Involvement of Phospholipase D in Sphingosine 1-Phosphate-induced Activation of Phosphatidylinositol 3-Kinase and Akt in Chinese Hamster Ovary Cells Overexpressing EDG3*

Phospholipase D (PLD), phosphatidylinositol 3-kinase (PI3K), and Akt are known to be involved in cellular signaling related to proliferation and cell survival. In this report, we provide evidence that PLD links sphingosine 1-phosphate (S1P)-induced activation of the G protein-coupled EDG3 receptor to stimulation of PI3K and its downstream effector Akt in Chinese hamster ovary (CHO) cells. S1P stimulation of EDG3-overexpressing CHO cells but not vector-transfected cells induced activation of PLD, PI3K, and Akt in a time- and dose-dependent manner. Akt phosphorylation was prevented by the PI3K inhibitors wortmannin and LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one), indicating that Akt activation was dependent on PI3K. S1P-induced activation of PI3K and Akt was abrogated by 1-butanol, which inhibited S1P-induced accumulation of phosphatidic acid by serving as a phosphatidyl group acceptor in the transphosphatidylation reaction catalyzed by PLD, whereas both PI3K and Akt activation were not inhibited by 2-butanol without such reaction. Co-expression of wild-type PLD2 with myc-Akt resulted in increased Akt activation in response to S1P. In contrast, co-expression of a catalytically inactive mutant of PLD2 eliminated the S1P-induced Akt activation. The treatment of EDG3-expressing CHO cells with exogenous Streptomyces chromofuscus PLD, which caused an accumulation of phosphatidic acid, resulted in increases in PI3K activity and the phosphorylation of Akt, the latter of which was completely abolished by LY294002. Furthermore, S1P-induced membrane ruffling, which was dependent on PI3K and Rac, was inhibited by 1-butanol, but not by 2-butanol. These results demonstrate that PLD participates in the activation of PI3K and Akt stimulation of EDG3 receptor.

Hydrolysis of phosphatidylcholine by phospholipase D

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1 The abbreviations used are: PA, phosphatidic acid; PBut, phosphatidylbutanol; PI3K, phosphatidylinositol 3-kinase; PLD, phospholipase D; PLDSc, Streptomyces chromofuscus PLD; S1P, sphingosine 1-phosphate; CHO, Chinese hamster ovary; PI3P, PI3-phosphate.
found to be a critical downstream effector of PI3K in cell survival signaling (38). In addition, we have recently demonstrated that S1P induces PI3K activation via EDG1, EDG3, and EDG5. S1P induced membrane ruffling and cell migration in a PI3K- and Rac-dependent manner (39). Interestingly, PLD has also been shown to be involved in this membrane ruffling (40). Thus, it is of interest to determine whether PLD is involved in S1P-induced activation of the PI3K/Akt pathway and, if so, how.

In the present study, we demonstrate that inhibition of PA accumulation and expression of a catalytically inactive mutant of PLD2 strongly inhibit EDG3-mediated stimulation of PI3K and Akt, whereas exogenous PLD alone induces stimulation of PI3K and Akt. These observations indicate a novel role for PLD in the PI3K signaling pathway.

EXPERIMENTAL PROCEDURES

Materials—Geneticin (G418) and Streptomyces chromofuscus PLD were obtained from Sigma, S1P was obtained from Matreya, Inc. (Pleasant Gap, PA), [9,10-3H]palmitic acid (54.0 Ci/mmol) and [γ-32P]ATP (3000 Ci/mmol) were obtained from PerkinElmer Life Sciences, and wortmannin and LY294002 were obtained from Calbiochem. Antibodies to phospholipid Akt (Ser-473) and Akt were obtained from New England Biolabs (Boston, MA), and anti-phosphoAkt (FY-20) antibody was obtained from Transduction Laboratories (Lexington, KY). Polyclonal anti-PLD1 and anti-PLD2 antibodies were prepared as described previously (25). Monoclonal anti-myc antibody (9E10)-producing hybridoma cells were obtained from American Type Culture Collection. Anti-rabbit and anti-mouse antibodies conjugated with horseradish peroxidase and chemiluminescence kit (ECL system) were obtained from Amersham Pharmacia Biotech. Expression plasmids of wild-type human PLD1 and mouse PLD2 and their catalytically inactive mutants (K898R and K758R, respectively) in pCGN were kindly supplied by Dr. Michael A. Frohman (Institute for Cell and Developmental Biology, University of Washington, Seattle, WA), and Myc- and HA-tagged Akt plasmids (pUSE-myc-Akt) were obtained from Upstate Biotechnology (Lake Placid, NY). All other reagents were obtained from standard commercial sources.

Cell Culture and Transfections—CHO-K1 cells were cultured in Ham’s F-12 medium supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin G, and 100 mg/ml streptomycin at 37 °C in a humidified, CO2-controlled (5%) incubator. Before each experiment, cells were switched to the serum-free Ham’s F-12 medium. Human EDG3 cDNA was cloned and ligated into expression vector pME18S as described previously (41). The stable transfectants of pME18S empty vector and pME18S-EDG3 were selected and maintained in the presence of 0.75 mg/ml G418 as described previously (42). For transient transfection experiments, cells were plated at 8–105 cells/plate in 60-mm plates and cultured for 24 h before transfection. Cells were incubated for 4 h with 2 µl of serum-free Ham’s F-12 medium containing 0.5 mg of total DNA (1 µg of pUSE-myc-Akt and 2 µg of expression plasmid for a pCGN-wild-type or an inactive mutant of PLDs, or an empty vector) and 15 µl of LipofectAMINE (Life Technologies, Inc.). The medium was changed to growth medium, and the cells were cultured for 24 h. The cells were serum-starved by further incubation in serum-free Ham’s F-12 medium for 24 h.

Measurement of PLD Activity—Subconfluent cells were labeled for 24 h with 1 µCi/ml [3H]palmitic acid in serum-free Ham’s F-12 medium. Cells were washed and preincubated in HEPES-Tyrode buffer containing 0.3% 1-butanol (v/v) for 10 min. After stimulation of cells with S1P, the reactions were terminated by removing the assay buffer, followed by the immediate addition of 1 ml of an ice-cold phosphate-buffered saline/methanol (2:5, v/v) mixture to the culture dishes. After extraction of cellular lipids, [3H]PA was measured by the same method as described for the PBut formation, except that incubation was performed using HEPES-Tyrode buffer without 1-butanol.

RESULTS

SIP-induced Phospholipase D Activation in EDG3-Over-expressing CHO-K1 Cells—We first examined whether EDG3 mediated S1P-induced PLD activation. S1P stimulation increased PBut formation dose-dependently with a maximal effective concentration value of 10−7 M in EDG3-CHO-K1 cells (Fig. 1A) and time-dependently with a plateau attained at 5 min (Fig. 1B). However, no significant increase in PBut formation was observed in response to S1P in vector-transfected control CHO-K1 cells. These results are indicative of EDG3 receptor-mediated PLD activation.

Involvement of PLD in S1P-induced PI3K Activation—SIP-induced stimulation of the PI3K activity in time- and dose-dependent manners with a peak response at 5 min and a maximal concentration of 1 µM S1P, respectively (Fig. 2, A and B). To assess the involvement of PLD in S1P-induced PI3K activation, we examined the effects of treatments with 1-butanol and 2-butanol on S1P-induced PI3K activation. In the presence of 1-butanol but not 2-butanol, PBut was efficiently produced at the expense of PA by the transphosphatidylidylation activity of
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PLD (Fig. 3A, top panel). This effect of 1-butanol was maximal at a concentration of 0.3% (v/v) (Fig. 3A, bottom panel). The presence of 1-butanol at 0.3% almost totally suppressed S1P-induced activation of PI3K (Fig. 3B). In contrast, 2-butanol was without any effect on S1P-induced PI3K activation. These results indicate that the product of PLD, PA, is involved in S1P-induced PI3K activation.

Dependence of S1P-induced Akt Activation on PI3K and PLD—As shown in Fig. 4, S1P induced marked phosphorylation of Akt at Ser-473 in a time-dependent manner (Fig. 4A). The stimulatory effect of S1P on Akt phosphorylation was also dose-dependent, with a maximum response observed at 1 μM (Fig. 4B). Pretreatment with the PI3K inhibitors wortmannin (100 nM) and LY294002 (10 μM) suppressed S1P-induced Akt activation by >80% (Fig. 4C), indicating that activation of Akt is mediated by PI3K. In contrast, PI3K inhibitors had no effect at all on S1P-induced PLD activation (Fig. 4D).

Treatment of cells with 1-butanol (0.3%) completely abolished S1P-induced phosphorylation of Akt (Fig. 5A), a finding that was consistent with inhibition by 1-butanol of S1P-induced PI3K activation (Fig. 3B). The inhibition by 1-butanol of S1P-induced Akt activation was dependent on the concentration of 1-butanol (Fig. 5B), with a concentration-response relationship similar to those for PBut formation and inhibition of PA accumulation (Fig. 3A). 2-Butanol was again ineffective in inhibiting S1P-induced Akt activation.

The involvement of PLD in S1P-induced Akt activation was further examined by transiently expressing wild-types and catalytically inactive mutants of PLD1 and PLD2. These catalytically inactive mutants have been shown to be devoid of activity both in vivo and in vitro when using phosphatidylcholine as a substrate, to be distributed intracellularly in a manner similar to that of the respective wild-type proteins, and to inhibit the activation of endogenous PLD when overexpressed (17). In the transfected cells, expression levels of transduced PLD gene products were higher than those of endogenous PLD1 and PLD2, as assessed by Western blotting analysis with specific anti-PLD1 and anti-PLD2 antibodies (Fig. 6A). We co-transfected cells with an expression vector for myc-tagged Akt and an expression vector for either the wild-type or the inactive mutant of PLD1 and PLD2 or the empty vector. We then immunoprecipitated myc-tagged Akt and performed an immunocomplex kinase assay using Crosstide as a substrate peptide. As shown in Fig. 6B, the expression levels of myc-Akt protein in transfected cells were nearly identical among the samples, as assessed by Western blotting using anti-Akt antibody. In vector-transfected cells, S1P stimulated Akt activity by approximately 2-fold (Fig. 6C). Expression of wild-type PLD1 slightly increased basal and S1P-stimulated PLD1 activities. The effect of wild-type PLD2 expression was more prominent, resulting in an approximately 2-fold increase in S1P-stimulated Akt activity. Furthermore, expression of a catalytically inactive mutant of PLD2 almost completely abolished S1P-induced Akt activation. Expression of a catalytically inactive mutant of PLD1 slightly inhibited basal and S1P-stimulated Akt activities. All of these results demonstrate that in EDG3-CHO-K1 cells, PA produced via the action of PLD2 rather than PLD1 participates in S1P-induced activation of Akt.

Inhibitory Effects of 1-Butanol on S1P-induced Membrane Ruffling—S1P induced membrane ruffling in EDG3-CHO-K1 cells (Fig. 7, a and b). This action of S1P was inhibited by...
The results are representative of three different experiments. The phosphorylated protein bands were subjected to densitometric analysis, and the results are expressed as the mean ± S.E. of three different experiments. D, before stimulation with 1 μM S1P for 5 min, [3H]palmitate-labeled EDG3-CHO-K1 cells were pretreated with or without wortmannin (WTM) (100 nM) or LY294002 (10 μM) for 30 min. Cell lysates were subjected to 10% SDS-polyacrylamide gel electrophoresis and blotted with anti-phospho-Akt (Ser-473) antibody. The extent of phosphorylation was quantitated by densitometer and expressed as the mean ± S.E. of three different experiments.

**DISCUSSION**

PLD is activated rapidly in response to diverse extracellular stimuli, including hormones, growth factors, neurotransmitters, cytokines, antigens, and certain physical stresses (1–5). The initial product of PLD, PA, is thought to serve a signaling function. However, the intracellular targets for this lipid messenger have not been clearly identified. In this study, we demonstrated that PLD stimulation is necessary for S1P-induced activation of PI3K and its downstream effector Akt, and is sufficient for inducing activation of both PI3K and Akt under certain conditions. This is the first report to indicate the involvement of PLD and PA in in vivo activation of PI3K and its effector Akt. This conclusion is based on the following three major findings: (a) S1P-induced activation of PI3K and Akt was inhibited by 1-butanol, which served as a phosphatidyl group acceptor in the PLD-catalyzed transphosphatidylation reaction to reduce the levels of PA. In contrast, 2-butanol, which did not serve as a phosphatidyl group acceptor, was ineffective; (b) S1P-induced activation of Akt was suppressed by the overexpression of a dominant negative PLD2 mutant; and (c) treatment of cells with exogenous PLDSc induced activation of PI3K and Akt, and Akt activation was completely abolished by a PI3K inhibitor, LY294002. Moreover, S1P-induced membrane ruffling, which was dependent upon PI3K (39), was abolished by 1-butanol, but not by 2-butanol. These observations point to an essential role for PLD and PA in PI3K activation induced by the G protein-coupled receptor agonist S1P.

Many studies have indicated that S1P induces cell proliferation, suppression of apoptosis, modulation of cell motility, and cell shape changes (18–24, 39, 46). The EDG receptors for S1P, including EDG3, have been shown to mediate S1P-evoked signaling events relevant to cell proliferation and survival, includ-
We have previously demonstrated that S1P-induced PLD activation is independent of extracellular signal-regulated kinase activation in NIH3T3 cells (25). In the present study, we have demonstrated that S1P-induced activation of PLD is required for activation of PI3K and Akt in EDG3-expressing CHO-K1 cells. It is widely accepted that the activation of Akt plays a pivotal role in cell survival and protection against apoptosis by phosphorylating BAD and caspase-9 and by regulating signaling via transcription factors such as the forkhead family and nuclear factor-κB (41, 47, 48). On the other hand, it has previously been shown that PLDs are also involved in cell survival activation in NIH3T3 cells (25). In the present study, we have demonstrated that S1P-induced activation of PLD is required for activation of PI3K and Akt in EDG3-expressing CHO-K1 cells. It is widely accepted that the activation of Akt plays a pivotal role in cell survival and protection against apoptosis by phosphorylating BAD and caspase-9 and by regulating signaling via transcription factors such as the forkhead family and nuclear factor-κB (41, 47, 48). On the other hand, it has previously been shown that PLDs are also involved in cell survival activation in NIH3T3 cells (25). In the present study, we have demonstrated that S1P-induced activation of PLD is required for activation of PI3K and Akt in EDG3-expressing CHO-K1 cells. It is widely accepted that the activation of Akt plays a pivotal role in cell survival and protection against apoptosis by phosphorylating BAD and caspase-9 and by regulating signaling via transcription factors such as the forkhead family and nuclear factor-κB (41, 47, 48). On the other hand, it has previously been shown that PLDs are also involved in cell survival
signaling events; for example, overexpression of PLD2 suppresses H2O2- and hypoxia-induced apoptosis in PC12 cells (34, 36). Our results reveal a novel link between PLD and the PI3K/Akt pathway in S1P-mediated survival signaling.

There is some existing evidence indicating a functional link between PLD and PI3K. For example, PLD and PI3K are regulated by receptor tyrosine kinases. Similar to PI3K, PLD is regulated by RaLα, a downstream target of Ras, in platelet-derived growth factor- and epidermal growth factor-stimulated cells (49, 50), and PLD2 is tyrosine-phosphorylated by forming a physical complex with the epidermal growth factor receptor (51). Recent studies have demonstrated that PLD1 and PI3K play a role in GLUT4 translocation between the plasma membranes and intracellular vesicles in insulin-stimulated cells (52). Furthermore, several studies have demonstrated through the use of PI3K inhibitors that PI3K is involved in agonist-stimulated PLD activation (53–56). These studies suggest, as a possible underlying mechanism, that the PI3K products phosphatidylinositol 3,4-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate might regulate the activity of the Ras-related low molecular mass GTPases Rho and Arf, which have been shown to be involved in the activation of PLD (1–5). We have observed that Clostridium difficile toxin B, which inactivates all Rho G proteins including Rho, Rac, and Cdc42 (57), blocks all S1P-induced activation of PLD, PI3K, and Akt in EDG3-CHO-K1 cells (data not shown). Our previous study, however, demonstrated that S1P-induced Rac activation occurs downstream from PI3K activation in EDG3-CHO-K1 cells (39). In the present study, PI3K inhibitors had no effect on S1P-induced PLD activation in EDG3-CHO-K1 cells (Fig. 4D), whereas 1-butanol inhibited S1P-induced PI3K activation (Fig. 3B). These observations indicate that PI3K exists downstream rather than upstream of PLD in EDG3-CHO-K1 cells.

Among the PI3Ks, two isoforms of PI3K catalytic subunits, p110α and p110β, form heterodimers with the p85/p55 adaptor subunits, whereas another catalytic subunit, p110γ, is associated with the p101 adaptor. p85/p55-associate p110α and p110β are stimulated by tyrosine kinase-coupled transmembrane receptors upon their recruitment to the plasma membrane by the assembly of phosphotyrosine-containing multimeric complexes. Ras is also thought to participate in the activation of p110α and p110β through its direct binding to these catalytic subunits. On the other hand, p110γ and p110β have been shown to be stimulated by the heterotrimeric G proteins (58, 59). Previous studies have shown that PA and lyso-PA inhibit PI3K activity in vitro systems, whereas these lipids activate phosphatidylinositol 4-kinase, phospholipase C, protein kinase C, and 5k tyrosine kinase under the same conditions (60, 61). On the other hand, recent studies have demonstrated that anionic phospholipids such as phosphatidylinositol 4,5-bisphosphate, PA, and phosphatidylycerine can bind to p110 (62). Therefore, it is an interesting possibility that PA produced by PLD activation participates in the recruitment of PI3K to the plasma membrane in S1P-stimulated cells. In the present study, we observed stimulation of PI3K activity in anti-phosphotyrosine antibody immunoprecipitates from S1P-stimulated EDG3-CHO-K1 cells (Fig. 3), indicating that S1P-stimulated PI3K activity is associated with a phosphotyrosine-containing protein or that PI3K is directly tyrosine-phosphorylated. A number of studies have demonstrated that exogenously added and endogenously generated PA induces enhancement of tyrosine phosphorylation in neutrophils and other cell types (63–65). A more recent study has demonstrated that exogenous PA induces tyrosine phosphorylation of the p85 regulatory subunit of PI3K in neutrophilic leukocytes (66). Therefore, it is tempting to speculate that PA-dependent tyrosine phosphorylation may be involved in recruitment to the plasma membrane and stimulation of the PI3K in the EDG3-mediated response to S1P. However, additional experiments are required to prove this hypothesis.

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