# Involvement of phospholipases D1 and D2 in sphingosine 1-phosphate-induced ERK (extracellular-signal-regulated kinase) activation and interleukin-8 secretion in human bronchial epithelial cells

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Sphingosine 1-phosphate (S1P), a metabolite of sphingomyelin degradation, stimulates interleukin-8 (IL-8) secretion in human bronchial epithelial (Beas-2B) cells. The molecular mechanisms regulating S1P-mediated IL-8 secretion are yet to be completely defined. Here we provide evidence that activation of phospholipases D1 and D2 (PLD1 and PLD2) by S1P regulates the phosphorylation of extracellular-signal-regulated kinase (ERK) and IL-8 secretion in Beas-2B cells. S1P, in a time- and dose-dependent manner, enhanced the threonine/tyrosine phosphorylation of ERK. The inhibition of S1P-induced ERK phosphorylation by pertussis toxin and PD 98059 indicated coupling of S1P receptors to $G_i$ and the ERK signalling cascade respectively. Treatment of Beas-2B cells with butan-1-ol, but not butan-3-ol, abrogated the S1P-induced phosphorylation of Raf-1 and ERK, suggesting that PLD is involved in this activation. The roles of PLD1 and PLD2 in ERK activation and IL-8 secretion activated by S1P were investigated by infecting cells with adenoviral constructs of wild-type and catalytically inactive mutants of PLD1 and PLD2. Infection of Beas-2B cells with the wild-type constructs resulted in the activation of PLD1 and PLD2 by S1P and PMA. Also, the enhanced production of [$^{32}$P]phosphatidic acid and [$^{32}$P]phosphatidylbutanol in the presence of butan-1-ol and the increased phosphorylation of ERK by S1P were blocked by the catalytically inactive mutants hPLD1-K898R and mPLD2-K758R. Transient transfection of Beas-2B cells with human PLD1 and mouse PLD2 cDNAs potentiated S1P-mediated IL-8 secretion compared with vector controls. In addition, PD 98059 attenuated IL-8 secretion induced by S1P in a dose-dependent fashion. These results demonstrate that both PLD1 and PLD2 participate in S1P stimulation of ERK phosphorylation and IL-8 secretion in bronchial epithelial cells.

Key words: adenoviral infection, ERK1/2, PLD isoenzymes, Raf-1 activation, S1P receptors.

## INTRODUCTION

Sphingosine 1-phosphate (S1P), a bioactive sphingolipid derived from the metabolism of sphingomyelin, has been implicated as a mediator of cellular functions such as proliferation, differentiation, apoptosis, tumour cell invasion, cell migration and angiogenesis [1]. S1P-mediated cellular responses are due to its action as an extracellular mediator and as an intracellular second messenger. To date, five G-protein-coupled cell surface receptors [$S1P_1$/Edg-1, $S1P_2$/Edg-5, $S1P_3$/Edg-3, $S1P_4$/Edg-6 and $S1P_5$/Edg-8 (Edg is defined as endothelial differentiation gene)] that bind to S1P with high affinity have been identified and partially characterized [2,3]. The S1P receptors are coupled differentially via $G_i$, $G_q$ and $G_{12/13}$ to multiple effector systems such as Rho, adenylate cyclase, protein kinase C (PKC), mitogen-activated protein kinases (MAPKs), phospholipases, phosphatidylinositol 3-kinase (PI 3-kinase) and non-receptor tyrosine kinases [4–7]. S1P also increases intracellular $Ca^{2+}$ levels via activation of phospholipase C- and inositol 1,4,5-trisphosphate-dependent $Ca^{2+}$ release from the endoplasmic reticulum [8]. Recently, S1P has been shown to exhibit pro-inflammatory effects by regulating interleukin-6 (IL-6) secretion in smooth muscle cells and osteoblasts, as well as IL-8 secretion in bronchial epithelial and ovarian cancer cells [9–12]. However, the signalling mechanisms responsible for induction of IL-6 or IL-8 secretion by S1P have not been fully identified.

Phospholipase D (PLD) catalyses the hydrolysis of phosphatidylcholine (PC) and other membrane phospholipids to phosphatidic acid (PA) and choline [13]. Two isoforms of PLD, PLD1 and PLD2, have been cloned and are being characterized for regulation and cellular function [14,15]. PA mediates the biological and physiological functions of PLD either directly or indirectly by its metabolism to lyso-PA or diacylglycerol. PA, a second messenger in mammalian cells, regulates protein tyrosine phosphatases, phosphatidylinositol 4-kinase, type I$\alpha$ phosphatidylinositol 4-phosphate 5-kinase, PI 3-kinase and NADPH oxidase [16–20]. Furthermore, PA generated by the PLD pathway binds to Raf-1, regulating its recruitment to the membrane and subsequent participation in the MAPK signalling cascade and signal transduction [21–23].

S1P activates PLD in several mammalian cell systems, including airway/alveolar epithelial-derived cell lines such as CFNPE9o$^-$ and A549 [24,25]. We recently demonstrated that S1P is a potent activator of PLD and enhances IL-8 secretion in the bronchial epithelial Beas-2B cell line [11]. In Beas-2B cells, PLD activation and IL-8 secretion were sensitive to pertussis toxin (PTx), indicating the coupling of S1P receptor signalling to the heterotrimeric G-protein $G_i$. Additionally, butan-1-ol, but

Abbreviations used: AP-1, activator protein-1; DMEM, Dulbecco's modified Eagle's medium; Edg, endothelial differentiation gene; ERK, extracellular-signal-regulated kinase; FBS, fetal bovine serum; IL-8, interleukin-8; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MEM, minimal essential medium; NF-$\kappa$B, nuclear factor-$\kappa$B; PA, phosphatidic acid; PBt, phosphatidylbutanol; PC, phosphatidylcholine; p.f.u., plaque-forming units; PI 3-kinase, phosphatidylinositol 3-kinase; $PIP_2$, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLD, phospholipase D; PTx, pertussis toxin; S1P, sphingosine 1-phosphate; the prefixes h and m denote human and mouse respectively.

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not butan-3-ol, attenuated S1P-mediated IL-8 production, suggesting the involvement of PLD-generated PA in IL-8 secretion [11]. However, the mechanism(s) of S1P signal transduction and the role of PLD in mediating IL-8 secretion in airway epithelial cells are not well defined. In the present study, we have characterized PLD isoenzyme (PLD1 and/or PLD2) activation by S1P and examined the roles of PLD1 and/or PLD2 in the S1P-induced activation of extracellular-signal-regulated kinase (ERK) and IL-8 secretion by transient infection of Beas-2B cells with adenoviral constructs of wild-type and catalytically inactive mutants of human (h)PLD1 and mouse (m)PLD2. We show here that S1P activated both PLD1 and PLD2 in Beas-2B cells, and overexpression of wild-type hPLD1 and mPLD2 enhanced ERK phosphorylation, while the catalytically inactive mutants attenuated ERK phosphorylation by S1P. Furthermore, S1P-induced IL-8 secretion was augmented in hPLD1- and mPLD2-overexpressing cells. Evidence is also provided for a role for PA generated by the PLD pathway in the phosphorylation of Raf-1. These data show that activation of PLD1 and PLD2 is involved in S1P-induced ERK phosphorylation and IL-8 secretion in human bronchial epithelial cells.

## MATERIALS AND METHODS

### Materials

S1P was obtained from BIOMOL Research Labs (Plymouth, PA, U.S.A.). PMA, fetal bovine serum (FBS), BSA (fraction V), opti-MEM1 medium, butan-1-ol, butan-3-ol, phosphate-free Dulbecco's modified Eagle's medium (DMEM), minimal essential medium (MEM) and DMSO were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). PD 98059, phosphatidylinositol 4,5-bisphosphate ($PIP_2$) and PTx were obtained from Calbiochem Corp. (La Jolla, CA, U.S.A.). Dioleoyl phosphatidylbutanol (PBt) and dioleoyl PC were purchased from Avanti Polar Lipids Corp. (Alabaster, GA, U.S.A.). [$^3$H]Choline-labelled PC and dioleoyl phosphatidylethanolamine were purchased from America Radiolabeled Chemicals (St. Louis, MO, U.S.A.). [$^{32}$P]$P_i$ (carrier-free) was obtained from New England Nuclear (Boston, MA, U.S.A.). Polyclonal antibodies against phospho-ERK were purchased from Cell Signaling (Beverly, MA, U.S.A.). Antibody for pan-ERK was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). Antibodies against Raf-1 and phospho-specific Raf-1 (S338) were purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Internal and N-terminal antibodies for PLD1 and PLD2 and IL-8 ELISA kit were purchased from Biosource International Inc. (Camarillo, CA, U.S.A.). FuGENE 6 transfection reagent was obtained from Roche (Indianapolis, IN, U.S.A.). Horseradish peroxidase-conjugated secondary antibodies were procured from Bio-Rad (Hercules, CA, U.S.A.). Enhanced chemiluminescence detection reagents were obtained from Amersham Pharmacia Inc. (Piscataway, NJ, U.S.A.). All other reagents were of analytical grade and obtained commercially.

### Cell culture

Human bronchial epithelial cells (Beas-2B; passage 32; kindly provided by Dr Sherer Sanders, Division of Pulmonary and Critical Care Medicine, Johns Hopkins University) were cultured in MEM supplemented with 5 % (v/v) FBS and antibiotics. Cells were grown in T-75 cm$^2$ vented flasks at 37 °C in 5 % $CO_2$/95 % air to 90 % confluence and subsequently propagated in 35 mm dishes. Cells at 60–70 % confluence were serum deprived by incubation for 12–18 h in MEM containing 0.2 % (v/v) FBS prior to stimulation with S1P or other agents. All experiments were carried out between passages 35 and 43.

### Adenovirus production of wild-type and catalytically inactive PLD1 and PLD2

hPLD1b (wild type and K898R mutant) and mPLD2 (wild type and K758R mutant) cDNAs from pCGN-hPLD1b and pCGN-mPLD2 [15] were subcloned into the pshuttle-CMV vector. Adenoviral DNA was produced by a double-recombination event between co-transformed adenoviral backbone plasmid, pAdEasy-1, and the shuttle vector carrying the PLD gene in *Escherichia coli* strain BJ5183. The recombinant plasmid was linearized and transfected into HEK293 cells to generate replication-defective adenovirus. Generation of purified virus [$10^{10}$ plaque-forming units (p.f.u.)/ml] was carried out by the University of Iowa Gene Transfer Vector Core.

### Transfection and viral infection

To Beas-2B cells grown to ~50 % confluence in 35 mm dishes was added purified adenovirus (50 p.f.u./cell) in complete MEM (volume 2 ml). After overnight culture, the virus-containing medium was replaced with fresh complete medium for different time periods, as indicated. For transient transfection, cells grown in 12-well plates were transfected with hPLD1 and mPLD2 plasmids (0.5 $\mu$g of DNA/well) with FuGENE 6 (3 $\mu$l/well) according to the manufacturer's recommendations. Cells were challenged with S1P or other agents 24–48 h after viral infection or transfection with plasmids.

### Measurement of PLD activation in intact cells

Beas-2B cells (80–90 % confluence) were labelled with [$^{32}$P]$P_i$ (5 $\mu$Ci/ml) in phosphate-free DMEM containing 0.2 % (v/v) FBS for 12–18 h at 37 °C in a 5 % $CO_2$/95 % air incubator. The radioactive medium was replaced with MEM, and cells were challenged with S1P in MEM containing 0.1 % BSA and 0.05 % butan-1-ol or butan-3-ol for specified time periods. Incubations were terminated by the addition of 1 ml of methanol/HCl (100:1, v/v) and lipids were extracted as described previously [26]. [$^{32}$P]PBt formed was quantified after separation of total radioactive lipids by TLC with an upper phase of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (65:10:15:50, by vol.) as the developing solvent system. Unlabelled dioleoyl PBt was added as a carrier during TLC separation, and radioactivity associated with PBt was determined by liquid scintillation counting. All values were normalized to $10^6$ d.p.m. in total lipid extract, and data are expressed as d.p.m./dish.

### Measurement of PLD1 and PLD2 activities *in vitro*

Briefly, Beas-2B cells infected with vector control, hPLD1 or mPLD2 adenoviral constructs were scraped into 1 ml of lysis buffer containing 25 mM Tris (pH 7.4), 1 mM EDTA and protease inhibitors, and sonicated by a probe sonicator. A 30 $\mu$l aliquot of cell lysate was mixed with 50 $\mu$l of lipid mix (5 $\mu$M PC, 88 $\mu$M phosphatidylethanolamine, 7 $\mu$M $PIP_2$ and [$^3$H]methylcholine-PC), 20 $\mu$l of 5 × assay buffer (250 mM Hepes, pH 7.4, 5 mM EGTA, 400 mM KCl, 5 mM dithiothreitol, 15 mM $MgCl_2$ and 10 mM $CaCl_2$) plus 1 $\mu$g of activated ADP-ribosylation factor. The mixture was incubated at 37 °C for 30 min. The reaction was stopped with 200 $\mu$l of 10 % (v/v)

trichloroacetic acid on ice and centrifuged, then the radioactivity in the supernatant was counted and PLD activity was expressed as pmol of [$^3$H]PC hydrolysed per 30 min.

### Measurement of IL-8 secretion

Beas-2B cells (~70% confluence) in 35 mm dishes were pretreated with specified agents for the indicated time periods, then media were aspirated and cells were challenged with MEM alone or MEM containing 0.1% BSA with or without S1P for 3 h. After this time the supernatant was carefully removed, centrifuged at 5000 ***g*** for 5 min at 4 °C and stored at −80 °C. The IL-8 concentration in the medium was measured by ELISA.

### Preparation of cell lysates and Western blot analysis

Beas-2B cells grown in 100 mm dishes (~70% confluence) were stimulated with S1P, rinsed with ice-cold PBS and lysed in RIPA buffer. Cell lysate was sonicated on ice and centrifuged at 10000 ***g*** for 5 min at 4 °C. Protein concentration was determined with a BCA protein assay kit (Pierce Chemical Co., Rockford IL, U.S.A.) using BSA as standard. The samples (10–20 $\mu$g of protein) were subjected to SDS/PAGE, and proteins from the gel were transferred to a PVDF membrane, blocked with 5% (w/v) non-fat dry milk in TBST (25 mM Tris base, pH 7.4, 137 mM NaCl and 0.1% Tween 20) for 1 h and incubated with primary antibodies against phospho-ERK, pan-ERK, PLD1 (internal plus N-terminal) or PLD2 (internal plus N-terminal) (1:1000 dilution) in TBST overnight at 4 °C. The membranes were washed four times with TBST at 20 min intervals and then incubated with either mouse or rabbit horseradish peroxidase-conjugated secondary antibody (1:3000 dilution) for 2 h at room temperature. The membranes were developed with an enhanced chemiluminescence detection system according to the manufacturer's instructions, and blots were quantified by densitometric scanning and image analysis with Molecular Analyst software. ERK phosphorylation was expressed as fold change normalized to total ERK.

### Assessment of Raf-1 phosphorylation

Beas-2B cells in 100 mm dishes were treated with medium alone or medium containing S1P (1 $\mu$M) in the absence or presence of butan-1-ol or butan-3-ol (0.05%) for 5 min. Cells were washed in ice-cold PBS containing 1 mM sodium orthovanadate, scraped into microcentrifuge tubes in RIPA buffer containing protease and phosphatase inhibitors and lysed as described above. Cell lysates, after boiling in SDS sample buffer, were resolved by SDS/PAGE (equal amounts of protein were loaded), transferred to a PVDF membrane and probed with phospho-specific anti-Raf-1 antibody, and immunocomplexes were detected by enhanced chemiluminescence.

### Statistical analysis

All results were subjected to statistical analysis using one-way ANOVA. Data are expressed as means ± S.D. of triplicate samples from at least three independent experiments.

## RESULTS

### S1P activates ERK1/2 in Beas-2B cells

Serum-deprived Beas-2B cells were challenged with S1P (1 $\mu$M) and ERK activation was measured by Western blot analysis with phospho-specific antibodies against tyrosine and threonine residues. Total ERK was measured with pan-ERK antibodies. As shown in Figure 1 (left panels), S1P stimulated ERK1/2 phosphorylation in a time-dependent manner. Enhanced phosphorylation of ERK1/2 (~2-fold) was detected within 2 min of exposure to S1P, while maximum phosphorylation (~5-fold) occurred at 15 min, and declined thereafter. However, even at 60 min after S1P treatment, phosphorylation of ERK1/2 was about 1.5-fold higher as compared with vehicle (Figure 1, left panels). Immunoprecipitates of ERK from S1P (1 $\mu$M)-challenged cells also exhibited enhanced phosphorylation of myelin basic protein with [$\gamma$-$^{32}$P]ATP (results not shown), indicating stimulation of the catalytic activity of ERK by S1P. Furthermore, the S1P-induced phosphorylation of ERK was dose-dependent. Phosphorylation of ERK1/2 was detected after 15 min of exposure to 100 nM S1P (2-fold increase over control), which increased to ~6-fold with 1 $\mu$M S1P (Figure 1, right panels).

### S1P-induced activation of ERK is sensitive to PTx and PD 98059

Previous studies have shown that, in many cell types, S1P receptors (formerly called Edg receptors) are coupled to $G_i$, $G_q$ or $G_{12/13}$. We therefore investigated the effect of PTx on S1P-mediated ERK1/2 activation. Beas-2B cells were pretreated with PTx (50 ng/ml) for 12 h at 37 °C before challenging with S1P (1 $\mu$M for 15 min). Figure 2 shows that PTx pretreatment almost completely blocked S1P-induced ERK1/2 phosphorylation, suggesting the involvement of $G_i$-coupled S1P receptors in the activation. Interestingly, PTx pretreatment also attenuated the basal phosphorylation of ERK1/2 in Beas-2B cells.

To further examine the signalling pathways involved in the activation of ERK, Beas-2B cells were pretreated with PD 98059, a specific inhibitor of MAPK/ERK kinase (MEK). As shown in Figure 3, PD 98059 at 10 $\mu$M suppressed both S1P- and PMA (TPA)-induced phosphorylation of ERK1/2, by 80% and 95% respectively. These results suggest that the S1P-induced activation of ERK is coupled to $G_i$ and MEK signalling in Beas-2B cells.

### Role of PLD in S1P-mediated ERK phosphorylation in Beas-2B cells

PLD, in addition to its catalytic activity, also exhibits a transphosphatidylation reaction. In the presence of short-chain primary (but not secondary or tertiary) alcohols, PLD catalyses the accumulation of phosphatidylalcohol from PA by a transphosphatidylation reaction. Thus the formation of phosphatidylalcohol, which removes the PA from second messenger functions, has been employed to imply a role for PLD in cell function. Earlier studies have shown S1P stimulation of PLD in Beas-2B cells [11]. To determine whether PLD activation by S1P regulates ERK phosphorylation, serum-deprived Beas-2B cells were stimulated with S1P (1 $\mu$M) for 15 min in the absence or presence of butan-1-ol or butan-3-ol. As shown in Figure 4, butan-1-ol (0.05%), but not butan-3-ol (0.05%), blocked S1P-induced ERK1/2 phosphorylation without affecting the basal levels of ERK, suggesting that PLD-generated PA is an upstream regulator of S1P-induced ERK1/2 activation.

To further support the finding that S1P-induced PLD activation is upstream of the ERK pathway, we examined the effect of PD 98059 on S1P-mediated [$^{32}$P]PBt formation. As shown in Figure 5, treatment with S1P (1 $\mu$M) or PMA (25 nM) for 15 min stimulated PLD activity by ~4- and 9-fold respectively over basal levels. Pretreatment of cells with PD 98059 (25 $\mu$M for 1 h) had no effect on either S1P- or PMA-induced [$^{32}$P]PBt generation (Figure 5). However, pretreatment with PTx (100 ng/ml for 1 h) effectively blocked (> 95%) S1P-induced, but not PMA-induced, [$^{32}$P]PBt accumulation. These results strongly indicate that ERK

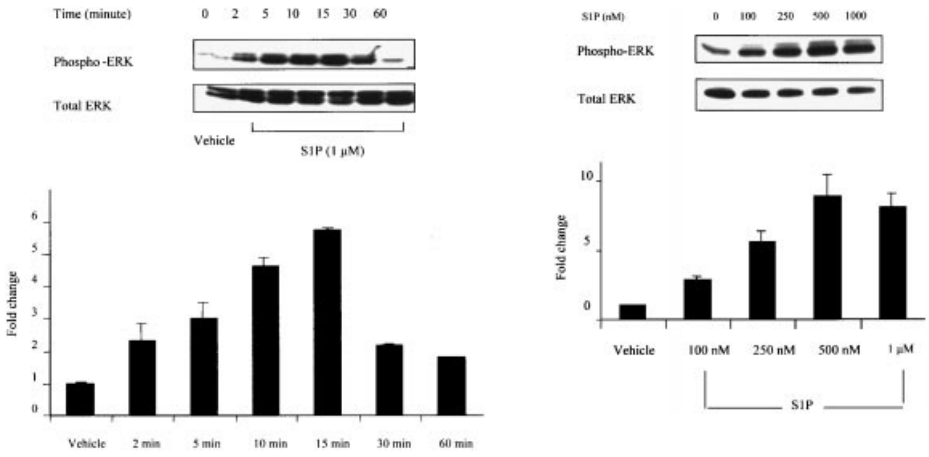


**Figure 1 S1P stimulates ERK phosphorylation in Beas-2B cells**

Left panels: Beas-2B cells were challenged with S1P (1 $\mu$M) for various time periods as indicated, and cell lysates were analysed by Western blotting for phosphorylation of ERK and total ERK with phospho-specific ERK and pan-ERK antibodies respectively. Right panels: Beas-2B cells were incubated with increasing concentrations of S1P as indicated for 15 min, and cell lysates were analysed by Western blotting with phospho-specific and pan-ERK antibodies as described in the Materials and methods section. Values are means $\pm$ S.D. from three independent experiments, and fold increases in phosphorylation were normalized to total ERK.

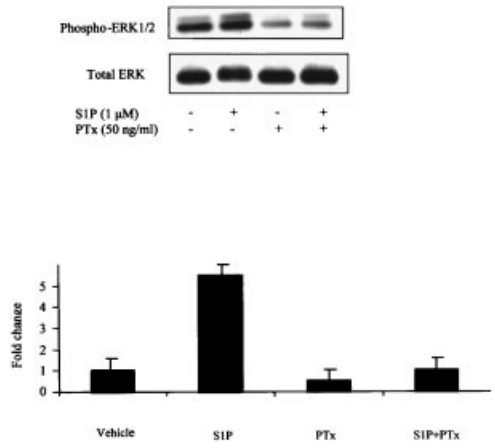


**Figure 2 PTx blocks S1P-induced ERK phosphorylation**

Beas-2B cells ($\sim$70% confluence in 100 mm dishes) were serum deprived [MEM containing 0.2% (v/v) FBS] for 18 h prior to PTx (50 ng/ml) treatment for 12 h. Cells were challenged with S1P (1 $\mu$M) for 15 min, then cell lysates were prepared as described in the Materials and methods section, subjected to SDS/PAGE and Western blotted with phospho-specific and pan-ERK antibodies. Values are means $\pm$ S.D. of three independent experiments in triplicate. Fold increases in ERK phosphorylation were normalized to total ERK.

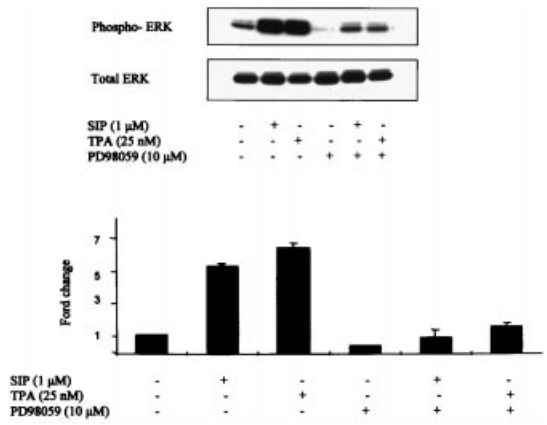


**Figure 3 PD 98059 attenuates S1P- and PMA-mediated ERK phosphorylation**

Beas-2B cells ($\sim$70% confluence in 100 mm dishes) were serum deprived for 18 h prior to treatment with PD 98059 for 60 min. Subsequently, cells were challenged with S1P (1 $\mu$M) or PMA (TPA; 25 nM) for 15 min in MEM with 0.1% BSA. Cell lysates were prepared as described in the Materials and methods section, and subjected to SDS/PAGE and Western blotting with phospho-specific ERK and pan-ERK antibodies. Shown is a representative gel. Values are means $\pm$ S.D. of triplicate determinations from three independent experiments.

is not involved in regulating S1P-mediated PLD activation in Beas-2B cells, and that activation of both ERK and PLD are $G_i$-dependent.

### Effect of PLD-generated PA on S1P-induced Raf-1 phosphorylation

As PA interacts directly with Raf-1, an essential component of ERK signalling [27], we investigated the effect of PLD-generated PA induced by S1P on the phosphorylation of Raf-1. Treatment of Beas-2B cells with S1P (1 $\mu$M) for 5 min induced a 2-fold increase in Raf-1 phosphorylation, as determined by Western blotting with a phosphoserine-specific Raf antibody. However, in the presence of 0.05% butan-1-ol (but not butan-3-ol), S1P-induced Raf-1 phosphorylation was significantly attenuated (Figure 6). This supports the notion that the S1P-induced generation of PA via PLD regulates the phosphorylation of Raf-1 in human bronchial epithelial cells, which in turn regulates downstream ERK1/2 activation.

### Overexpression of hPLD1 and mPLD2 adenoviral constructs in Beas-2B cells

To further examine the role of PLD isoenzymes (PLD1 and PLD2) in S1P-mediated ERK phosphorylation and IL-8 secretion, we generated adenoviral constructs of wild-type and

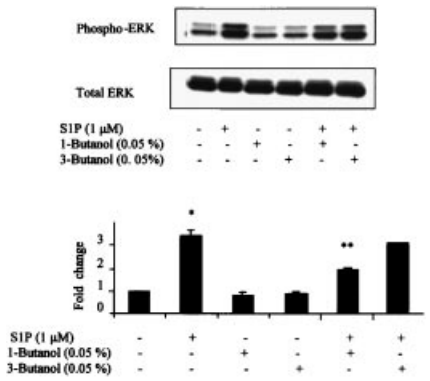


**Figure 4 Butan-1-ol, but not butan-3-ol, attenuates S1P-induced ERK phosphorylation**

Beas-2B cells (~70% confluence in 100 mm dishes) were serum deprived for 18 h in MEM containing 0.2% (v/v) FBS. Cells were pretreated with MEM or MEM plus butan-1-ol or butan-3-ol (0.05%) for 15 min and then challenged with S1P (1 $\mu$M) in 0.1% BSA for an additional 15 min. Cell lysates were subjected to SDS/PAGE and Western blotting with phospho-specific and pan-ERK antibodies. Values are means $\pm$ S.D. of triplicate determinations from three independent experiments. Fold increases in ERK phosphorylation were normalized to total ERK in the cell lysates. $^{*}P < 0.05$ compared with vehicle control; $^{**}P < 0.05$ compared with S1P treatment.

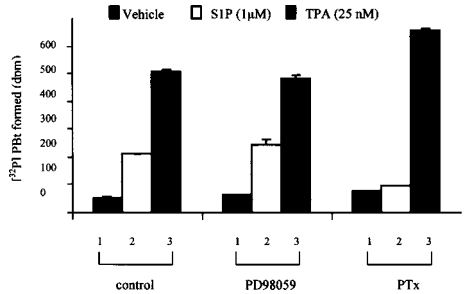


**Figure 5 Effects of PD 98059 and PTx on S1P- and PMA-induced PLD activation**

Beas-2B cells (~80% confluence in 35 mm dishes) were labelled with [$^{32}$P]$P_i$ (5 $\mu$Ci/ml) in phosphate-free DMEM for 18 h, then radioactive medium was aspirated and cells were pretreated with PD 98059 (25 $\mu$M) or PTx (100 ng/ml) for 1 h. Cells were challenged with MEM or MEM containing either S1P (1 $\mu$M) or PMA (TPA; 25 nM) in 0.1% BSA and 0.05% butan-1-ol for 15 min. Lipids were extracted under acidic conditions and the [$^{32}$P]PBt formed was quantified after separation by TLC. Values are means $\pm$ S.D. of three independent experiments in triplicate, and are normalized to $10^6$ d.p.m. in total lipids.

catalytically inactive mutants of hPLD1 and mPLD2. Beas-2B cells were infected with the adenoviral constructs (50 p.f.u./cell) for 12, 24 and 48 h. Cell lysates were analysed for increased expression of the PLD1 and PLD2 proteins, enhanced generation of PA and PBt in response to agonists, and PLD1 and PLD2 activities *in vitro*. As shown in Figure 7(A), infection of Beas-2B cells with adenoviral constructs of wild-type hPLD1 and mPLD2 enhanced the expression of PLD1 and PLD2 proteins in a time-dependent manner, with optimal expression observed at 48 h after infection. Therefore this time point was chosen for all of the overexpression studies. The functional significance of hPLD1 and mPLD2 overexpression was tested by determining the accumulation of [$^{32}$P]PA and [$^{32}$P]PBt in the presence of butan-1-ol via PLD activation. Overexpression of hPLD1 and mPLD2 wild types in Beas-2B cells increased the accumulation of [$^{32}$P]PA under basal conditions and in response to S1P challenge compared with vector control cells (Figure 7B). In cells infected with the wild-type constructs, PMA-induced PLD activation, as determined by [$^{32}$P]PBt formation, was potentiated. Inhibition of [$^{32}$P]PBt generation was observed in mutant infected cells compared with vector controls (Figure 7C). *In vitro* PLD1 and PLD2 activities, as measured by [$^{3}$H]PC hydrolysis, were increased in hPLD1- and mPLD2-overexpressing cells, while cells infected with hPLD1 and mPLD2 mutants exhibited lower activity (Figure 7D). These results demonstrate the overexpression of PLD1 and PLD2 proteins by the adenoviral constructs and the functional consequences of the overexpressed proteins in bronchial epithelial cells.

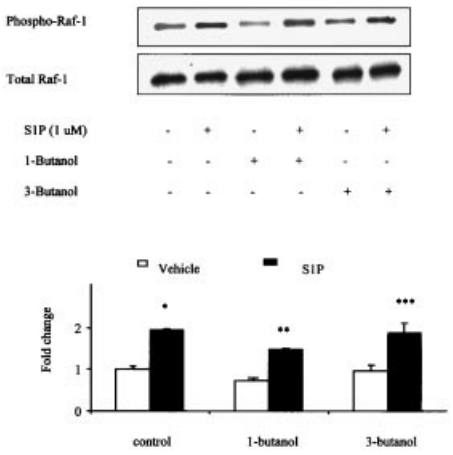


**Figure 6 Effects of butan-1-ol and butan-3-ol on S1P-stimulated Raf-1 phosphorylation**

Beas-2B cells were serum starved for 18 h, then stimulated with S1P (1 $\mu$M) for 5 min in the presence of butan-1-ol or butan-3-ol (0.05%, v/v). Cell lysates were subjected to SDS/PAGE, and Raf-1 phosphorylation was measured by Western blotting with a phosphoserine-specific antibody. The extent of Raf-1 phosphorylation was normalized to total Raf-1. Values are means $\pm$ S.D. of triplicate determinations from three independent experiments. $^{*}P < 0.05$ compared with vehicle control; $^{**}P < 0.05$ compared with S1P treatment; $^{***}P > 0.05$ compared with S1P treatment (i.e. not significantly different).

### S1P activates both PLD1 and PLD2 in Beas-2B cells

As S1P stimulates PLD in human bronchial epithelial cells [11,24], we investigated which of the isoenzymes of PLD are activated by S1P in Beas-2B cells. In vector-infected cells, S1P stimulated [$^{32}$P]PBt accumulation by 5-fold (vehicle, $581 \pm 27$ d.p.m.; S1P, $3006 \pm 94$ d.p.m.). Overexpression of hPLD1 (vehicle, $1608 \pm 220$ d.p.m.; S1P, $7760 \pm 439$ d.p.m.) or mPLD2 (vehicle, $6109 \pm 253$ d.p.m.; S1P, $15737 \pm 217$ d.p.m.) enhanced both basal and S1P-induced [$^{32}$P]PBt accumulation compared with vector controls. However, overexpression of the catalytically inactive mutants of PLD1 (K898R; vehicle, $791 \pm 49$ d.p.m.; S1P, $2257 \pm 140$ d.p.m.) and mPLD2 (K758R; vehicle, $786 \pm 186$ d.p.m.; S1P, $1760 \pm 106$ d.p.m.) partially blocked S1P-mediated [$^{32}$P]PBt formation without altering basal activity (Figure 8).

### Overexpression of PLD1 and PLD2 enhances S1P-induced ERK phosphorylation

To further establish a role for PLD1 and PLD2 in S1P-mediated ERK phosphorylation, Beas-2B cells were infected with wild-type and catalytically inactive mutants of hPLD1 and mPLD2 adenoviral constructs. Overexpression of wild-type hPLD1

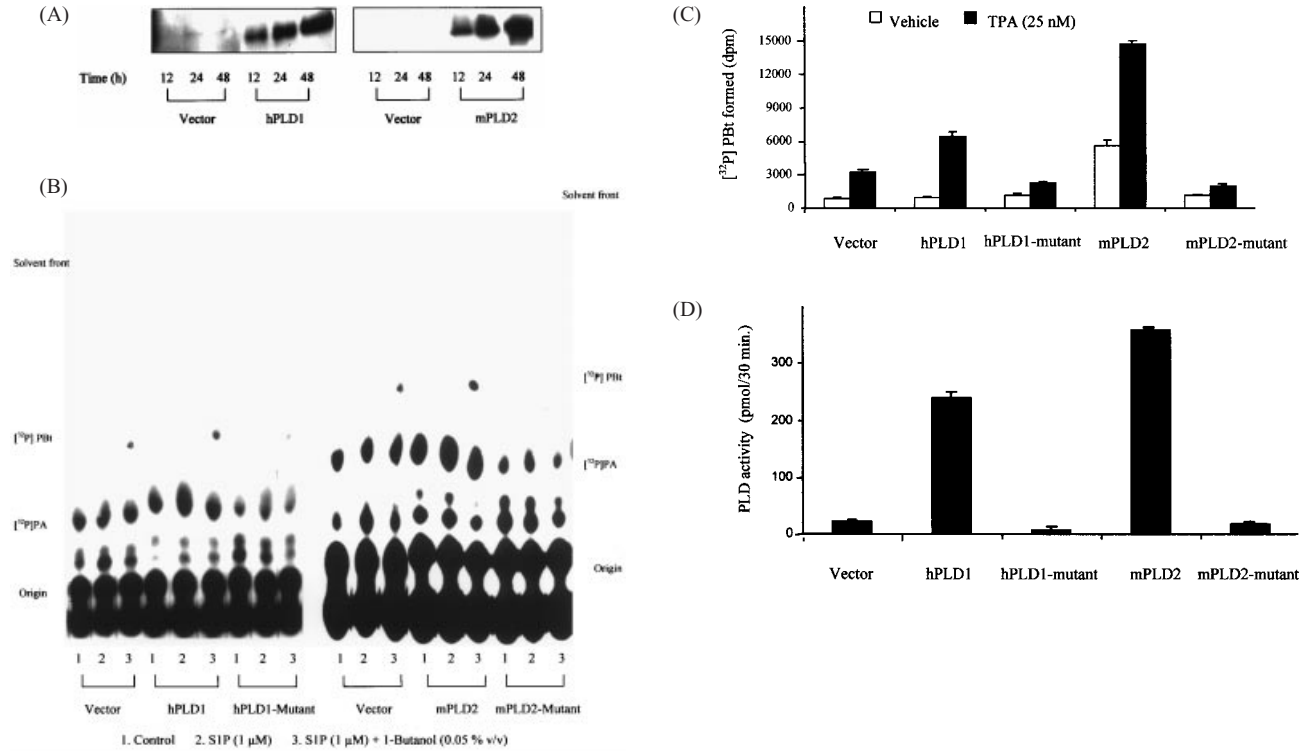


**Figure 7 Overexpression of adenoviral constructs of wild-type and mutant hPLD1 and mPLD2 in Beas-2B cells**

(**A**) Beas-2B cells (~50% confluence in 60 mm dishes) were infected with vector or with wild-type hPLD1 or mPLD2 adenoviral constructs (50 p.f.u./cell; $3 \times 10^5$ cells per dish) in complete MEM for 12, 24 and 48 h. At the indicated time points, cell lysates were prepared as described in the Materials and methods section, and subjected to SDS/PAGE and Western blotting with internal plus N-terminal PLD1 and PLD2 antibodies. (**B**) Beas-2B cells in 35 mm dishes were infected with vector or with wild-type or mutant hPLD1/mPLD2 adenoviral constructs (50 p.f.u./cell; $1 \times 10^5$ cells per dish) for 24 h. The cells were labelled with [$^{32}$P]$P_i$ (5 $\mu$Ci/ml) in phosphate-free DMEM for 12–18 h, then radioactive medium was aspirated and cells were challenged with MEM or MEM plus S1P (1 $\mu$M) in 0.1% BSA in the absence or presence of 0.05% butan-1-ol for 2 min. Lipids were extracted under acidic conditions and separated by TLC as described in the Materials and methods section. Labelled phospholipids were visualized by autoradiography. (**C**) Beas-2B cells (~50% confluence in 35 mm dishes) were infected with vector or with wild-type or mutant hPLD1/mPLD2 adenoviral constructs and labelled with [$^{32}$P]$P_i$ as indicated in (**B**). Cells were challenged with MEM or MEM plus PMA (TPA; 25 nM) plus 0.05% butan-1-ol for 15 min. Lipids were extracted under acidic conditions and [$^{32}$P]PBt formed was quantified after separation by TLC. Values are means ± S.D. of three independent experiments in triplicate. (**D**) Beas-2B cells (~70% confluence in 100 mm dishes) were infected with vector or with wild-type or mutant hPLD1/mPLD2 adenoviral constructs for 24 h. Cell lysates prepared in assay buffer, as indicated in the Materials and methods section, were assayed for PLD1 and PLD2 activities *in vitro* in the presence of $PIP_2$ and other cofactors, as described in the Materials and methods section. PLD activity was expressed as pmol of [$^3$H]choline released from [$^3$H]PC/30 min. Values are means ± S.D. of triplicate samples.

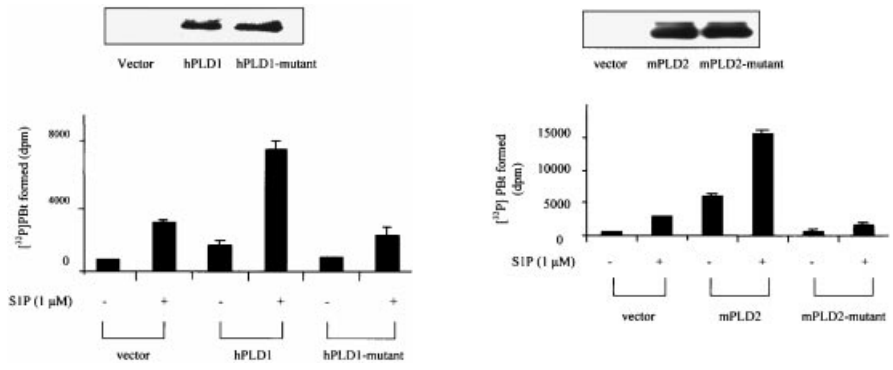


**Figure 8 Effects of overexpression of wild-type and catalytically inactive mutants of hPLD1 and mPLD2 on S1P-induced [$^{32}$P]PBt formation**

Left panels: Beas-2B cells (~70% confluence in 35 mm dishes) were infected with wild-type or mutant hPLD1 (50 p.f.u./cell; $2 \times 10^5$ cells per dish) for 24 h and subsequently labelled with [$^{32}$P]$P_i$ for 18 h as described in the Materials and methods section. Cells were rinsed in MEM and challenged with MEM or MEM plus S1P (1 $\mu$M) containing 0.1% BSA and 0.05% butan-1-ol for 15 min. Lipids were extracted under acidic conditions and [$^{32}$P]PBt formed was quantified after separation of the total labelled lipids by TLC. Values are means ± S.D. of triplicate determinations from three independent experiments, normalized to $10^6$ d.p.m. in total lipid extracts. Right panels: Beas-2B cells in 60 mm dishes were infected with vector or with wild-type or mutant mPLD2, labelled with [$^{32}$P]$P_i$ and challenged with S1P (1 $\mu$M) for 15 min as described above. [$^{32}$P]PBt formed was quantified after separation by TLC and data were normalized to $10^6$ d.p.m. in total lipids. Values are means ± S.D. of three independent experiments in triplicate.

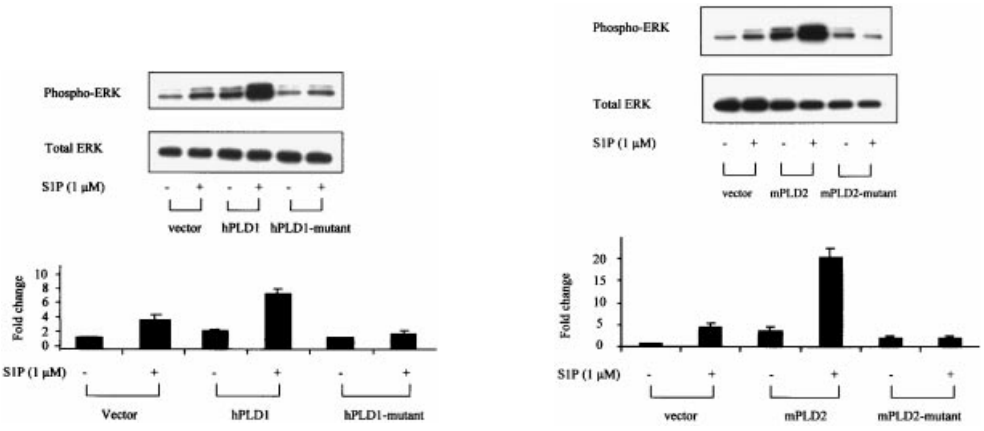


**Figure 9 Effects of overexpression of wild-type and mutant hPLD1 and mPLD2 on S1P-induced ERK phosphorylation**

Left panels: Beas-2B cells (~70% confluence in 60 mm dishes) were infected with vector or with wild-type or mutant hPLD1 for 24 h. Cells were serum deprived for 3 h before challenging with MEM or MEM plus S1P (1 $\mu$M) containing 0.1% BSA for 15 min. Right panels: Beas-2B cells were infected with vector or with wild-type or mutant mPLD2 as described above. Cell lysates were subjected to SDS/PAGE and Western blotting with phospho-specific and pan-ERK antibodies. Values are means ± S.D. of three independent experiments in triplicate. Fold increases in ERK phosphorylation were normalized to total ERK in the cell lysates.

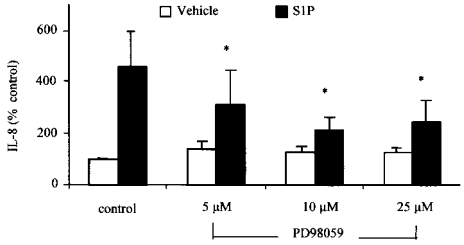


**Figure 10 PD 98059 attenuates S1P-mediated IL-8 secretion**

Beas-2B cells grown to ~90% confluence in 60 mm dishes were serum deprived for 3 h. Cells were pretreated with PD 98059, at the indicated concentrations, for 1 h prior to challenge with MEM or MEM plus S1P (1 $\mu$M) containing 0.1% BSA for 15 min. The medium was carefully removed, centrifuged at 5000 ***g*** for 5 min and the supernatant stored at −80 °C for IL-8 measurement by ELISA. Values are means ± S.D. of three independent experiments in triplicate. $^{*}P < 0.05$ compared with S1P treatment alone.

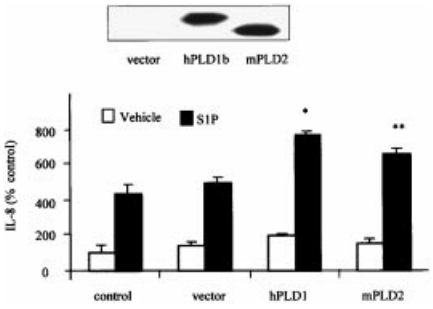


**Figure 11 Overexpression of wild-type hPLD1 or mPLD2 potentiates S1P-induced IL-8 secretion**

Beas-2B cells grown to ~50% confluence in 12-well plates were transiently transfected with hPLD1 or mPLD2 cDNA (0.5 $\mu$g/well) using FuGENE 6 (3 $\mu$g/well), with Opti-MEM as the transfection agent. After 5 h of exposure to the plasmids, the medium was replaced with 1 ml of complete MEM/10% (v/v) FBS. After 48 h of transfection, cells were rinsed in MEM and challenged with MEM or MEM plus S1P (1 $\mu$M) in 0.1% BSA for 3 h. The supernatants (1 ml) were removed, centrifuged at 5000 ***g*** for 5 min and stored at −80 °C for IL-8 determination by ELISA. Values are means ± S.D. of three independent experiments in triplicate. * and ** denote a significant difference ($P < 0.05$) compared with S1P treatment of the vector control.

(vehicle, 2.1 ± 0.9-fold; S1P, 7.3 ± 1.9-fold) and mPLD2 (vehicle 3.9 ± 1.5-fold; S1P, 20.6 ± 4.4-fold) increased both basal and S1P-mediated phosphorylation of ERK1/2 compared with vector control cells (vehicle, 1.0; S1P, 4.7 ± 1.8-fold). Infecting the cells with the catalytically inactive mutants of hPLD1 (vehicle, 0.9 ± 0.1-fold; S1P, 1.8 ± 0.6-fold) and mPLD2 (vehicle, 2.2 ± 0.92-fold; S1P, 2.2 ± 0.98-fold) abolished ERK1/2 phosphorylation mediated by S1P (Figure 9). These results further confirm the involvement of both PLD1 and PLD2 in ERK phosphorylation induced by S1P in Beas-2B cells.

### Role of ERK in S1P-induced IL-8 secretion in Beas-2B cells

We have previously demonstrated that S1P-induced IL-8 secretion is attenuated by butan-1-ol, but not by butan-3-ol, in Beas-2B cells, suggesting regulation of IL-8 secretion by the PLD signalling cascade [11]. As activation of PLD1 and PLD2 by S1P also enhances ERK phosphorylation, it was important to examine the link between ERK phosphorylation and IL-8 secretion mediated by PLD. As shown in Figure 10, S1P (1 $\mu$M) treatment of Beas-2B cells for 3 h stimulated IL-8 secretion by 4-fold compared with control cells exposed to MEM alone. Pretreatment of cells with PD 98059 (10 $\mu$M), a selective inhibitor of MEK1/2 upstream of ERK, attenuated S1P-mediated IL-8 secretion (~40% of control).

To investigate the role of PLD1 and PLD2 in S1P-induced IL-8 secretion, Beas-2B cells were infected with vector control or wild-type adenoviral constructs prior to S1P stimulation. Surprisingly, even in the vector control (LacZ), a high level of IL-8 secretion was observed (results not shown). Therefore we transiently overexpressed PLD1 and PLD2 in Beas-2B cells with mammalian expression plasmids using FuGENE 6. As shown in Figure 11, overexpression of PLD1 and PLD2 potentiated S1P-

mediated IL-8 secretion compared with the vector control. These data further establish a role for both PLD1 and PLD2 in S1P-induced IL-8 secretion in bronchial epithelial cells.

## DISCUSSION

S1P, a potent bioactive sphingolipid, has emerged as an important extracellular and intracellular signalling molecule in mammalian cells [1]. S1P mediates a number of cellular responses, including cell proliferation, chemotaxis and angiogenesis [28–30]. Many of the cellular responses to S1P are mediated by activation of protein kinases, changes in intracellular $Ca^{2+}$, stimulation of phospholipase C and PLD and transactivation of growth factor receptors [1,3,31]. We have recently shown that S1P is also a potent activator of IL-8 secretion in human bronchial epithelial cells in culture, which was partially blocked by inhibitors of PKC and Rho kinase [11]. Here we report that: (1) S1P activates both PLD1 and PLD2 isoforms in Beas-2B cells; (2) S1P-induced phosphorylation of ERK is regulated by PLD1 and PLD2; and (3) S1P-mediated IL-8 secretion is reliant on PLD1- and PLD2-dependent ERK activation. The role of S1P and its receptors in bronchial epithelial cell signal transduction has not been well defined. In the present study, S1P-mediated PLD1 and PLD2 activation, ERK phosphorylation and IL-8 secretion are probably mediated by the S1P family of G-protein-coupled receptors.

S1P has been shown to activate PLD in a number of different mammalian cell types [32–34]. However, most of these studies did not identify the isoform(s) of PLD activated by S1P. As the efficiency of transient transfection of bronchial epithelial cells with FuGENE 6 or Lipofectamine is low ($\sim$5–10%), we developed adenoviral constructs of wild-type hPLD1 and mPLD2 and the catalytically inactive K898R PLD1 and K758R mPLD2 mutants. Infection of Beas-2B cells with these adenoviral constructs (50 p.f.u./cell) for 12, 24 and 48 h produced $>$ 90% efficiency of infection, as determined by $\beta$-galactosidase staining of vector-infected cells (results not shown) and enhanced protein expression (Figure 7A). Using the adenoviral constructs, we report here for the first time that S1P rapidly and transiently activates both PLD1 and PLD2 in human bronchial epithelial cells. Furthermore, the catalytically inactive mutants of hPLD1 and mPLD2 blocked the S1P-mediated activation of PLD1 and PLD2 respectively. Interestingly, the inactive mutants of both PLD1 and PLD2 showed inhibitory effects on endogenous PLD activity (Figure 7D) and a downstream target such as ERK phosphorylation. The mechanism(s) of inhibition by the inactive mutants is unclear; however, this may occur through competition for PLD binding to activators such as $PIP_2$, ADP-ribosylation factor and Rho. Recent studies in COS-7 cells suggest that PLD1 and PLD2 can exist as homodimers and heterodimers; this may, in part, explain the suppression of endogenous PLD activity by the inactive mutants of PLD1 and PLD2 [35]. The signalling pathways involved in S1P-mediated PLD1 and PLD2 stimulation have not been characterized. In C2C12 skeletal muscle cells, S1P-induced PLD activation was sensitive to PTx, low concentrations of GF 109201X and rottlerin. These data suggest that, in C2C12 cells, activation of PLD by S1P is linked to a PTx-sensitive G-protein-coupled S1P receptor, and the participation of both PKC$\alpha$ and PKC$\delta$ in PLD stimulation [36]. In the present study, S1P-induced PLD activation in Beas-2B cells also showed sensitivity to PTx and inhibition by Y27632 and bisindolylmaleimide, suggesting the involvement of Rho kinase and PKC [11]. Further studies with the adenoviral constructs of wild-type and catalytically inactive mutants of PLD1 and PLD2 should reveal the signalling pathways that are activated by S1P in regulating PLD1 and PLD2 in human bronchial epithelial cells.

Enhancement of phosphorylation of ERK by S1P has been shown in a variety of cell types [37–43]. In Beas-2B cells, PLD activation and ERK phosphorylation in response to S1P were inhibited by PTx, suggesting coupling of S1P receptor(s) to $G_i$-proteins. Also, PD 98059, an inhibitor of MEK1/2, attenuated S1P-induced ERK phosphorylation, suggesting regulation of the Ras/Raf/MEK/ERK signalling cascade in Beas-2B cells. Furthermore, PD 98059 did not attenuated S1P- or PMA-induced PLD activation, suggesting ERK stimulation downstream of PLD in Beas-2B cells. A role for PKC, PI 3-kinase and Src has been demonstrated in agonist-induced ERK activation [41]; however, in Beas-2B cells, inhibitors of PKC, PI 3-kinase or Src had no effect on ERK phosphorylation induced by S1P (results not shown). There have been several reports in the literature demonstrating the participation of PLD in ERK phosphorylation. In NIH 3T3 cells, lyso-PA-stimulated ERK phosphorylation was increased on PLD1 overexpression [42]. In Rat fibroblasts overexpressing the epidermal growth factor receptor, epidermal growth factor-induced ERK activation was augmented by increased PLD activity, whereas decreased PLD activity due to dominant-negative PLD gene expression attenuated ERK phosphorylation [43]. Also, insulin-stimulated ERK phosphorylation was decreased in HIRcB cells overexpressing PLD mutants [23].

To elucidate the role of PLD in the S1P-induced phosphorylation of ERK, we took advantage of the PLD-catalysed transphosphatidylation of PA to phosphatidylalcohol, in the presence of a short-chain primary alcohol, as an indication of PLD/PA-dependent cellular responses. We found that, in Beas-2B cells, butan-1-ol (but not butan-3-ol) blocked S1P-induced ERK phosphorylation. Furthermore, neither butan-1-ol nor butan-3-ol, at concentrations of 0.05%, increased basal phosphorylation of ERK (Figure 4). The participation of PLD1 and PLD2 in ERK phosphorylation was also evident from the adenoviral infection of Beas-2B cells with wild-type and catalytically inactive (hPLD1-K898R and mPLD2-K789R) mutants. In Beas-2B cells, overexpression of both hPLD1 and mPLD2 wild types increased [$^{32}$P]PBt formation by severalfold compared with vector controls after stimulation with S1P (Figure 8). Also, overexpression of PLD1 and PLD2 wild types enhanced basal and S1P-mediated ERK phosphorylation, while the mutants blocked the response to S1P (Figure 9). These results provide the first direct evidence for PLD1 and PLD2 activation and involvement in ERK phosphorylation mediated by S1P in bronchial epithelial cells. While PD 98059 blocked S1P-induced ERK phosphorylation, it did not affect PLD activation (Figure 5), suggesting that PLD activation is upstream of MEK and ERK in Beas-2B cells. However, in vascular smooth muscle cells, noradrenaline-induced PLD stimulation is dependent on ERK, indicating involvement of the Ras/MEK/ERK pathway in stimulation by PLD [44].

One of the critical components of the ERK activation cascade is Raf-1. Recent studies indicate that an association between PA and Raf is necessary, in some cell types, for recruitment of Raf-1 to the membrane. However, PA does not activate Raf-1 *in vitro* or *in vivo*, suggesting participation of other signalling molecules in the activation of the Raf-1 kinase [23]. Distinct binding domains for PA and phosphatidylserine at the C-terminal domain of Raf-1 have been identified [21]. A physiological role of PA binding to Raf in the translocation of Raf to the plasma membrane and subsequent activation of Raf-1 has been demonstrated [23]. The present study did not address the binding or translocation of Raf to the membrane in S1P-stimulated Beas-2B cells. S1P enhanced the phosphorylation of Raf-1 at serine-338, as determined by Western blotting with a phospho-specific

antibody (Figure 6), and the addition of butan-1-ol (but not butan-3-ol) attenuated the S1P-enhanced phosphorylation of Raf-1. Earlier studies demonstrated that phosphorylation of Raf-1 at serine-338/339, in addition to tyrosine-340/341, is an essential regulatory event for the biological and enzymic activity of Raf-1 [45–47]. Although it has been established that activated Raf-1 phosphorylates MEK1, which in turn phosphorylates and activates ERK1/2, it is unclear whether S1P-induced phosphorylation of Raf-1 at serine-338/339 regulates ERK. As PA generated by PLD signalling recruits Raf-1 to the plasma membrane for subsequent activation by Ras, our results also indicate a role for PA in Raf-1 activation.

We have previously demonstrated that S1P is a potent activator of IL-8 secretion in Beas-2B cells, with PLD involvement in the signalling pathway [11]. In addition to regulation by PLD, inhibitors of PKC and Rho kinase also attenuated IL-8 secretion by S1P, indicating the involvement of multiple signalling pathways in the production and secretion of IL-8 [11]. Attempts to use adenoviral constructs of PLD1 and PLD2 wild types in S1P-induced IL-8 secretion were difficult, as the adenoviral vector controls (LacZ) increased the basal secretion of IL-8 compared with uninfected control cells. However, transient overexpression of PLD1 and PLD2 wild-type plasmids with FuGENE 6 showed increased IL-8 secretion compared with vector controls, confirming the participation of PLD1 and PLD2 in S1P-induced IL-8 secretion.

Agonist-induced IL-8 secretion in epithelial and other cells is regulated at the transcriptional level by the transcription factors activator protein-1 (AP-1) and nuclear factor-$\kappa$B (NF-$\kappa$B). AP-1 and NF-$\kappa$B regulate IL-8 gene transcription both individually and synergistically [48]. MAPK activation increases both AP-1 expression and activity [49]. Our results provide evidence that PLD activity regulates S1P-induced IL-8 secretion via, at least in part, ERK1/2 activation. Regulation of NF-$\kappa$B activation is very complex, involving phosphorylation and dissociation of the inhibitory protein I$\kappa$B via I$\kappa$B kinase 1/2 and/or phosphorylation of the RelA (p65) component of NK-$\kappa$B by PKC$\zeta$ and p21 Ras [50]. PKC$\zeta$ is not activated by $Ca^{2+}$ or diacylglycerol; however, it is activated by several acidic phospholipids, including phosphatidylinositol 3,4,5-trisphosphate and PA [51]. Therefore it is possible that PA generated by the PLD pathway activates NF-$\kappa$B via PKC$\zeta$ in Beas-2B cells. In human pulmonary endothelial cells, hyperoxia-induced NF-$\kappa$B activation was partially blocked by butan-1-ol, but not butan-3-ol, suggesting a possible role for PA in NF-$\kappa$B activation (V. Natarajan, N. L. Parinandi, M. A. Kleinberg, R. Cummings, L. Wang and P. Usatyuk, unpublished work).

S1P-induced IL-8 secretion via PLD/PA and ERK signalling is of physiological relevance in inflammatory diseases of the lung and airway, such as asthma. IL-8 is a potent chemoattractant and activator of neutrophils at sites of acute inflammation [52], and elevated levels of IL-8 have been reported in the bronchoalveolar lavage of patients with chronic lung diseases, asthma, pulmonary sarcoidosis and the acute respiratory distress syndrome [9,53,54]. Recent studies show elevated levels of S1P in the airways of asthmatics, but not control subjects, following segmental antigen challenge in association with smooth muscle cell proliferation and IL-6 secretion [55]. Thus S1P-induced PLD activation may function as a pro-inflammatory signal in airway epithelial and other cells, regulating the secretion of chemotactic factors and cytokines.

In summary, our results demonstrate that both PLD1 and PLD2 are activated by S1P in airway epithelial cells. Furthermore, PA generated by the PLD signal transduction pathway regulates IL-8 secretion via Raf-dependent ERK phosphorylation. Further studies identifying downstream targets of PA-dependent kinases, including PKC$\zeta$ regulation of NF-$\kappa$B activation, are in progress.

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