Involvement of phospholipase D in insulin-like growth factor-I-induced activation of extracellular signal-regulated kinase, but not phosphoinositide 3-kinase or Akt, in Chinese hamster ovary cells

Yoshiko BANNO^{*1}, Yoh TAKUWA[†], Momoko YAMADA[‡], Noriko TAKUWA[†], Kenji OHGUCHI[§], Akira HARA[‡] and Yoshinori NOZAWA[§]

^{*}Department of Biochemistry, Gifu University School of Medicine, Tsukasamachi-40, Gifu 500-8705, Japan, [†]Department of Physiology, Kanazawa University Graduate School of Medicine, Takaramachi, Kanazawa, Japan, [‡]Department of Biochemistry, Gifu Pharmaceutical University, Mitahora, Gifu, Japan, and [§]Gifu International Institute of Biotechnology, Mitake, Gifu, Japan

Available evidence suggests the involvement of phospholipase D (PLD) in cell proliferation and survival. Phosphoinositide 3-kinase (PI 3-kinase)/Akt and extracellular signal-regulated kinases (ERKs) are signalling molecules that have essential roles in cell proliferation and survival. We previously demonstrated that sphingosine 1-phosphate (S1P)-induced PLD activation via the G-protein-coupled receptor endothelial differentiation gene (EDG) 3/S1P₃ was involved in S1P-induced stimulation of PI 3-kinase and Akt. In the present study, we examined the involvement of two PLD isozymes, PLD1 and PLD2, in insulin-like growth factor (IGF)-I receptor tyrosine kinase-mediated stimulation of PI 3-kinase/Akt and ERKs. IGF-I and to a lesser degree S1P stimulated PI 3-kinase activity in Chinese hamster ovary cells overexpressing EDG3/S1P₃. IGF-I-induced ERK phosphorylation was suppressed by butan-1-ol, but not butan-2-ol, whereas no effect of butanol was observed in IGF-I-induced Akt activation in S1P₃-overexpressing Chinese hamster ovary

cells. Overexpression of wild-type PLD1 and PLD2 substantially potentiated S1P-, but not IGF-I-, induced activation of PI 3-kinase and Akt, whereas overexpression of the catalytically inactive mutant of PLD1 or PLD2 did not affect the responses to either agonist. On the other hand, overexpression of wild-type PLD1 and PLD2 potentiated IGF-I- and, to much smaller extents, S1P-induced ERK stimulation. ERK activation by IGF-I as well as S1P was dependent on Ras, but Akt activation by IGF-I was not dependent on Ras. These results suggest that PLDs are involved in growth factor regulation of at least two signalling pathways, PI 3-kinase/Akt and ERKs, depending on the class of cell-surface receptors.

Key words: CHO-K1 cells, extracellular signal-regulated kinase (ERK), insulin-like growth factor-I (IGF-I), phosphoinositide 3-kinase (PI 3-kinase), sphingosine 1-phosphate.

INTRODUCTION

Phospholipase D (PLD) cleaves phosphatidylcholine in response to a variety of cell stimuli to generate phosphatidic acid, which is associated with a number of cellular responses including regulated secretion, cytoskeletal reorganization, proliferation and meiosis [1,2]. Phosphatidic acid is also known to activate directly phosphatidylinositol-4-phosphate-5-kinase type I [3], mammalian target of rapamycin ('mTOR') [4] and many other enzymes *in vitro* and *in vivo* [5,6]. However, the physiological roles of phosphatidic acid are not fully elucidated. Two mammalian PLD genes, PLD1 and PLD2, have been cloned [7]. PLD1 is regulated by low-molecular-mass GTP-binding proteins such as ADP-ribosylation factor and the Rho family GTP-binding protein and also by protein kinase C. On the other hand, the mechanisms that regulate PLD2 activity are still undefined *in vivo*, although PLD2 is constitutively active and requires PtdIns(4,5)P₂ *in vitro* [1,2,5–7]. Recent studies have shown that overexpression of PLD1 and PLD2 in transformed cells stimulates cell proliferation and generates a survival signal that overcomes the apoptotic signals [6,8]. On the other hand, Lee et al. [9] have demonstrated that PLD2-overexpression reduced

extracellular signal-regulating kinase (ERK) activation and negatively regulated mitogen signalling. However, little information is available about the mechanism of PLD activation in cell survival signalling.

Much evidence suggests that sphingosine 1-phosphate (S1P) is a member of a new class of lipid second messengers derived from sphingolipid metabolism [10]. Recently, five closely related G-protein-coupled receptors (GPCRs) of the endothelial differentiation gene (EDG) family (namely EDG1/S1P₁, EDG3/S1P₃, EDG5/S1P₂, EDG6/S1P₄ and EDG8/S1P₅) were identified as plasma membrane receptors for S1P [11,12]. The potential roles of S1P in cellular growth and survival have also been suggested by its ability to decrease ceramide- and serum-withdrawal-induced apoptosis [13,14]. ERK, as well as Akt, is implicated in cell proliferation and survival signalling. Recent studies have demonstrated that PLD2, as well as PLD1, plays a role in insulin and lysophosphatidic acid receptor-mediated activation of ERK via Raf-1 translocation to membrane [15,16]. Our previous study has demonstrated that PLD was involved in EDG3/S1P₃-mediated, S1P-induced phosphoinositide 3-kinase (PI 3-kinase)/Akt activation in Chinese hamster ovary (CHO) cells [17]. It is known that insulin-like growth factor (IGF) strongly activates

Abbreviations used: CHO, Chinese hamster ovary; EDG, endothelial differentiation gene; ERK, extracellular signal-regulated kinase; GPCR, G-protein-coupled receptor; IGF, insulin-like growth factor; PBut, phosphatidylbutanol; PI 3-kinase, phosphoinositide 3-kinase; PLD, phospholipase D; PLD1KR, K898R mutant of PLD1; PLD2KR, K758R mutant of PLD; PLD1WT and PLD2WT, wild-type PLD1 and PLD2 respectively; S1P, sphingosine 1-phosphate.

¹ To whom correspondence should be addressed (e-mail banno@cc.gifu-u.ac.jp).

Figure 1 Overexpression of PLD1 and PLD2, and stimulation of PLD activity by agonists in S1P₃-CHO cells

S1P₃-CHO cells stably transfected with vector, PLD1WT, PLD1KR, PLD2WT or PLD2KR were maintained in Ham's F12 supplemented with 10% fetal bovine serum containing 0.75 mg/ml G418 and 0.5 mg/ml Zeocin. **(A)** Cells were harvested and the level of PLD expression was monitored by Western blot analysis as described in the Materials and methods section. After 16 h in the absence of serum, cells were labelled with [³H]palmitic acid for 3 h. [³H]Palmitate-labelled vector- and PLD-overexpressed CHO cells were stimulated with 1 μ M S1P for 5 min, 100 nM PMA for 30 min **(B)** or 100 ng/ml IGF-I for 5 min **(C)**. PBut formation was measured as described in the Materials and methods section. The results of Western blot are representative of three independent experiments. The data on [³H]PBut formation are means $\pm$ S.E.M. from three different experiments.

PI 3-kinase in CHO cells [18]. It was shown previously that GPCR-mediated activation of PI 3-kinase mechanistically differs from that mediated by receptor tyrosine kinases [19,20]. In this study, we have examined whether PLDs are involved in IGF-I-induced activation of PI 3-kinase/Akt and ERK.

MATERIALS AND METHODS

Materials

Geneticin (G418) and PMA were obtained from Sigma (St. Louis, MO, U.S.A.); pCMV-zeo and Zeocin were from Invitrogen (Groningen, The Netherlands); S1P was from Matreya (Pleasant Gap, PA, U.S.A.); [9,10-³H]palmitic acid (54.0 Ci/mmol) and [γ -³²P]ATP (3000 Ci/mmol) were from DuPont-NEN (Boston, MA, U.S.A.). Antibodies against phospho-specific Akt (Ser⁴⁷³), phospho-specific ERK1/2 (Tyr²⁰⁴), Akt and ERK1/2 were obtained from New England BioLabs (Boston, MA, U.S.A.), and anti-phosphotyrosine antibody (PY-20) was obtained from Transduction Laboratories (Lexington, KY, U.S.A.). Anti-PI 3-kinase p110 α , p110 β and p110 γ antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Polyclonal anti-PLD1 and -PLD2 antibodies were prepared as described previously [21]. Anti-rabbit and anti-mouse antibodies conjugated with horseradish peroxidase and the chemiluminescence kit (ECL[®] system) were obtained from Amersham Biosciences (Little Chalfont, Bucks., U.K.). Expression plasmids of wild-type human PLD1 and mouse PLD2 and their cata-

lytically inactive mutants K898R (PLD1KR) and K758R (PLD2KR), respectively, in pCGN were kindly supplied by Dr Michael A. Frohman (Institute for Cell and Developmental Biology, State University of New York, New York, NY, U.S.A.). All other reagents were obtained from standard commercial sources.

Cell culture and transfections

CHO-K1 cells overexpressing S1P₃ (S1P₃-CHO cells) were cultured in Ham's F-12 medium supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin G, 100 μ g/ml streptomycin and 0.75 mg/ml G418 as described previously [22]. For stable transfection of PLD1 and PLD2, S1P₃-CHO cells were plated at 8×10^5 in 60 mm plates and cultured for 24 h prior to transfection. Cells were incubated for 4 h with 2 ml of serum-free Ham's F-12 medium containing 1.05 μ g of total plasmid DNA (1 μ g of pCGN-wild-type or inactive mutants of PLDs and 0.05 μ g of pCMV-Zeo), and 5 μ l of LIPOFECTAMINE[™] (Gibco Life Technologies). Stable transfections were selected in the presence of 0.7 mg/ml G418 and 0.5 mg/ml Zeocin, and expanded for further analysis under selective conditions. Replication-deficient adenovirus encoding N¹⁷-Ras was generated and amplified as described previously [22]. The cells were infected with adenovirus and cultured for 48 h. The cells were serum-starved in serum-free Ham's F-12 medium for 24 h before stimulation assay.

Measurement of PLD activity

Subconfluent cells were labelled for 24 h with 1 μ Ci/ml [3 H]palmitic acid in serum-free Ham's F-12 medium. Cells were washed and preincubated in Hepes-Tyrod buffer containing 0.3% butan-1-ol (v/v) for 10 min. Following stimulation of cells with S1P or PMA, the reactions were terminated by removing the assay buffer, followed by immediate addition of 1 ml of an ice-cold PBS/methanol (2:5, v/v) mixture to the culture dishes. After extraction of cellular lipids, [3 H]phosphatidylbutanol (PBut) was separated by TLC and measured as described previously [21].

Western blot analysis

Cells were grown in 100 mm dishes to subconfluence and further incubated in serum-free Ham's F-12 medium for 24 h. Washed cells were then stimulated, harvested in an ice-cold lysis buffer (1% Nonidet P-40, 0.5% sodium cholate, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 20 mM Hepes, 3 mM MgCl₂, 1 mM PMSF, 20 μ g/ml leupeptin, 20 mM β -glycerophosphate, 1 mM NaF and 1 mM sodium orthovanadate, pH 7.4) and sonicated. Protein concentrations were assayed using the Bradford protein assay reagent (Bio-Rad) with BSA as a standard. Total cell lysates (50 μ g of protein) were subjected to SDS/PAGE (10% gel) and transferred to PVDF membranes (Millipore). The membranes were blocked with 5% BSA. Phosphorylation of Akt or ERK and the total amounts of Akt and ERK were determined by immunoblotting with rabbit polyclonal anti-phospho-specific Akt (Ser⁴⁷³) or anti-phospho-specific ERK1/2, and anti-Akt or anti-ERK antibodies respectively, and with horseradish peroxidase-linked secondary antibody. After repeated washings, the antibodies were detected using the ECL Western blotting detection system.

PI 3-kinase assay

PI 3-kinase activity was carried out as described previously [23]. The immunoprecipitation was performed with PI 3-kinase p110 α , p110 β , p110 γ or phosphotyrosine (PY-20) antibodies, and the PI 3-kinase activity was assayed using phosphatidylinositol as substrate.

RESULTS AND DISCUSSION

To understand roles of PLD isozymes in S1P- and IGF-I-induced survival signalling, we established S1P₃-CHO cells overexpressing PLD isoforms. The cells that overexpressed PLD isozymes were generated by stable transfection with expression vectors containing the cDNAs encoding wild-type PLD1 and PLD2 (PLD1WT and PLD2WT respectively), and catalytically inactive PLD1KR and PLD2KR, and subsequent selection performed as described in the Materials and methods section. Cells transfected with an empty vector served as controls in all subsequent experiments. Western blot analysis with specific anti-PLD1 and anti-PLD2 antibodies showed overexpression of each of PLD1WT, PLD1KR, PLD2WT and PLD2KR (Figure 1A).

To examine functional activities of expressed PLD isozymes, we determined cellular PLD activity by measuring the formation of PBut in the presence of butan-1-ol via the transphosphatidyl-ation reaction in [3 H]palmitic acid-prelabelled cells. As shown in Figure 1(B), the unstimulated basal PBut formation in the cells overexpressing PLD1WT was 2-fold higher than in the vector control cells. We previously showed that both S1P and PMA stimulated PLD activity in CHO cells [17]. In PLD1WT-overexpressing cells S1P- and PMA-stimulated PBut formation was 5–6-fold potentiated compared with the vector control cells.

Figure 2 S1P- or IGF-I-induced PI 3-kinase activation in vector control or PLD-overexpressing S1P₃-CHO cells

Prior to stimulation with agonists, S1P₃-CHO cells were incubated in serum-free medium for 24 h. (A) The vector cell lysates were then subjected to Western blot analysis with PI 3-kinase p110 α , p110 β or p110 γ antibodies. (B) The vector cells were stimulated without or with 1 μ M S1P or 100 ng/ml IGF-I for 5 min, and subjected to immunoprecipitation with anti-PI 3-kinase p110 α or p110 β , or PY-20 (P-Tyr) antibodies. (C) The vector control and PLD-overexpressing cells were stimulated without or with 1 μ M S1P or 100 ng/ml IGF-I for 5 min, and subjected to immunoprecipitation with anti-PY-20 (P-Tyr) antibody. The immunocomplexes were assayed for PI 3-kinase activity as described in the Materials and methods section. The results shown are representative of three independent experiments.

In PLD2WT-overexpressing cells as well, both the basal and stimulated PBut formation was 2.5–4-fold potentiated compared with the vector control cells (Figure 1B). In contrast, in the cells overexpressing either PLD1KR or PLD2KR the basal PBut formation was slightly suppressed, although stimulated PBut formation was essentially similar in magnitude to that observed in the vector control cells. In the vector control cells IGF-I (100 ng/ml) stimulated PBut formation only slightly (1.5-fold), which was less than that observed with S1P (Figures 1B and 1C). In the cells overexpressing either PLD1WT or PLD2WT, IGF-I-induced PBut formation was substantially potentiated but was still lower than that with S1P stimulation, whereas in the cells overexpressing either PLD1KR or PLD2KR the level of IGF-I-induced PBut formation was similar to that in the vector control cells.

Our previous study demonstrated that S1P-induced PLD activation via the GPCR S1P₃ was involved in S1P-induced stimulation of the PI 3-kinase/Akt survival signalling pathway [17]. However, it is not known whether PLD is also involved in receptor tyrosine kinase-mediated activation of PI 3-kinase and its downstream effector Akt. We therefore examined the involvement of PLD1 and PLD2 in IGF-I-induced stimulation of PI 3-kinase and Akt. S1P₃-CHO cells exhibited abundant PI 3-kinase activities associated with anti-p110 α and anti-p110 β immunoprecipitates, but not with anti-p110 γ immunoprecipitates (Figure 2A). IGF-I (100 ng/ml) induced robust stimulation of PI 3-kinase activities associated with anti-p110 α , anti-p110 β or anti-phosphotyrosine immunoprecipitates (Figure 2B). In contrast, the optimal concentration of S1P (1 μ M) weakly stimulated PI 3-kinase activities associated with only anti-p110 β and anti-phosphotyrosine immunoprecipitates. IGF-I-stimulated PI 3-kinase activity associated with anti-phosphotyrosine immunoprecipitates in either PLD1WT- or

Figure 3 Effect of overexpression of PLD1 or PLD2 on agonist-stimulated phosphorylation of Akt

Prior to stimulation with agonists, S1P₃-CHO cells were incubated in the serum-free medium for 24 h. The vector control or PLD1WT- or PLD2WT-overexpressing cells were stimulated without or with 1 μ M S1P or 100 ng/ml IGF-I for 5 min, and the cell lysates were then subjected to Western blot analysis using antibodies of phospho-specific Akt or Akt as described in the Materials and methods section. The results from the Western blots are representative of three independent experiments. The data expressed as arbitrary units are means $\pm$ S.E.M. from three different experiments.

Figure 4 Effect of overexpression of PLD1 or PLD2 on agonist-stimulated ERK phosphorylation

Prior to stimulation with agonists, S1P₃-CHO cells were incubated in the serum-free medium for 24 h. The vector control or PLD-overexpressing cells were stimulated without or with 1 μ M S1P or 100 ng/ml IGF-I for 5 min, and the cell lysates were then subjected to Western blot analysis using antibodies of phospho-specific ERK or ERK as described in the Materials and methods section. The results of Western blots are representative of three independent experiments. The data are mean $\pm$ S.E.M. from three different experiments.

PLD2WT-overexpressing cells was not different from that in the vector control cells (Figure 2C). In contrast, S1P-stimulated PI 3-kinase activity in cells overexpressing either PLD1WT or PLD2WT was considerably enhanced compared with the vector control cells.

We next examined the effect of overexpression of PLDs on stimulation of Akt. In the vector control cells, IGF-I (100 ng/ml) stimulated Akt 4-fold more strongly than S1P (1 μ M), as estimated by Western blot analysis of Ser⁴⁷³ phosphorylation of

Akt (Figure 3). In the cells overexpressing PLD1WT or PLD2WT, IGF-I-induced Akt stimulation was similar to that in the vector control cells (Figure 3, right-hand panel), which was in agreement with the results of IGF-I-induced PI 3-kinase stimulation (Figure 2C). On the other hand, S1P-induced Akt stimulation in the cells overexpressing PLD1WT or PLD2WT was potentiated compared with the vector control cells (Figure 3, left-hand panel), which was consistent with our previous observation that transient overexpression of either PLD1WT or

Figure 5 Effects of butan-1-ol and butan-2-ol on IGF-I-induced Akt and ERK activation in S1P₃-CHO cells

Before stimulation with 100 ng/ml IGF-I for 5 min, S1P₃-CHO cells were incubated with butan-1-ol (0.3%) or butan-2-ol (0.3%) for 20 min. The cell lysates were subjected to 10% PAGE and blotted with anti-phospho-Akt, -Akt, -phospho-ERK or -ERK antibodies as described in the Materials and methods section. The results of the Western blots are representative of three independent experiments. The extent of phosphorylation was quantified by densitometer and expressed as the means $\pm$ S.E.M. from three different experiments.

PLD2WT potentiated S1P-induced Akt stimulation [17]. Thus different from the case of S1P stimulation of the GPCR S1P₃, neither PLD1 nor PLD2 seems to be involved in IGF-I receptor tyrosine kinase-mediated stimulation of PI 3-kinase and the downstream effector Akt.

There are some reports suggesting that PLDs are also involved in extracellular ligand-induced activation of ERKs, another

survival signalling pathway [15,16]. Therefore, we next examined the involvement of PLD1 and PLD2 in IGF-I- and S1P-induced ERK activation in CHO cells. In the vector control cells both S1P (1 μ M) and IGF-I (100 ng/ml) induced ERK stimulation as estimated with Western analysis of Tyr²⁰⁴ phosphorylation of ERK1 and ERK2 (Figure 4). S1P was more effective in stimulating ERKs than IGF-I. S1P-induced ERK activation in the cells expressing either PLD1WT or PLD2WT was slightly (15–20%) augmented compared with the vector control cells (Figure 4, left-hand panel). On the contrary, IGF-I stimulation of ERKs in cells expressing either PLD1WT or PLD2WT was markedly (2.5–3-fold) enhanced compared with the vector control cells (Figure 4, right-hand panel). This potentiating effect of PLDs on IGF-I stimulation of ERKs was in contrast with the cases of IGF-I stimulation of PI 3-kinase and Akt (Figures 2 and 3).

To confirm the involvement of PLD in this survival signalling, the effects of butanol on IGF-I-induced Akt and ERK signalling pathways were examined in the vector control cells. As shown in Figure 5, the IGF-I-induced ERK activation was suppressed by butan-1-ol, but not butan-2-ol (Figure 5, right-hand panel). On the other hand, neither butanol had an effect on IGF-I-induced Akt phosphorylation (Figure 5, left-hand panel). Our previous study demonstrated that S1P-induced stimulation of the PI 3-kinase/Akt was suppressed by butan-1-ol, but not butan-2-ol [17]. S1P-induced ERK activation was inhibited, but to a lesser extent, by butan-1-ol (results not shown). These results were completely in agreement with the observations obtained using overexpression of PLDs, suggesting that PLD is involved in the IGF-I-induced ERK activation pathway, but not in the Akt pathway, in S1P₃-CHO cells.

To understand the mechanisms underlying the different involvement of PLDs in S1P- and IGF-I-mediated stimulation of ERK, we examined the involvement of PI 3-kinase and the small GTPase Ras, which are known upstream intermediates for cell-surface receptor-mediated ERK activation. Although the PI

Figure 6 Effect of LY29004 and dominant-negative Ras on IGF-I-induced Akt or ERK activation in S1P₃-CHO cells

Prior to stimulation with agonists, S1P₃-CHO cells were incubated in the serum-free medium for 24 h. (A) S1P₃-CHO cells were treated with or without 20 μ M LY29004 for 15 min, and stimulated with 100 ng/ml IGF-I for 5 min. (B) S1P₃-CHO cells were transiently transfected with Asn¹⁷-H-Ras (N¹⁷-Ras; dominant negative) expression vector, and then stimulated without or with 100 ng/ml IGF-I for 5 min. Cell lysates were subjected to Western blot analysis using antibodies for phospho-specific Akt, phospho-specific ERK, Akt or ERK as described in the Materials and methods section. The results of Western blots are representative of three independent experiments. The data expressed as arbitrary units are means $\pm$ S.E.M. from three different experiments.

3-kinase inhibitor LY29004 abrogated IGF-I-induced Akt activation, it did not inhibit ERK activation (Figure 6A). LY29004 had also no effect on S1P-mediated ERK activation (results not shown). We showed previously that expression of a dominant negative mutant of H-Ras, N¹⁷-Ras, abolished S1P-induced ERK activation in S1P₃-CHO cells [24], indicating dependence of S1P-induced ERK1/2 stimulation on Ras. We observed in the present study that adenovirus-mediated expression of N¹⁷-Ras abolished IGF-I-induced ERK activation (Figure 6B, right-hand side), indicating that IGF-I stimulation of ERK1/2 is also Ras-dependent. These observations may suggest that PLDs are involved in IGF-I-induced, but not S1P-induced, Ras activation, or that PLDs participates specifically in IGF-I-induced ERK stimulation at a site(s) downstream or independent of Ras. Furthermore, to investigate the mechanism underlying the distinct dependence on PLDs of IGF-I-induced stimulation of ERKs and PI 3-kinase/Akt, we examined the effect of N¹⁷-Ras expression on IGF-I stimulation of Akt. As shown in Figure 6(B) (left-hand side), N¹⁷-Ras expression did not affect IGF-I-induced Akt activation, indicating that IGF-I-stimulation of Akt is independent of Ras. Thus Akt and ERKs differ in their requirement of Ras for IGF-I-induced activation. This observation may favour the possibility that PLDs could be involved in IGF-I-induced Ras activation and consequently in IGF-I-induced ERK activation.

In conclusion, the present study demonstrated that PLDs are differentially involved in the GPCR S1P₃ and IGF-I receptor tyrosine kinase-mediated activation of the survival signalling pathways, PI 3-kinase/Akt and ERKs. Thus PLDs affect the activities of at least two cellular targets for regulating the cell survival signalling pathways. The involvement of PLDs in the stimulatory regulation of PI 3-kinase and ERKs is dependent on the cell context, particularly the class of activated cell-surface receptors which a growth factor acts on to convey stimulatory signals into the survival signalling pathway. The exact molecular mechanisms underlying the observations remain to be fully clarified.

We thank Dr Michael A. Frohman (Institute for Cell and Developmental Biology, State University of New York, New York, NY, U.S.A.) for providing the plasmid encoding wild-type and catalytically inactive mutants of PLD1 and PLD2. This work was supported in part by Grants-in-Aid for Scientific Research on Priority Areas 10212204, a Special Coordination Fund for Promoting Science and Technology from the Science and Technology Agency of Japan, and Grants-in-Aid for Scientific Research (B) 12470042 and (C) 12680633 from the Ministry of Education, Science, Sports, and Culture of Japan.

REFERENCES

- Exton, J. H. (1998) Phospholipase D. *Biochim. Biophys. Acta* **1436**, 105–115
- Liscovitch, M., Czarny, X., Fiucci, M. and Tang, G. (2000) Phospholipase D: molecular and cell biology of a novel gene family. *Biochem. J.* **345**, 401–415
- Honda, A., Nogami, M., Yokozeki, T., Yamazaki, M., Nakamura, H., Watanabe, H., Kawamoto, K., Nakayama, K., Morris, A. J., Frohman, M. A. and Kanaho, Y. (1999) Phosphatidylinositol 4-phosphate 5-kinase α is a downstream effector of the small G protein ARF6 in membrane ruffle formation. *Cell* **99**, 521–532
- Gang, Y., Vilella-Bach, M., Bachmann, R., Flanagan, A. and Chen, J. (2001) Phosphatidic acid-mediated mitogenic activation of mTOR signaling. *Science* **294**, 1942–1945
- Cockcroft, S. (2001) Signaling roles of mammalian phospholipases D1 and D2. *Cell. Mol. Life Sci.* **58**, 1674–1687
- Banno, Y. (2002) Regulation and possible role of mammalian phospholipase D in cellular functions. *J. Biochem. (Tokyo)* **13**, 301–306
- Frohman, M. A. and Morris, A. J. (1999) Phospholipase D structure and regulation. *Chem. Phys. Lipids* **98**, 127–140
- Joseph, T., Bryant, A., Frankel, B., Wooden, R., Kerkhoff, E., Rapp, U. R. and Foster, D. A. (2002) Phospholipase D overcomes cell cycle arrest induced by high-intensity Raf signaling. *Oncogene* **21**, 3651–3658
- Lee, C. S., Bae, Y.-S., Lee, S. D., Suh, P.-G. and Ryu, S. H. (2001) ATP-induced mitogenesis is modulated by phospholipase D2 through extracellular signal regulated protein kinase dephosphorylation in rat pheochromocytoma PC12 cells. *Neurosci. Lett.* **313**, 117–120
- Stumff, H. L., Peterson, C., Liu, H., Milstien, S. and Spiegel, S. (2002) Sphingosine-1-phosphate and lipid phosphohydrolases. *Biochim. Biophys. Acta* **1582**, 8–17
- Pyne, S. and Pyne, N. J. (2000) Sphingosine 1-phosphate signalling in mammalian cells. *Biochem. J.* **349**, 385–402
- Hla, T., Lee, M. J., Ancellin, N., Liu, C. H., Thangada, S., Thompson, B. D. and Kluk, M. (1999) Sphingosine-1-phosphate: extracellular mediator or intracellular second messenger? *Biochem. Pharmacol.* **58**, 201–217
- Goetzl, E. J., Kong, Y. and Mei, B. (1999) Lysophosphatidic acid and sphingosine 1-phosphate protection of T cells from apoptosis in association with suppression of Bax. *J. Immunol.* **162**, 2049–2056
- An, S., Zheng, Y. and Bleu, T. (2000) Sphingosine 1-phosphate-induced cell proliferation, survival, and related signaling events mediated by G protein-coupled receptors EDG3 and EDG5. *J. Biol. Chem.* **275**, 288–296
- Rizzo, M. A., Shome, K., Vasudevan, C., Stolz, D. B., Sung, T. C., Frohman, M. A., Watkins, S. C. and Romero, G. (1999) Phospholipase D and its product, phosphatidic acid, mediate agonist-dependent Raf-1 translocation to the plasma membrane and the activation of the mitogen-activated protein kinase pathway. *J. Biol. Chem.* **274**, 1131–1139
- Hong, J.-H., Oh, S.-O., Lee, M., Kim, Y.-R., Kim, D. U., Hur, G. M., Lee, J. H., Lim, K., Hwang, B. D. and Park, S. K. (2001) Enhancement of lysophosphatidic acid-induced ERK phosphorylation by phospholipase D1 via the formation of phosphatidic acid. *Biochem. Biophys. Res. Commun.* **281**, 1337–1342
- Banno, Y., Takuwa, Y., Akao, Y., Okamoto, H., Osawa, Y., Naganawa, T., Nakashima, S., Suh, P.-G. and Nozawa, Y. (2001) Involvement of phospholipase D in sphingosine 1-phosphate-induced activation of phosphatidylinositol 3-kinase and AKT in Chinese hamster ovary cells overexpressing EDG3. *J. Biol. Chem.* **276**, 35622–35628
- Okamoto, H., Takuwa, N., Yokomizo, T., Sugimoto, N., Sakurada, S., Shigematsu, H. and Takuwa, Y. (2000) Inhibitory regulation of Rac activation, membrane ruffling, and cell migration by the G protein-coupled sphingosine-1-phosphate receptor EDG5 but not EDG1 or EDG3. *Mol. Cell Biol.* **20**, 9247–9261
- Murga, C., Fukuhara, C. and Gutkind, S. J. S. (2000) A novel role for phosphatidylinositol 3-kinase β in signaling from G protein-coupled receptors to Akt. *J. Biol. Chem.* **275**, 12069–12073
- Xu, Q. and Wu, Z. (2000) The insulin-like growth factor-phosphatidylinositol 3-kinase-Akt signaling pathway regulates myogenin expression in normal myogenic cells but not in rhabdomyosarcoma-derived RD cells. *J. Biol. Chem.* **275**, 36750–36757
- Banno, Y., Fujita, H., Ono, Y., Nakashima, S., Ito, Y., Kuzumaki, N. and Nozawa, Y. (1999) Differential phospholipase D activation by bradykinin and sphingosine 1-phosphate in NIH 3T3 fibroblasts overexpressing gelsolin. *J. Biol. Chem.* **274**, 27385–27391
- Okamoto, H., Takuwa, N., Yatomi, Y., Gonda, K., Shigematsu, H. and Takuwa, Y. (1998) EDG3 is a functional receptor specific for sphingosine 1-phosphate and sphingosyl-phosphorylcholine with signaling characteristics distinct from EDG1 and ARG16. *J. Biol. Chem.* **273**, 27104–27110
- Takuwa, N., Fukui, Y. and Takuwa, Y. (1999) Cyclin D1 expression mediated by phosphatidylinositol 3-kinase through mTOR-p70(S6K)-independent signaling in growth factor-stimulated NIH 3T3 fibroblasts. *Mol. Cell Biol.* **19**, 1346–1358
- Okamoto, H., Takuwa, N., Tatomi, Y., Gonda, K., Shigematsu, H. and Takuwa, Y. (1999) EDG3 is a functional receptor specific for sphingosine 1-phosphate and sphingosylphosphorylcholine with signaling characteristic distinct from EDG1 and AGR16. *Biochem. Biophys. Res. Commun.* **260**, 203–208

Received 2 September 2002/10 October 2002; accepted 15 October 2002

Published as BJ Immediate Publication 18 October 2002, DOI 10.1042/BJ20021368