluorescein isothiocyanate-conjugated anti-rat IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA); and Alexa 488-conjugated anti-rabbit IgG antibody and rhodamine phalloidin (Molecular Probes, Eugene, OR). Establishment of PC12 Clonal Cell Lines and Cell Culture—pTRE plasmids (40 μg each) encoding WT- and LD-PLD isozymes were co-transfected with 2 μg of pTK-Hyg (Clontech) into the PC12 Tet-Off cell line (Clontech) by electroporation. Selection was performed in DMEM containing 10% fetal calf serum, 5% horse serum, 100 μg/ml G418, 200 μg/ml hygromycin B, and 2 μg/ml doxycycline. Colonies were recovered and cultured in medium A consisting of DMEM supplemented with 10% fetal calf serum, 5% horse serum, 100 μg/ml G418, and 100 μg/ml hygromycin B in the presence or absence of 2 μg/ml doxycycline for 3 days. The expression levels of PLDs in the membrane fraction (20 μg of protein) were analyzed by Western blotting. Transfectant clones exhibiting substantial tetracycline responses were selected and maintained in medium A in the presence of 2 μg/ml doxycycline.

To assess neurite outgrowth, PC12 clonal cells were cultured on Type I collagen-coated coverslips for 2 days in medium A, followed by further culture for 1 day in medium B consisting of DMEM supplemented with 1% horse serum, 100 μg/ml G418, and 100 μg/ml hygromycin B in the presence or absence of doxycycline, unless otherwise mentioned. In most experiments in which PLD activity was assayed, the PC12 clonal cells were cultured for 3 days in medium A with or without doxycycline on Type I collagen-coated dishes (IWAKI, Chiba, Japan).

Transient Expression of MEK-CA and PLD2—In the experiment for inhibition of NGF-induced neurite outgrowth by transiently expressed LD-PLD2 on MEK-CA-induced neurite outgrowth, MEK-CA was transiently expressed in PC12 clonal cell lines. WT- and LD-PLD2 were inducibly expressed in these PC12 cells by culturing them in the absence of doxycycline for 2 days. Non-induced controls were cultured in the presence of doxycycline. These PC12 clones were transfected with pSRs-HA-MEK-CA using LipofectAMINE Plus followed by 1 day of culture in medium A. Assay for PLD Activity—PLD activity was assessed by measuring accumulation of the unambiguous PLD activity marker [32P]phosphatidylethanolamine ([32P]PTE), which is generated in the presence of ethanol. PC12 clonal cells cultured for 3 days in medium A with or without doxycycline, or parental cells transiently expressing WT-PLD2 and/or MEK-CA, were labeled with 25 μCi/ml [32P]Pi at 37 °C for 1.5 h in Pi-free minimal essential medium (Invitrogen) supplemented with 25 mM Hepes, pH 7.5, and 1% bovine serum albumin (BSA). After being washed, the labeled cells were incubated without or with 50 ng/ml NGF (Alomone Laboratories, Jerusalem, Israel) in the presence of 1% ethanol at 37 °C for 30 min. Lipids were extracted with chloroform/methanol/acetone (65:35:6 by volume). The [32P]PTE produced was analyzed by a BAS2000 Bio-imaging analyzer (Fuji Photo Film, Tokyo, Japan) and expressed as a percentage of total [32P]phospholipids. The data are represented as the mean ± difference of duplicate determinations representative of at least three independent experiments with similar results.

Western Blotting—Western blotting was performed as previously reported with minor modification (35). In brief, proteins separated by SDS-PAGE were transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% BSA in buffer consisting of 50 mM Tris-HCl, pH 8.0, 2 mM CaCl₂, 0.5 mM NaCl, and 0.1% sodium dodecyl sulfate, incubated with primary antibodies in blocking solution, and then with HRP-conjugated secondary antibodies in blocking solution containing 0.2% SDS and 2% Nonidet P-40. Immunoreactive proteins were detected with an ECL immunoblotting detection reagent (Amersham Biosciences).

Immunofluorescent Microscopy—After being treated as described in the legends, PC12 cells were fixed, permeabilized, and blocked as previously reported (23). In brief, cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) on ice for 30 min. After permeabilization in 0.1% Triton X-100 and 0.1% Tween 20, cells were blocked with 1 mg/ml BSA in PBS, incubated with primary antibodies in PBS supplemented with 0.05% Tween 20 and then with fluorescein isothiocyanate-conjugated anti-rat IgG antibody (1:200 dilution) and Alexa 488-conjugated anti-rabbit IgG antibody (1:1000 dilution). F-actin was stained with rhodamine phalloidin (1:1000 dilution). Cells were imaged using an Axiosvert S100 Zeiss fluorescence microscope (Zeiss, Göttingen, Germany). Intra-cellular localization of overexpressed WT-PLD2 in PC12 cells was deter-
Involvement of PLD2 in NGF-induced Neurite Outgrowth of PC12 Cells—To begin examining the potential involvement of PLD in NGF-stimulated neurite outgrowth, we established PC12 clonal cell lines that inducibly express WT- and LD-PLD1 and -PLD2. We obtained two clonal cell lines that inducibly express WT-PLD2 to different levels (Fig. 1A).

Upon PLD2 induction, clone 8, which exhibited the higher level of inducible WT-PLD2 expression, flattened and extended a limited number of short neurites despite the absence of NGF stimulation, whereas clone 11, which expressed less WT-PLD2, remained spherical with protrusions slightly longer than those observed in non-induced cells (Fig. 1B). These protrusions did not extend into neurites, even if the culture period was prolonged further (data not shown).

NGF stimulation for 1 day in the absence of PLD2 induction similarly resulted in formation of relatively short neurites (Fig. 1C, top row). Upon PLD2 induction, however, NGF stimulation for 1 day elicited formation of extremely elongated neurites (Fig. 1C, bottom row). Neurites continued to extend in length in control cells treated with NGF over a 6-day period (Fig. 1D). At each time point examined, the neurites in PLD2-induced cells were longer than those in control cells. In contrast to the exaggeration of neurite length, neither neurite number per cell nor neurite branching were affected by WT-PLD2 induction (data not shown). Basal activity of PLD increased in a WT-PLD2 expression-level-dependent fashion and NGF stimulation further increased the activity (Fig. 1E), demonstrating that NGF receptor signaling is coupled to PLD2 activation. These findings suggested that PLD2 enzymatic activation could be involved in NGF-induced neurite outgrowth.

The results observed with PLD2 induction could also reflect its functioning as a scaffolding molecule or nonspecific sequestration of signaling components. To investigate whether the enhancement by PLD2 of the NGF-induced neurite outgrowth depends upon its lipase activity, the consequence of inducing an inactive PLD2 allele (LD-PLD2) was examined (Fig. 2). LD-PLD2 was inducibly expressed by 3- to 5-fold over endogenous PLD2 (Fig. 2A). Induction of LD-PLD2 expression slightly but significantly suppressed both basal and NGF-stimulated PLD activation (Fig. 2B). LD-PLD2-induced neurite outgrowth was also inhibited by induction of LD-PLD2 (Fig. 2, C and D). Suppression of the NGF-induced neurite outgrowth by LD-PLD2 was additionally demonstrated using transient expression to rule out long term effects of low level LD-PLD2 expression on the clonal cell line as a possible explanation (Fig. 2, E and F). Neurite number per cell and neurite branching were not affected by LD-PLD2 expression (data not shown), again suggesting that PLD2 is involved in neurite elongation but not neurite initiation. The observation that the inactive PLD2 allele did not promote exaggerated neurite extension demonstrates that the facilitation of exaggerated NGF-induced neurite outgrowth by wild-type PLD2 requires its lipase activity. The observation that the inactive PLD2 allele significantly suppressed neurite growth in comparison to control cells further suggested that endogenous PLD2 participates in neurite extension.

There are two PLD isoforms, PLD1 and PLD2. To evaluate whether stimulation of the NGF-induced neurite outgrowth is specific to PLD2, we examined PC12 cell clonal cell lines that inducibly expressed WT- and LD-PLD1 (Fig. 3A). In contrast to PLD2, inducible expression of WT- and LD-PLD1 was without effect on the NGF-induced neurite outgrowth and on basal and NGF-stimulated PLD activities (Fig. 3, B and C). These results, taken together with the observations described above, led us to conclude that PLD2, but not PLD1, plays a role in the NGF-triggered neurite extension.

The potential involvement of endogenous PLD2 was further explored by examining the consequence of pharmacological suppression of PLD-catalyzed PA production on control and...
induced cells (Fig. 4). Because NGF-induced neurite outgrowth was exaggerated similarly by WT-PLD2 expression in clones 8 and 11 (Fig. 1D), only clone 11 was used in further studies. When the cells were incubated with 1-butanol, which prevents PLD-catalyzed PA production by preferentially inducing PLD-mediated transphosphatidylation (production of phosphatidylbutanol), NGF-induced neurite outgrowth in both control and PLD2-expressing cells was suppressed (Fig. 4A). In contrast, 2-butanol, which cannot be used as a substrate for PLD-catalyzed transphosphatidylation, instead stimulated NGF-stimulated neurite outgrowth (Fig. 4A). Alcohols can have many effects on cells, and we don’t have a mechanistic explanation for how 2-butanol promotes neurite extension. Nevertheless, the 1-butanol-specific inhibition of neurite extension provides additional support for the hypothesis that signaling from PLD2 to NGF-induced neurite outgrowth is mediated by its product PA.

**PLD2 Involvement in ERK-Mediated Neurite Outgrowth**

Activation of ERK is essential for NGF-induced neurite outgrowth from PC12 cells (29–31), and ERK has previously been connected to PLD signaling in other contexts. However, the relationship between the ERK MAP kinase cascade and PLD2 activation remains unclear. In Rat-1 fibroblasts overexpressing the human insulin receptor, PLD2 activates the ERK MAP kinase pathway through the translocation of Raf-1 to the plasma membrane upon insulin stimulation (36). In contrast, however, for PC12 cells and rabbit vascular smooth muscle cells stimulated by hydroxyperoxide and norepinephrine, respectively, PLD2 activation has been reported to be mediated by ERK (28, 37). We therefore set out to investigate whether PLD2 functions upstream or downstream of ERK in the NGF-induced neurite extension signaling pathway in PC12 cells. As shown in Fig. 5, induction of WT- and LD-PLD2 expression did not affect NGF-induced ERK phosphorylation/activation, eliminating the possibility that PLD2 is an upstream signaling mediator in this setting.

To address the possibility that PLD2 functions downstream of the ERK MAP kinase cascade, we employed the MEK-specific inhibitor PD98059. As reported previously (29, 38), the inhibitor completely suppresses NGF-induced ERK phosphorylation/activation (Fig. 6A). Moreover, the inhibitor blocked NGF-induced neurite outgrowth from control and PLD2-expressing cells (Fig. 6, B and C), and it suppressed NGF-stimulated PLD2 activation (Fig. 6D). The inhibition was not due to a direct effect of the inhibitor on PLD2, because it failed to suppress PLD2 activity as assessed using an in vitro system (data not shown). These results demonstrate that ERK directly or indirectly activates PLD2 and that ERK stimulation of PLD2 is required for neurite outgrowth.
Finally, we employed the constitutively active MEK, MEK-CA, to confirm the epistatic relationship of ERK and PLD2 using another approach. Transient co-expression of MEK-CA and WT-PLD2 into parental cells resulted in robust PLD2 activation (Fig. 7). Furthermore, neurite outgrowth stimulated by transient expression of MEK-CA was significantly enhanced by induction of WT-PLD2 expression (Fig. 8, A and B). In contrast, LD-PLD2 expression inhibited MEK-CA-induced neurite outgrowth (Fig. 8, A and B), as did suppression of PLD-mediated PA production by 1-butanol (Fig. 8, C and D).
independent experiments; an asterisk red C and HA-MEK-CA (green) (A), and total neurite length per cell was determined as described in Fig. 1D (B). C and D, the PC12 clonal cell line (clone 11) was transiently expressed with MEK-CA after cultured in the absence (induction −) or presence of doxycycline (induction +) for 2 days. After being further incubated in the absence (None) or presence of 0.4% 1-butanol (1-BtOH) or 2-butanol (2-BtOH) for 1 days, cells were stained for F-actin (red) and HA-MEK-CA (green) (C), and total process length was determined as in Fig. 1D (D). Scale bar in A and C, 10 μm. Presented in B and D are the results of the mean ± S.E. from three independent experiments; an asterisk denotes statistical significance (p < 0.05).

The inhibitory effect of 1-butanol was not attributable to direct inhibition of the MAP kinase cascade, because 1-butanol did not affect the phosphorylation of ERK under these conditions (data not shown). These results, taken together, provide unambiguous evidence that PLD2 activity is up-regulated by activation of ERK, which in turn enhances neurite outgrowth from PC12 cells.

**PLD2 Co-localizes with F-actin in Growth Cones during Neurite Outgrowth**—Because actin-based dynamic reorganization of the membrane in growth cones is one of critical events that takes place during chemoattractant-stimulated neurite outgrowth (24), we examined whether PLD2 and F-actin co-localize in the growth cone. Consistent with the report by Colley et al. (18) with non-neuronal cells, inducibly expressed WT-PLD2 localized at the plasma membrane of soma in non-stimulated PC12 cells and co-localized with F-actin (Fig. 9). Interestingly, at 12 h after the initiation of NGF stimulation, PLD2 was found to be enriched at the growth cone where it again co-localized with F-actin (Fig. 9). These results suggest the possibility that the mechanism through which PLD2 signals to neurite outgrowth in PC12 cells involves actin cytoskeletal reorganization in the growth cone.

![Image](http://example.com/image.png)

**DISCUSSION**

The ERK MAP kinase cascade plays a crucial role in NGF-induced neurite outgrowth from PC12 cells (29–31). However, the downstream sequence of events that links ERK signaling to neurite outgrowth has remained poorly understood. In the present study, we provide unambiguous evidence that PLD2 functions as a downstream signaling molecule for ERK in this pathway, providing insight not only into the connection between ERK and neurite remodeling, but as well into the regulatory mechanisms that control PLD2 activation in physiological settings.

We report here that PLD2 activation downstream of ERK is absolutely required for NGF-induced neurite outgrowth. Activation of PLD2 by itself, however, does not appear to suffice to maximally trigger neurite outgrowth. This conclusion is reached from the observations that overexpression of WT-PLD2 in the PC12 cell line clone 8, in which basal PLD activity was increased to a much greater extent than that observed for NGF-stimulated control cells, induced only limited neurite extension in the absence of NGF (Fig. 1, B and E) but then promoted profoundly exaggerated neurite outgrowth in the presence of NGF. Accordingly, it is likely that PLD2 signaling synergizes with other pathway(s) in parallel to fully promote neurite outgrowth from PC12 cells. Recently, Harada et al. (40) have reported that the neuron-specific cyclin-dependent kinase 5 activator p35 is induced by NGF stimulation of PC12 cells in an ERK-dependent manner and that p35 induction is essential but insufficient to drive NGF-induced neurite outgrowth. This report, taken together with the result shown in Fig. 8 that the MEK-induced neurite outgrowth was dramatically enhanced by co-expression of WT-PLD2, suggest that the pathway may branch downstream from ERK to activate both PLD2 and p35 in parallel. It would be of interest to investigate whether ERK-dependent
PC12 cells, because PI(4,5)P2 levels of non-induced control pathway that regulates NGF-induced neurite outgrowth is the key target signaling molecule of PA in the signaling al.

In contrast, Rizzo et al. elegantly demonstrated in insulin-stimulated Rat-1 fibroblasts that PLD2 activates ERK MAP kinase, which is opposite to what we found for our NGF-stimulated PC12 cells (Fig. 5). This paradox suggests that the relationship between PLD2 and ERK MAP kinase signaling may depend upon the cell types and/or agonists under investigation.

Is the activation of PLD2 by ERK direct or indirect? Because purified recombinant PLD2 cannot be activated and phosphorylated in vitro by immunologically precipitated ERK2 (which had been overexpressed in HeLa cells and activated by hydroxypertoxide stimulation, data not shown), it is likely that PLD2 activation by ERK is indirect. PLD2 activation may involve other signaling inputs as well, because the inhibition of the NGF-stimulated PLD2 activity by the PD MEK inhibitor was not complete (Fig. 6D).

A key question raised by our findings concerns the molecular mechanisms through which PA produced by PLD2 regulates neurite outgrowth. In non-neuronal cells, PA regulates molecule(s) involved in actin cytoskeleton reorganization and membrane trafficking (45, 46), both of which are essential events for neurite outgrowth. Reorganization of the actin cytoskeleton plays crucial roles in axonal outgrowth and guidance of primary cultured neurons (24, 42). Furthermore, actin filaments have been shown to accumulate in the growth cones at the tips of neurites during neuronal development (24, 47). These findings lead us to speculate that PA enhances neurite outgrowth through reorganization of the actin cytoskeleton. This is supported by the observation that overexpressed WT-PLD2 in neuronal cells where PA stimulates budding from the Golgi apparatus by recruiting the cytosolic COP protein apparatus by recruiting the cytosolic COP protein.

The mechanisms that control PLD2 activation in vitro are poorly understood in comparison to those that control PLD1 (17, 44). In the present study, we show that PLD2 activity is regulated by ERK in PC12 cells (Fig. 6). This conclusion is in agreement with reports by Parmentier et al. (28) and Banno et al. (37) on norepinephrine-stimulated vascular smooth muscle cells and hydroxysteroid-stimulated PC12 cells, respectively. In contrast, Rizzo et al. elegantly demonstrated in insulin-stimulated Rat-1 fibroblasts that PLD2 activates ERK MAP kinase, which is opposite to what we found for our NGF-stimulated PC12 cells (Fig. 5). This paradox suggests that the relationship between PLD2 and ERK MAP kinase signaling may depend upon the cell types and/or agonists under investigation.

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