THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 41, Issue of October 10, pp. 39931–39940, 2003
Printed in U.S.A.

Involvement of Phospholipase D2 in Lysophosphatidate-induced Transactivation of Platelet-derived Growth Factor Receptor-β in Human Bronchial Epithelial Cells*

Received for publication, March 21, 2003, and in revised form, July 24, 2003
Published, JBC Papers in Press, July 29, 2003, DOI 10.1074/jbc.M302896200

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Lysophosphatidate (LPA) mediates multiple cellular responses via heterotrimeric G protein coupled LPA-1, LPA-2, and LPA-3 receptors. Many G protein-coupled receptors stimulate ERK following tyrosine phosphorylation of growth factor receptors; however, the mechanism(s) of transactivation of receptor tyrosine kinases are not well defined. Here, we provide evidence for the involvement of phospholipase D (PLD) in LPA-mediated transactivation of platelet-derived growth factor receptor-β (PDGF-Rβ). In primary cultures of human bronchial epithelial cells (HBEpCs), LPA stimulated tyrosine phosphorylation of PDGF-Rβ and threonine/tyrosine phosphorylation of ERK1/2. The LPA-mediated activation of ERK and tyrosine phosphorylation of PDGF-Rβ was attenuated by tyrphostin AG 1296, an inhibitor of PDGF-R kinase, suggesting transactivation of PDGF-R by LPA. Furthermore, LPA-, but not PDGF β-chain homodimer-induced tyrosine phosphorylation of PDGF-Rβ was partially blocked by pertussis toxin, indicating coupling of LPA-R(s) to G_i. Exposure of HBEpCs to LPA activated PLD. Butan-1-ol, which acts as an acceptor of phosphatidate generated by the PLD pathway, blocked LPA-mediated transactivation of PDGF-Rβ. This effect was not seen with butan-3-ol, suggesting PLD involvement. The role of PLD1 and PLD2 in the PDGF-Rβ transactivation by LPA was investigated by infection of cells with adenoviral constructs of wild type and catalytically inactive mutants of PLD. LPA activated both PLD1 and PLD2 in HBEpCs; however, infection of cells with cDNA for wild type PLD2, but not PLD1, increased the tyrosine phosphorylation of PDGF-Rβ in response to LPA. Also, the LPA-mediated tyrosine phosphorylation of PDGF-Rβ was attenuated by the catalytically inactive mutant mPLD2-K758R. Infection of HBEpCs with adenoviral constructs of wild type hPLD1, mPLD2, and the inactive mutants of hPLD1 and mPLD2 resulted in association of PLD2 wild type and inactive mutant proteins with the PDGF-Rβ compared with PLD1. These results show for the first time that transactivation of PDGF-Rβ by LPA in HBEpCs is regulated by PLD2.

Lysophosphatidate (LPA)[1] is a potent bioactive lipid that is implicated in cell proliferation, differentiation, suppression of apoptosis, tumor metastasis, and cytoskeletal reorganization (1–5). In plasma, LPA is present at <0.2 μM, but activated platelets produce high amounts of LPA which can reach 1–10 μM, making it the most abundant mitogen/survival factor present in serum (6). Mammalian cells contain small quantities of intracellular LPA; however, many biological actions of LPA are mediated by specific G protein-coupled receptors (GPCRs), LPA-1, LPA-2, and LPA-3 (formerly called EDG-2, -4, and -7). LPA, akin to sphingosine 1-phosphate (S1P), binds with high affinity to its receptors that are coupled to multiple types of heterotrimeric G proteins (G_i, G_q, and $G_{12/13}$). Ligation of LPA to LPA-Rs (LPA-Rs) leads to a myriad of signal transduction pathways including release of $[Ca^{2+}]_i$, activation of protein kinase C, phosphatidylinositol-3-kinase, mitogen-activated protein kinases (ERK1 and ERK2), tyrosine kinases, and phospholipases (7). LPA-induced release of $[Ca^{2+}]_i$ and decreased cAMP are pertussis toxin (PTx)-sensitive, suggesting involvement of G_i (8). LPA-mediated activation of ERK1/2 requires p21 Ras (9) and is dependent on phosphatidylinositol 3-kinase signaling through G_i (10). Furthermore, LPA activated the nonreceptor tyrosine kinase, Src, and Pyk2 (11, 12). LPA-induced Rho activation through $G_{12/13}$ results in stress fibers, assembly of focal adhesion, and nuclear factor-κB-dependent gene transcription (13).

LPA activates PLD in various mammalian cells, including fibroblasts, smooth muscle cells, and prostate cancer cells (14–16). PLD isoenzymes, PLD1 and PLD2, hydrolyze phosphatidylcholine to generate phosphatidate (PA), and they are activated by hormones, growth factors, neurotransmitters, cytokines, and reactive oxygen species (17). The second messenger functions of PA are mediated directly or after its conversion to either diacylglycerol or LPA by the action of PA phosphohydrolase or phospholipase A1/A2, respectively. PA derived through PLD regulates protein tyrosine phosphatases, ERK1/2 phosphorylation, phosphatidylinositol 4-kinase, phosphatidylinositol 3-kinase, phosphatidylinositol 4-phosphate

* This work was supported by National Institutes of Health Grant HL 71152 (to V. N.) and by Canadian Institutes of Health Research Grant MOP 10504 (to D. N. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: LPA, lysophosphatidate; BSA, bovine serum albumin; EGF, epidermal growth factor; ERK, extracellular-signal regulated kinase; GPRC, G protein-coupled receptor; HBEpCs, human bronchial epithelial cells; LPA-R, LPA receptor; PA, phosphatidate; PBt, phosphatidylbutanol; PDGF, platelet-derived growth factor; PDGF-R, platelet-derived growth factor receptor; pfu, plaque-forming unit(s); PLD, phospholipase D; PTx, pertussis toxin; RT-PCR, reverse transcription-PCR; S1P, sphingosine 1-phosphate; BEBM, bronchial epithelial basal medium; BEGM, bronchial epithelial growth medium; PDGF-BB, platelet-derived growth factor β-chain homodimer.

5-kinase, phagocytic NADPH oxidase, actin polymerization, coatomer assembly, vesicle trafficking, and cytokine secretion (18–21). However, the relationships between PLD1 and PLD2 activation and cellular responses have not been well studied.

In contrast to LPARs, the protein growth factor receptors are receptor tyrosine kinases, and their activation stimulates signaling pathways, involving ERK, phosphatidylinositol 3-kinase, protein kinase C, and phospholipases (1, 22, 23). Receptors for LPA/S1P and growth factors (EGF and PDGF) are coexpressed in many cell types including endothelial, smooth muscle, and epithelial cells (24–26). It is now becoming clearer that cross-talk among lipid mediators, cytokines, and growth factors regulates the amplitude and specificity of cellular responses such as secretion, proliferation, and differentiation. For instance, several agonists of GPCR, such as thrombin, angiotensin II, bradykinin, S1P, and LPA, not only induce tyrosine phosphorylation of intracellular signaling proteins, but also transactivate the growth factor receptors, EGF and PDGF (27–33). This transactivation amplifies mitogenic signals from a variety of stimuli. However, relatively little is known regarding the role and regulation of LPA-mediated transactivation of PDGF-Rs in mammalian cells.

In the present study, we investigated potential signaling pathways activated by LPA which regulate the transactivation of PDGF-Rβ in human bronchial epithelial cells (HBEpCs). LPA-induced phosphorylation of PDGF-Rβ in HBEpCs was sensitive to PTx. LPA also stimulated both PLD1 and PLD2 in HBEpCs; however, PLD2 but not PLD1 activation regulated LPA-mediated transactivation of PDGF-Rβ. Furthermore, overexpression of wild type and catalytically inactive mutant of mPLD2 compared with hPLD1 preferentially formed a complex with PDGF-Rβ in HBEpCs. Our results show for the first time that generation of PA by PLD2 activation is involved in LPA-induced PDGF-Rβ transactivation and downstream signaling in HBEpCs.

EXPERIMENTAL PROCEDURES

Materials

LPA was obtained from BIOMOL Research Labs (Plymouth, PA). 12-*O*-Tetradecanoylphorbol-13-acetate, bovine serum albumin (fraction V), butan-1-ol, butan-3-ol, and dimethyl sulfoxide were purchased from Sigma. Recombinant PDGF-BB, tryphostins AG 1296 and PTx were obtained from Calbiochem. PBt and monooleoyl LPA were purchased from Avanti Polar Lipid Corp (Alabaster, GA). $^{32}P_i$ (carrier-free) was obtained from PerkinElmer Life Sciences. RedTaq DNA polymerase was purchased from Sigma. A cDNA synthesis kit was purchased from Invitrogen, and a total RNA isolation kit was obtained from Qiagen (Valencia, CA). Polyclonal antibody against phospho-ERK was obtained from Cell Signaling (Beverly, MA). Antibodies for total PDGF-Rβ and phospho-PDGF-Rβ (Tyr-716) were purchased from Upstate Biotechnology (Lake Placid, NY), and antibodies against total ERK were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Phosphatidylcholine-specific PLD1, human N-terminal, and internal as well as PLD2, mouse internal, and N-terminal polyclonal antibodies were purchased from BIOSOURCE International (Camarillo, CA). BEBM and supplement kit were purchased from BioWhittaker (Walkersville, Frederick, MD). Primary HBEpCs, passage 2, were procured from Clonetics (San Diego, CA).

Cell Culture

Primary HBEpCs were cultured in BEBM serum-free medium containing all of the growth factors supplied by Clonetics (BEGM) at 37 °C in 5% CO_2 and 95% air. Cells were grown in to ~90% confluence in T-75-cm^2 vented flasks and subsequently propagated in 35-mm dishes. Cells were grown for 48 h without changing the medium before stimulation with LPA or PDGF-BB in conditioned BEBM (medium collected from cells cultured for 48 h) containing 0.1% BSA. All experiments were carried out between passages 2 and 5.

Transfection and Viral Infection

For transient transfection, HBEpCs grown in 6-well plates (~60 confluence), were transfected with PDGF-Rβ or PLD2 plasmids (1 μg of cDNA was mixed with 6 μl of FuGENE 6/well in 1 ml of BEGM) according to the manufacturer's recommendations. After 5 h, the transfection medium was aspirated and 2 ml of regular BEGM was added, and cells were incubated for 24–48 h. Infection of HBEpCs (~60% confluence) in 35-mm dishes was carried out with purified hPLD1 and mPLD2 adenoviral particles (50 pfu/cell) in 1 ml of BEGM for 24 h, and the virus-containing medium was replaced with complete BEGM for different time periods. For coimmunoprecipitation experiments, cells were infected with PLD1 or PLD2 adenoviral particles (50 pfu/cell) for 3 h and then transfected with PDGF-Rβ cDNA for 4 h. After transfection, the medium was removed, conditioned medium was added, and cells were grown overnight before challenging with LPA or PDGF-BB.

Oligonucleotides for RT-PCR Analysis

LPA Receptors—Total RNA was isolated from HBEpCs using an RNEasy kit (Qiagen). cDNA was generated using a cDNA synthesis kit according to manufacturer's recommendation and subjected to PCR at 94 °C for 2 min following 30 cycles of 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 3 min, followed by 72 °C for 10 min. The following oligonucleotide primers were used for RT-PCR analysis of LPA-Rs.

For LPA-1R: forward, 5′-GTAATGGTGGTTCTCTATGCTCAC-3′; reverse 5′-GGACAGCACACGTCTAGAAG-3′.

For LPA-2R: forward, 5′-GTCGAGCCTGCTTGTCTTC-3′; reverse, 5′-CCAGAGCAGTACCACCTG-3′.

For LPA-3R: forward, 5′-GGAATTGCCTCTGCAACATCT-3′; reverse, 5′-GAGTAGATGATGGGGTTCA-3′.

For β-actin: forward, 5′-GACTACCTCATGAAGATC-3′; reverse, 5′-GATCCACATCTGCTGGAA-3′.

The PCR products were separated by electrophoresis, and primers used in these reactions yielded PCR products of 197, 205, 382, and 513 bp, respectively.

PLD1 and PLD2—PCRs were performed for 30 cycles at 94 °C for 2 min followed by denaturation at 94 °C for 1 min, annealing at 60 °C for 2 min, and elongation at 72 °C for 3 min. The following oligonucleotides primers were used for RT-PCR.

PLD1: forward, 5′-TGGGCTCACCATGAGAA-3′; reverse, 5′-GTCA-TGCCAGGGCATCCGGGG-3′.

PLD 2: forward, 5′-TCCATCCAGGCCATTCTGCAC-3′; reverse, 5′-CGTTGCTCTCAGCCATGTCTTG-3′.

PCR products were analyzed by agarose gel electrophoresis, and the primers used in these reactions yielded products of 642 bp/PLD1a, 528 bp/PLD1b, and 468 bp/PLD2.

Preparation of Cell Lysates, Immunoprecipitation, and Western Blotting

HBEpCs grown on 60- or 100-mm dishes (~90% confluence) were stimulated with LPA or PDGF-BB, rinsed three times with ice-cold phosphate-buffered saline containing 1 mM orthovanadate and lysed in RIPA buffer (20 mM Tris-HCl, pH 7.4, 1% Triton X-100 (v/v), 150 mM NaCl, 5 mM EDTA, 100 mM NaF, and 1 mM Na_3VO_4). Cell lysates were sonicated (3 × 15 s) on ice, centrifuged at 5,000 × *g* for 5 min at 4 °C, and protein concentrations were determined with BCA protein assay kit (Pierce Chemical Co.) using BSA as standard. Equal amounts of protein (300–500 μg) were precleared with 20 μl of protein A/G-agarose for 30 min at 4 °C, centrifuged at 5,000 × *g* for 10 min, supernatants were incubated with 2 μg/ml anti-PDGF-Rβ antibody overnight at 4 °C followed by the addition of 40 μl of protein A/G-agarose and additional incubation for 2 h at 4 °C. The cell lysates were centrifuged at 5,000 × *g* for 5 min, washed three times with ice-cold phosphate-buffered saline containing 1 mM orthovanadate, protein-agarose complexes were dissociated by boiling for 5 min in 2× Lammeli buffer, and samples were centrifuged in a microfuge at 5,000 × *g* for 5 min. The supernatants were subjected to SDS-PAGE on 10% gels, transferred to polyvinylidene difluoride membranes, blocked with 5% (w/v) non-fat dry milk in TBST (25 mM Tris, pH 7.4, 137 mM NaCl, and 0.1% Tween 20) for 1 h and incubated with primary antibodies against PDGF-Rβ or phospho-PDGF-Rβ (1:1,000 dilution) in TBST overnight at 4 °C. The membranes were washed three times with TBST at 30-min intervals and then incubated with either mouse or rabbit horseradish peroxidase-conjugated secondary antibody (1:3,000 dilution) for 2–4 h at room temperature. The membranes were developed with the enhanced chemiluminescence detection system according to manufacturer's instruction, and blots were quantified by densitometry and image analysis with Molecular Analyst software. Changes in PDGF-Rβ phosphorylation were expressed as -fold change normalized to total cellular PDGF-Rβ protein.

Measurement of Phospholipase D in Intact Cells

HBEpCs in 35-mm dishes were labeled with 30 μCi/ml $^{32}P_i$ BMGM for 18–24 h at 37 °C in 5% CO_2 and 95% air incubator. Cells were pretreated with vehicle alone or specific agents for specified times prior to challenge with LPA. Cells were then challenged with LPA in vehicle containing 0.1% BSA or other agents and in the presence of 0.05% butan-1-ol. In some experiments, incubations were also carried out in the presence of 0.05% butan-3-ol that served as additional controls. Incubations were terminated by the addition of 1 ml of methanol:HCl (100:1 v/v), cells were scraped into glass tubes, and lipids were extracted by the addition of 1 ml of methanol:HCl (100:1 v/v), 2 ml of chloroform, and 0.8 ml of 1 M HCl (21). Formation of [^{32}P]PBt, as a result of PLD activation and transphosphatidylation of [^{32}P]PA, to butan-1-ol but not butan-3-ol, was separated from the total lipid extract by thin layer chromatography on 1% potassium oxalate plates with the upper phase of ethyl acetate:2,2,4-trimethyl pentane:glacial acetic acid: water (65:10:15:50 v/v) as the developing solvent system. Unlabeled PBt was added as carrier during separation of labeled lipids that were visualized by exposure to iodine vapor. Radioactivity associated with PBt was counted by liquid scintillation counting, and all values were normalized to 10^6 dpm in total lipid extract. [^{32}P]PBt formed in control and agonist challenged samples was expressed as dpm/dish or percent control.

Statistical Analysis

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

RESULTS

Expression of LPA-Rs in HBEpCs—Recent cloning of GPCRs for LPA has identified three high affinity receptors, namely LPA-1, LPA-2, and LPA-3 in mammalian cells and tissues. In the lung and human airway smooth muscle cells, mRNA for all three LPA-Rs has been shown by RT-PCR (46). However, the relative expression and functional role of LPA-Rs in airway cells are unknown. Therefore, we determined the mRNA and protein expression of LPA-R subtypes in primary cultures of HBEpCs using RT-PCR, Western blotting, and immunocytochemistry. As shown in Fig. 1*A*, mRNA for all the three LPA-Rs was identified in HBEpCs. The presence of LPA-1 (50 kDa), LPA-2 (45 kDa), and LPA-3 (40 kDa) receptor proteins was also confirmed by Western blotting with specific antibodies to the three receptor proteins in HBEpCs (Fig. 1*B*) (provided by Glaxo Smith Kline, King of Prussia, PA). Furthermore, the Western blot results were complemented by immunocytochemical localization using LPA-R antibodies. All three LPA-Rs were also detected in HBEpCs by immunocytochemistry (Fig. 1*C*). LPA-3 was barely detectable compared with LPA-1 and LPA-2 by immunofluorescent microscopy. Both LPA-1 and LPA-2 were not only localized at the plasma membrane, but were also distributed in the cytoplasm of the cell. The distribution of LPA-1 appeared punctated and located in the perinuclear membrane. The staining of LPA-3 was more pronounced at the perinuclear membrane compared with the plasma membrane or cytoplasm distribution. These results indicate that LPA-Rs are not only present at the plasma membrane, but also distributed in the cytoplasm and perinuclear membranes.

LPA-induced ERK1/2 Activation Is Sensitive to AG 1296 and PTx in HBEpCs—Previous studies have demonstrated stimulation of mitogenic signaling pathways for S1P, LPA, and growth factors in human airway smooth muscle and epithelial cells (1, 21, 34, 35). To examine ERK activation further, HBEpCs were challenged with 1 μM LPA or 20 ng/ml PDGF-BB, and phosphorylation of ERK was analyzed by Western blotting with phospho-specific antibodies against threonine/tyrosine residues. As shown in Fig. 2, LPA and PDGF stimulated ERK1/2 phosphorylation by 2.2- and 1.8-fold, respectively. Enhanced phosphorylation of ERK, normalized to total ERK, was detected as early as 2 min of exposure to either LPA

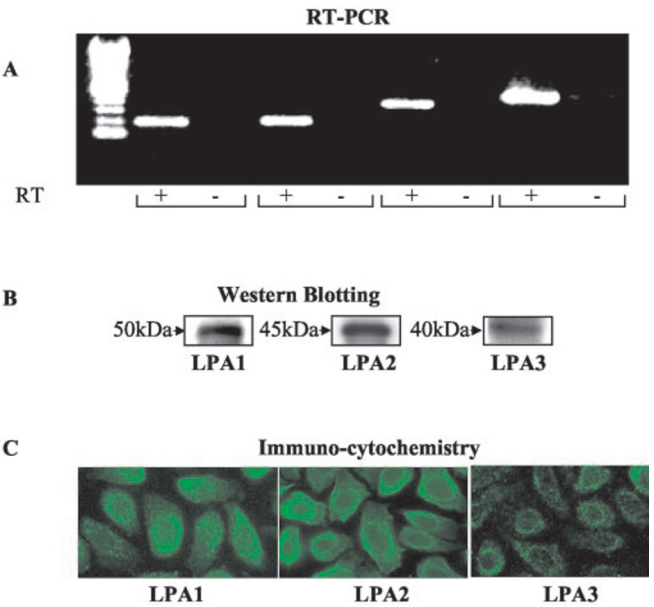

FIG. 1. **Detection of LPA-Rs by RT-PCR, Western blotting, and immunocytochemistry.** *A*, total RNA was extracted from primary HBEpCs, and transcription of the genes encoding LPA-Rs (1, 2, and 3) was assessed by RT-PCR (− indicates in the absence of RNA, and + indicates in the presence of 2 μg of RNA) with primers to the indicated receptors and β-actin. RT-PCR products were visualized by ethidium bromide staining after separation on agarose gels. *B*, cell lysates (30 μg of protein) were subjected to SDS-PAGE and analyzed by Western blotting with LPA-1, LPA-2, or LPA-3 receptors. Each Western blot is representative of three independent experiments. *C*, HBEpCs grown on coverslips to ~90% confluence were subjected to immunostaining with LPA-R antibodies and examined by fluorescent microscopy. Each immunofluorescence image is representative of three separate experiments.

or PDFG-BB, whereas maximum phosphorylation (~ 3–4-fold) occurred at 15 min and declined thereafter (results not shown). As LPA and PDGF stimulated ERK, we next investigated the mechanisms of ERK activation. Treatment of cells with PD98059, a known inhibitor of mitogen-activated protein kinase/ERK kinase, blocked (> 95%) both LPA- and PDGF-BB-mediated phosphorylation (results not shown). These results suggest that LPA- and PDGF-BB-induced activation of ERK is coupled to mitogen-activated protein kinase/ERK kinase signaling in HBEpCs. Pretreatment of cells with the PDGF-R-selective tyrosine kinase inhibitor tyrphostin AG 1296 completely blocked PDGF-BB-stimulated ERK phosphorylation, whereas LPA activation of ERK was only partially blocked. This suggests that PDGF-R kinase activity is required for part of the LPA activation of the ERK cascade (Fig. 2). Previous studies showed that in Beas-2B cells, S1P-induced activation of ERK was sensitive to PTx (21), indicating the involvement of G_i-coupled S1P receptors. In HBEpCs pretreated with 100 ng/ml PTx for 3 h, the LPA-stimulated ERK1/2 phosphorylation was attenuated by about 95% (Fig. 3). These results demonstrate that LPA-Rs are coupled to PTx-sensitive G_i in the ERK signaling cascade.

LPA Induction of PDGF-Rβ Phosphorylation Is Coupled through $G\alpha_i$—The inhibition of LPA-induced ERK activation by AG 1296 suggests that LPA-R stimulation results in PDGF-R transactivation. Therefore, we assessed PDGF-Rβ phosphorylation in response to LPA. HBEpCs were challenged with LPA, and PDGF-Rβ phosphorylation was studied by Western blotting with phospho-specific PDGF-Rβ antibody (specific for tyrosine 716). As shown in Fig. 4, stimulation of HBEpCs with varying concentrations of LPA (0.1–5 μM) increased PDGF-Rβ tyrosine phosphorylation. Enhanced phosphorylation of

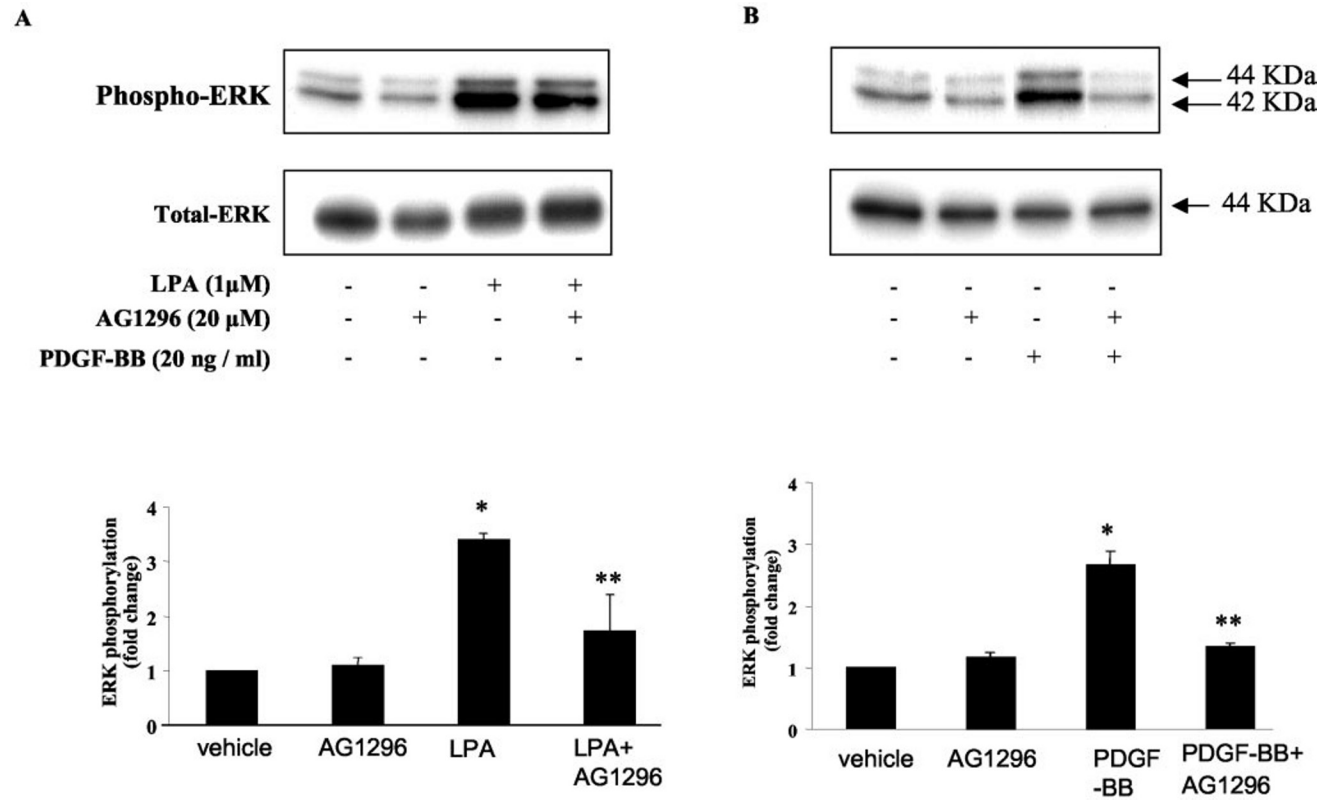

FIG. 2. **Effect of tyrphostin AG 1296 on LPA- and PDGF-BB-mediated ERK phosphorylation.** HBEpCs (passage 2, ~ 90% confluence in 35-mm dishes) were pretreated with BEBM or BEBM plus 5 μM AG 1296 for 1 h and then stimulated with 1 μM LPA (*A*) or 20 ng/ml PDGF-BB (*B*) for 15 min. Cell lysates (30 μg of protein) from *A* and *B* were subjected to SDS-PAGE and Western blotted with phospho-specific and pan-ERK antibodies. Values are the means ± S.D. from three independent experiments in triplicate, and -fold changes in ERK phosphorylation were normalized to total ERK. The histograms are shrunk horizontally to fit under the blots. *, $p < 0.05$ compared with vehicle control; **, $p < 0.05$ compared with LPA or PDGF-BB treatment.

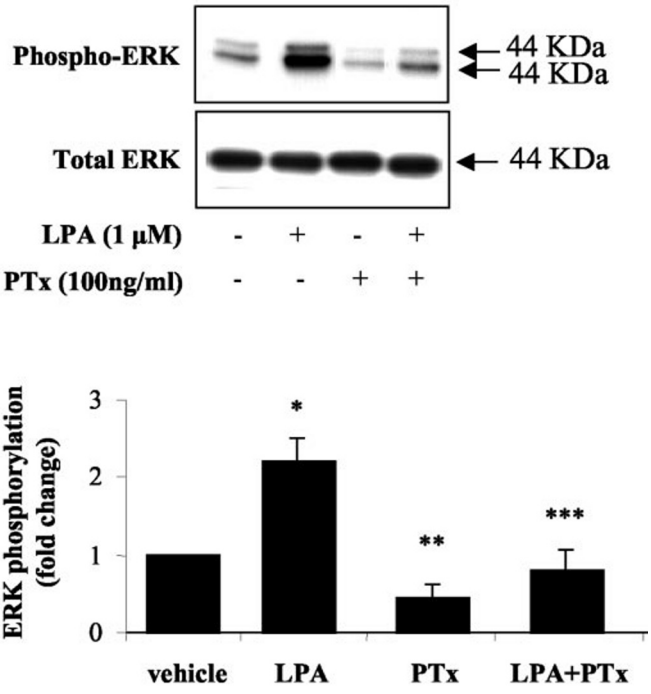

FIG. 3. **PTx blocks LPA-induced ERK phosphorylation.** HBEpCs (passage 2, ~ 90% confluence in 35-mm dishes) were pretreated with 100 ng/ml PTx for 3 h. Cells were challenged with 1 μM LPA for 15 min, then cell lysates were subjected to SDS-PAGE and Western blotted with phospho-specific and pan-ERK antibodies. Values are the means ± S.D. of three independent experiments in triplicate. -Fold changes in ERK phosphorylation were normalized to total ERK. *, $p < 0.05$ compared with vehicle control; **, $p < 0.05$ compared with vehicle control; ***, $p < 0.05$ compared with LPA treatment.

PDGF-Rβ after 15 min of exposure to 0.1 μM LPA (~4-fold increase over control) increased to ~7-fold with 0.5 μM LPA (Fig. 4). Furthermore, LPA stimulated PDGF-Rβ phosphorylation in a time-dependent manner (Fig. 5). Enhanced tyrosine phosphorylation of PDGF-Rβ (~2-fold) was detected within 2 min of challenge to LPA, whereas maximum phosphorylation (~10-fold) was observed at 5 min and declined thereafter up to 30 min (Fig. 5). In addition to tyrosine 716, PDGF-Rβ is also tyrosine phosphorylated at other sites by PDGF. We therefore compared the Western blots of phospho-specific tyrosine 716 antibodies with immunoprecipitation of PDGF-Rβ and Western blotting with anti-phosphotyrosine antibody. HBEpCs were transfected with PDGF-Rβ wild type plasmid for 18 h, and cells were stimulated with 1 μM LPA for different times. Cells lysates (1.0 mg of protein) were immunoprecipitated with PDGF-Rβ antibody, and precipitates were analyzed by Western blotting with anti-phosphotyrosine antibody. As shown in Fig. 5, overexpression of cells with wild type PDGF-Rβ plasmid and stimulation with LPA provided a similar time-dependent tyrosine phosphorylation of PDGF-Rβ. Phosphorylation of PDGF-Rβ was detected after 5 min of exposure to 100 nM LPA (~ 3-fold increase over control), which increased to ~ 7-fold with 500 nM LPA and plateaued at higher concentrations of LPA (1 and 5 μM). These results clearly demonstrate phosphorylation of PDGF-Rβ by LPA in HBEpCs.

To ascertain further the role of LPA-R stimulation by LPA in transactivation of PDGF-Rβ, we investigated the effect of tyrphostin AG 1296 on tyrosine phosphorylation of PDGF-Rβ. The LPA-induced tyrosine phosphorylation of PDGF-Rβ (~6-fold increase over control in 10 min) was attenuated by tyrphostin AG 1296 in a dose-dependent manner with a ~50% inhibition at 1 μM AG 1296 (Fig. 6*A*). However, the basal phosphorylation

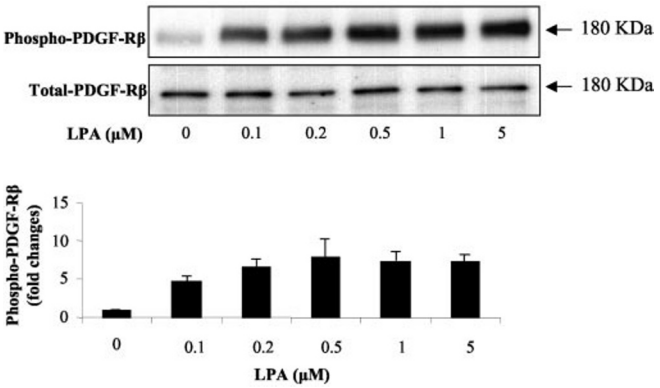

FIG. 4. **Dose-dependent phosphorylation of PDGF-Rβ by LPA.** HBEpCs (passage 3, ~ 90% confluence) in 35-mm dishes were challenged with increasing concentrations of LPA as indicated for 5 min, and cell lysates were analyzed by Western blotting with phospho-specific PDGF-Rβ (tyrosine 716) and PDGF-Rβ antibodies as described under "Experimental Procedures." Values are the means ± S.D. from three independent experiments, and -fold increases in PDGF-Rβ phosphorylation were normalized to total PDGF-Rβ. The histogram is expanded vertically.

of PDGF-Rβ, in the absence of LPA, was not drastically altered by AG 1296 (Fig. 6*A*). As expected, pretreatment of cells with 1 μM AG 1296 for 1 h also abolished the PDGF-BB (20 ng/ml)-mediated PDGF-Rβ phosphorylation (Fig. 6*B*). These results provide further evidence that LPA causes activation of the PDGF-Rβ in HBEpCs.

LPA-Rs are coupled to a variety of heterotrimeric G proteins (7). Previously, we and others showed that G_i coupling played an important role in S1P- and LPA-mediated signal transduction in Beas-2B cells (21) and fibroblasts (36). To determine the contribution of G_i in LPA-induced PDGF-Rβ transactivation, HBEpCs were pretreated with 100 ng/ml PTx for 12 h prior to stimulation with 1 μM LPA for 15 min. This treatment dramatically inhibited tyrosine phosphorylation of PDGF-Rβ (~70% inhibition compared with stimulation by LPA alone), whereas PTx had no effect on basal phosphorylation of PDGF-R (Fig. 7). As a control, we established that PTx had no significant effect on PDGF-BB-mediated tyrosine phosphorylation of PDGF-Rβ (results not shown). These results suggest that LPA-induced transactivation of PDGF-Rβ is partly regulated by coupling of an LPA-R to G_i.

Role of PLD in LPA-induced PDGF-Rβ Transactivation—The internalization of growth factor receptors in response to EGF or PDGF involves endocytosis (37, 38). Because PLD has been implicated in protein trafficking and membrane fusion, we hypothesized that LPA-mediated PLD stimulation and PA generation may be involved in PDGF-Rβ transactivation in HBEpCs. RT-PCR revealed the presence of mRNA for PLD1 and PLD2, whereas Western blotting with specific antibodies confirmed the expression of PLD1 (116 kDa) and PLD2 (100 kDa) proteins in primary cultures of HBEpCs (Fig. 8). As shown in Table I, exposure of ^{32}P-labeled HBEpCs to 1 μM LPA for 10 min, in the presence of 0.05% butan-1-ol, significantly enhanced [^{32}P]PBt accumulation, an index of PLD activation (39). However, under similar incubation condition, 50 ng/ml PDGF-BB did not enhance [^{32}P]PBt formation (Table I). Furthermore, the PDGF-R kinase inhibitor AG 1296 had no significant effect on LPA- or 12-*O*-tetradecanoylphorbol-13-acetate-induced [^{32}P]PBt formation (Table I). These results demonstrate that activation of PLD by LPA is not mediated through PDGF-R in HBEpCs.

The role of LPA-induced PLD activation in transactivation of the PDGF-Rβ was investigated by using the ability of primary, but not secondary or tertiary, alcohols to divert PA formation to accumulation of phosphatidylalcohol. HBEpCs were stimulated with 1 μM LPA for 15 min in the absence or presence of 0.1% butan-1-ol or butan-3-ol. As shown in Fig. 9, butan-1-ol, but not butan-3-ol, blocked LPA-induced PDGF-Rβ phosphorylation (LPA, ~ 5-fold increase compared with vehicle; butan-1-ol plus LPA, ~ 3-fold change) without affecting the basal phospho-PDGF-R levels. These results demonstrate the involvement of PLD in LPA-induced transactivation of PDGF-Rβ in HBEpCs.

Role of PLD2 Rather Than PLD1 in LPA-induced PDGF-Rβ Phosphorylation—To investigate further the involvement of PLD isoenzymes in LPA-induced PDGF-Rβ transactivation, we overexpressed wild type and catalytically inactive mutants of hPLD1 and mPLD2. HBEpCs (~50% confluence) were infected with the adenoviral constructs (50 pfu/cell) for 12, 24, and 48 h, and cell lysates were analyzed for enhanced expression of PLD1 and PLD2 proteins by Western blot analysis using N-terminal plus internal anti-PLD1 or anti-PLD2 antibodies. As shown in Fig. 10*A*, infection of HBEpCs with adenoviral constructs increased the expression of PLD1 and PLD2 proteins in a time-dependent fashion with expression of the proteins seen as early as 12 h and maximum expression observed at 48 h after infection. The functional roles of hPLD1 and mPLD2 overexpression were tested by determining which of the isoenzymes of PLD are activated by LPA in HBEpCs. In both control and vector-infected cells, 1 μM LPA stimulated [^{32}P]PBt formation by 4–5-fold compared with an increase of 7.5-fold in cells that overexpressed hPLD1. Overexpression of mPLD2 increased basal PLD activity by about 3.6-fold, and this activity was increased further by about 5-fold by stimulation of cells with LPA (Fig. 10*B*). By contrast, overexpression of catalytically inactive mutant of mPLD2 (K758R), but not hPLD1 (K898R), partially blocked LPA-mediated [^{32}P]PBt accumulation (Fig. 10*B*). These results show that LPA stimulates PLD2 >PLD1, and the catalytically inactive mutant of mPLD2 attenuated LPA-mediated [^{32}P]PBt formation in HBEpCs.

We then used these cells that expressed wild type and catalytically inactive mutants of hPLD1 and mPLD2 to study their respective roles in activation of the PDGF-Rβ. As shown in Fig. 11*A*, overexpression of either wild type hPLD1 or its mutant K898R had no effect on LPA-mediated PDGF-R phosphorylation. However, in cells infected with cDNA for wild type mPLD2, the basal (~1.5-fold change) as well as LPA-mediated (~3.0-fold change) phosphorylations of the PDGF-Rβ were significantly higher compared with the cells infected with empty vector (Fig. 11*B*). Overexpression of the catalytically inactive mutant of mPLD2 attenuated both the basal and LPA-induced phosphorylation of PDGF-Rβ (Fig. 11*B*). These results establish the involvement of PLD2, rather than PLD1, in LPA-mediated PDGF-R transactivation in HBEpCs.

To study this action further, we investigated possible interaction among PLD1, PLD2, and PDGF-Rβ in the absence or presence of LPA. HBEpCs were infected with adenoviral constructs of wild type hPLD1, mPLD2, and catalytically inactive mutants hPLD1 (K898R) and mPLD2 (K758R) for 12 h followed by a second transfection with wild type PDGF-Rβ for another 24 h. Immunoprecipitation of the PDGF-Rβ from vehicle and LPA-challenged cells resulted in coimmunoprecipitation of wild type mPLD2 and catalytically inactive mutant mPLD2-K758R expressed proteins compared with vector control (Fig. 12*A*). Relative to mPLD2, the amount of hPLD1 that coimmunoprecipitated with PDGF-Rβ was almost negligible in vehicle and LPA-stimulated HBEpCs (Fig. 12*A*). The Western blots for PLD1 and PLD2 in PDGF-Rβ immunoprecipitates were carried out simultaneously and for the same exposure times to ensure identical development of the samples. In contrast to coimmu-

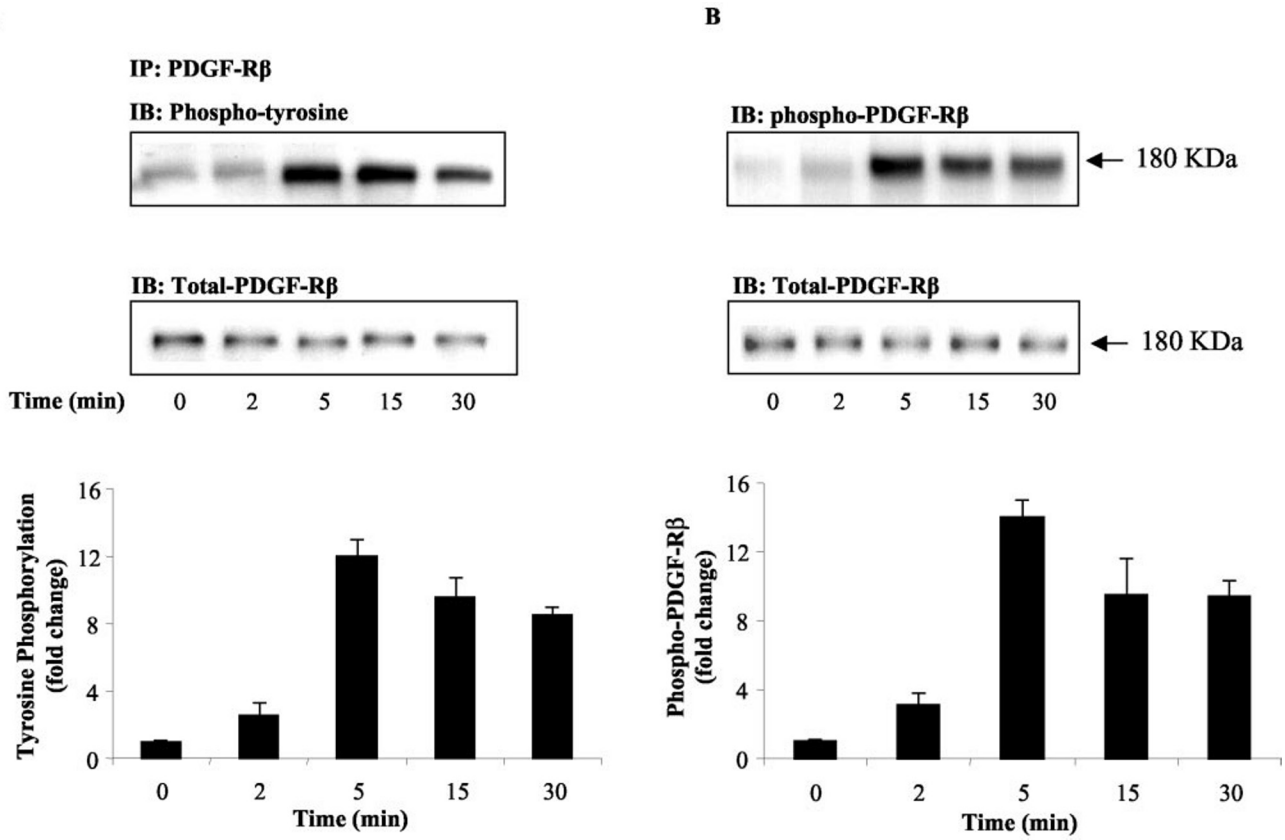

FIG. 5. **LPA stimulates phosphorylation of PDGF-Rβ.** *A*, HBEpCs (passage 3, ~80% confluence in 35-mm dishes) were transfected with PDGF-Rβ plasmid DNA for 4 h, then medium containing DNA-transfection reagent complex was replaced with conditioned BEBM, and cells were cultured for another 24 h and challenged with 1 μM LPA for various times as indicated. To cell lysates (1 mg of protein), 2 μg/ml anti-PDGF-Rβ antibody was added, incubated for 18 h at 4 °C followed by the addition of 20 μl of Sepharose A/G and additional incubation for 2 h at 4 °C. The immunocomplex was centrifuged at 5,000 × *g* for 10 min, and the pellet was washed three times with ice-cold RIPA buffer containing 1 mM orthovanadate and protease inhibitors. The immunoprecipitates (*IP*) were subjected to SDS-PAGE, transferred onto polyvinylidene difluoride membrane, and probed with anti-tyrosine antibody. Membranes were stripped and reprobed with anti-PDGF-Rβ antibody for total PDGF-Rβ. *B*, cell lysates were also analyzed by Western blotting with phospho-specific anti-PDGF-Rβ antibody (against tyrosine 716). Values are the means ± S.D. from three independent experiments, and -fold changes in PDGF-R phosphorylation were normalized to total PDGF-Rβ. The histograms are shrunk horizontally to fit under the blots.

noprecipitation with PDGF-Rβ, a comparable expression of PLD1 and PLD2 proteins was observed in total cell lysates from control and LPA-treated cells (Fig. 12*B*). Although stimulation of cells with PDGF-BB increased tyrosine phosphorylation of PDGF-Rβ, overexpression of either the wild type mPLD2 or catalytically inactive PLD2 mutant had no effect on PDGF-Rβ phosphorylation (data not shown). Presumably, PDGF did not increase association of PLD2 with PDGF-Rβ. These results show that in HBEpCs, PDGF-BB mediated phosphorylation of PDGF-Rβ is independent of PLD stimulation in HBEpCs, and PLD2, but not PLD1 forms a physical complex with PDGF-Rβ which is independent of the catalytic activity of PLD2.

DISCUSSION

LPA mediates cellular responses through its GPCRs. Many responses to LPA such as proliferation, anti-apoptotic, and tumor invasiveness are mediated by signaling pathways of protein kinase C, Src kinase, ERK, phospholipases, and Ca^{2+} (40). Many GPCRs, including those for platelet-activating factor, S1P, LPA, thrombin, insulin-like growth factor I, chemokine CXCR-1/2, β_2-adrenergic agonists, and angiotensin II (28, 30, 31, 41–45) activate ERK1/2 involving transactivation of receptor tyrosine kinases. Earlier studies showed that LPA induces protein-tyrosine phosphorylation in fibroblasts and exhibits growth factor-like properties via ERK activation (36). The role of LPA and its receptors in bronchial epithelial cell signal transduction and cellular responses has not been well characterized. Here we report for the first time that LPA transactivates PDGF-R in HBEpCs, resulting in stimulation of ERK1/2. Furthermore, we demonstrated a novel mechanism that this transregulation of the PDGF-R by LPA is mediated by coupling to G_i and PA generated by PLD2, but not PLD1.

LPA-1, LPA-2, and LPA-3 receptors are present in the lung (46). RT-PCR studies have confirmed mRNA for all the three receptors in isolated human aortic smooth muscle cells (47). In HBEpCs, we detected the mRNA for all the three receptors by RT-PCR, protein expression by Western blotting with LPA-R specific antibodies, and immunocytochemistry (Fig. 1). Interestingly, the immunocytochemical analysis revealed that the LPA-Rs apparently have differential subcellular localizations. LPA-Rs were detected not only on the plasma membrane, but also in the cytoplasm, endoplasmic reticulum, and perinuclear location. Western blot analysis using antibodies directed against internal domains of LPA1–3 of the LPA1–3 receptors revealed considerable specificity with LPA-2 antibody exhibiting a single band corresponding to the expected weight of each receptor (data not shown). The intracellular distribution of LPA-Rs indicates that they may be targets of the LPA generated after agonist stimulation (48). More than one LPA-R could be involved in LPA-induced ERK1/2 activation. Further studies on the types of LPA-Rs that are coupled to different GPCRs and native LPA-R localization will help in understanding intracel-

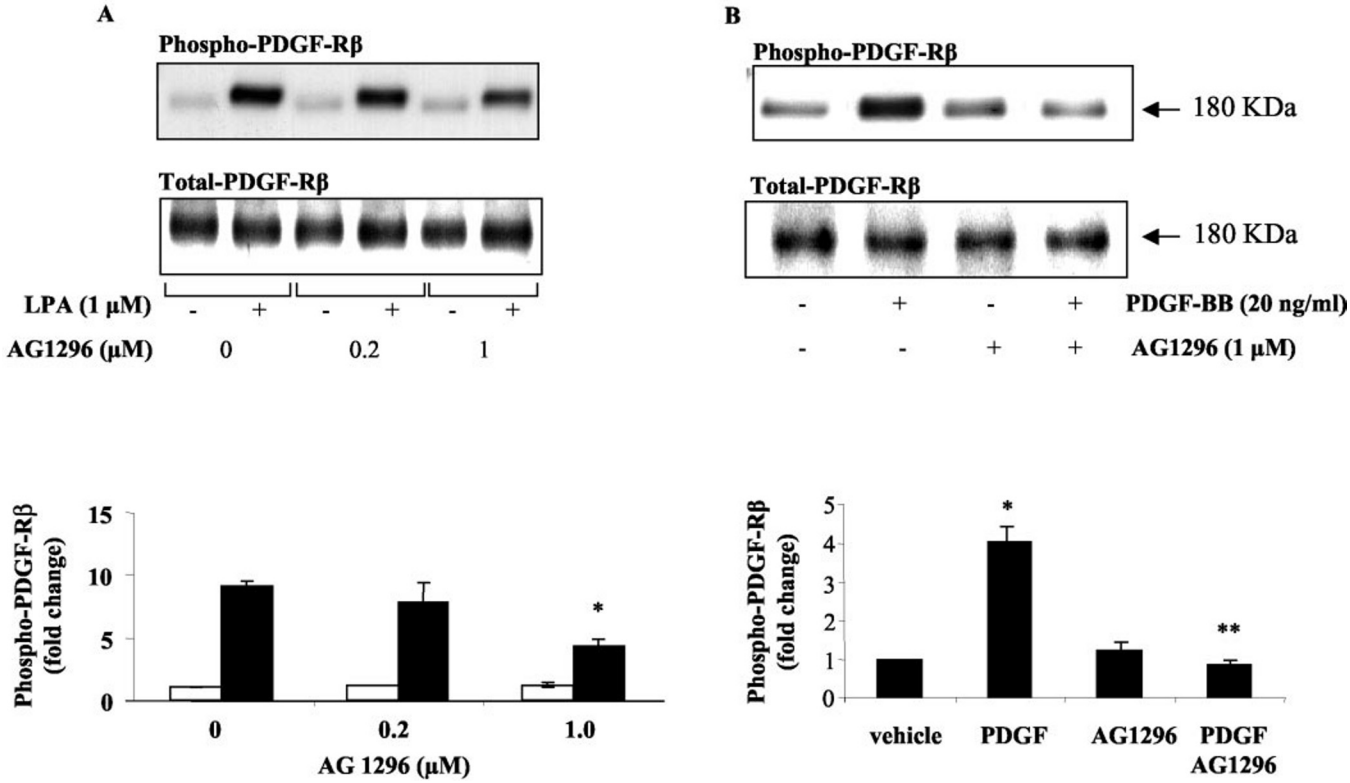

FIG. 6. **AG 1296 attenuates LAP- and PDGF-BB mediated PDGF-Rβ phosphorylation.** HBEpCs (passage 2, ~80% confluence) in 35-mm dishes were treated with varying concentrations of tyrphostin AG 1296 (*A*) or 1 μM AG 1296 (*B*) for 1 h. Subsequently the cells challenged with 1 μM LPA as in *A* or 20 ng/ml PDGF-BB as in *B* for 5 min in BEBM with 0.1% BSA. Cell lysates were subjected to SDS-PAGE and Western blotting with phospho-specific PDGF-Rβ (tyrosine 716) and PDGF-Rβ antibodies. Values are the means ± S.D. of triplicate determinations, and -fold increases in PDGF-Rβ phosphorylation were normalized to total PDGF-Rβ. *, $p < 0.05$ compared with vehicle control; **, $p < 0.05$ compared with PDGF treatment.

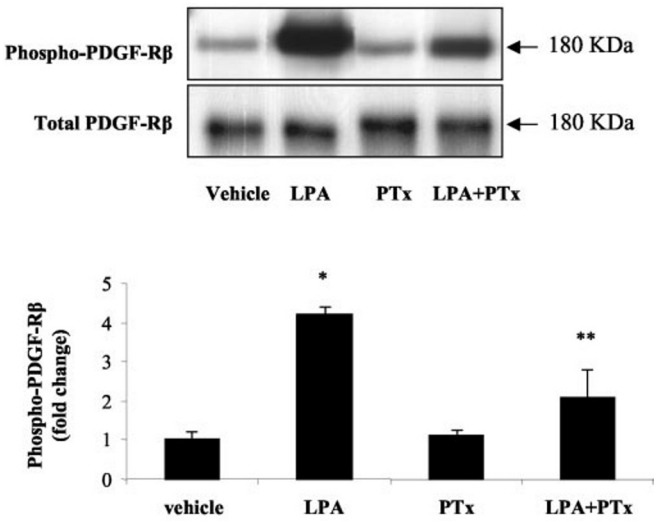

FIG. 7. **PTx blocks LPA-induced PDGF-Rβ transactivation.** HBEpCs (passage 3, ~90% confluence) in 35-mm dishes were treated with 100 ng/ml PTx for 12 h prior to challenge with 1 μM LPA for 15 min. Cell lysates were prepared and analyzed for PDGF-Rβ phosphorylation with phospho-specific PDGF-Rβ (tyrosine 716) and PDGF-Rβ antibodies. Values are the means ± S.D. of three independent experiments, and -fold changes in PDGF-Rβ phosphorylation were normalized to total PDGF-Rβ. *, $p < 0.05$ compared with vehicle control; **, $p < 0.05$ compared with LPA treatment.

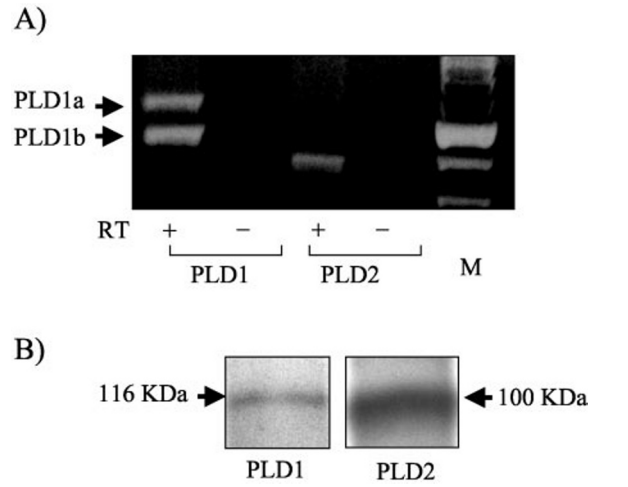

FIG. 8. **Detection of PLD1 and PLD2 in HBEpCs by RT-PCR and Western blotting.** *A*, total RNA was extracted from primary HBEpCs, and transcription of the genes encoding PLD1 and PLD2 was assessed by RT-PCR (− indicates in the absence of RNA, and + indicates in the presence of 2 μg of RNA) with primers as indicated under "Experimental Procedures." RT-PCR products were visualized by ethidium bromide staining after separation on agarose gels. *B*, cell lysates (40 μg of protein) were subjected to SDS-PAGE on 6% gels and analyzed by Western blotting with PLD1 (internal + N-terminal antibodies, 1:500 dilution) and PLD2 (internal + N-terminal antibodies, 1:1,000 dilution). The Western blot is representative of three independent experiments.

lular targets of LPA and its action.

LPA-Rs are coupled to heterotrimeric G proteins: G_i, G_q/G_{11}, and $G_{12/13}$, increasing the complexity in signaling pathways inducing multiple cellular responses. In HASMs, ERK activation by LPA was blocked by PTx treatment (1, 49). In fibroblasts, LPA-stimulated cell growth and decreased in cAMP was sensitive to PTx, suggesting coupling of LPA-Rs to G_i (8). Similarly, LPA-mediated activation of the Ras/mitogen-activated protein kinase cascade and cell migration occurred in a PTx-

TABLE I
Effect of AG 1296 on PLD activation by LPA, PDGF-BB, and 12-O-tetradecanoylphorbol-13-acetate (TPA) in HBEpCs

HBEpCs were labeled with $^{32}P_i$ (20 μCi/ml) in BEBM serum-free medium for 18 h. The radioactive medium was aspirated, and cells were pretreated with AG 1296 (10 μM) for 1 h prior to addition of either LPA (1 μM) or PDGF-BB (50 ng/ml) or 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (25 nM) for 15 min in the presence 0.1% BSA and 0.05% butan-1-ol. Lipids were scraped under acidic conditions with methanol:HCl and extracted with chloroform as described under "Experimental Procedures." The formation of [^{32}P]PBt, an index of PLD activation, was quantified by scintillation counting after separation of the labeled lipids by thin layer chromatography using 1% potassium oxalate plates developed with the upper phase of ethyl acetate:iso-octane:glacial acetic acid:water (65:10:15:50, v/v). Values are the means ± S.D. of three independent experiments in triplicate. Counts (dpm) in [^{32}P]PBt were normalized to 1×10^6 dpm in total lipid extracts.

Pretreatment	Treatment	[^{32}P] PBt formed (dpm)
Vehicle	Vehicle	550 ± 71
Vehicle	LPA (1 μM)	3,081 ± 382
Vehicle	PDGF-BB (50 ng/ml)	682 ± 21
Vehicle	TPA (25 ng/ml)	3,708 ± 234
AG 1296 (10 μM)	Vehicle	422 ± 33
AG 1296 (10 μM)	LPA (1 μM)	2,796 ± 208
AG 1296 (10 μM)	PDGF-BB (50 ng/ml)	538 ± 46
AG 1296 (10 μM)	TPA (25 ng/ml)	3,432 ± 136

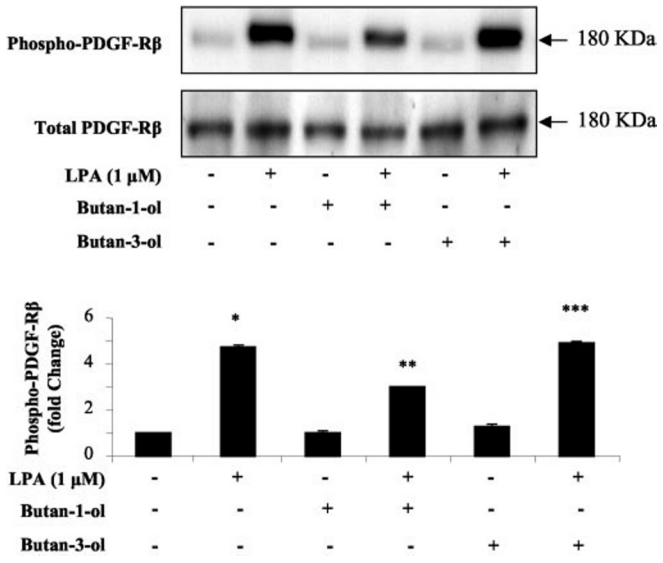

FIG. 9. **Butan-1-ol, but not butan-3-ol, attenuates LPA-induced PDGF-Rβ phosphorylation.** HBEpCs (passage 2, ~80% confluence) in 35-mm dishes were pretreated with BEBM or BEBM plus 0.1% butan-1-ol or 0.1% butan-3-ol for 15 min and then challenged with 1 μM LPA in 0.1% BSA for an additional 15 min. Cell lysates (20–30 μg of protein) were subjected to SDS-PAGE and Western blotting with phospho-specific PDGF-Rβ (tyrosine 716) and PDGF-Rβ antibodies. Values are the means ± S.D. from three independent experiments, and -fold changes PDGF-Rβ phosphorylation were normalized to total PDGF-Rβ in total cell lysates. *, $p < 0.05$ compared with vehicle control; **, $p < 0.05$ compared with LPA treatment; ***, $p > 0.05$ compared with LPA treatment.

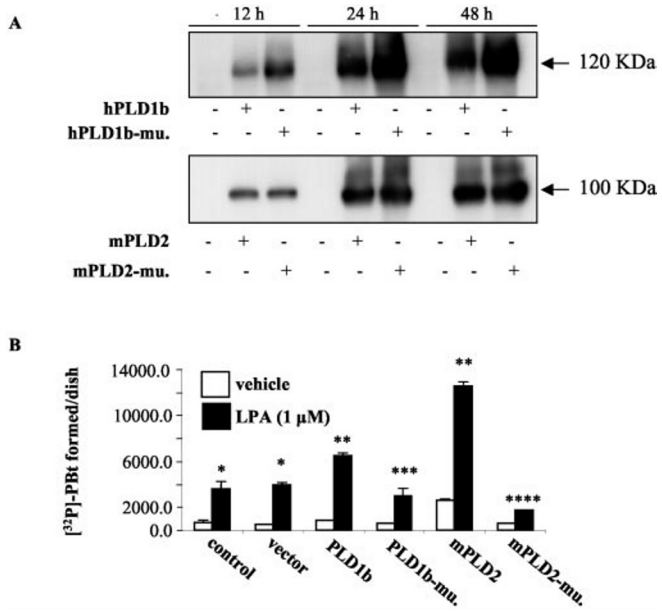

FIG. 10. **Effects of overexpression of wild type and catalytically inactive mutants of hPLD1 and mPLD2 on LPA-induced [^{32}P]PBt formation.** *A*, HBEpCs (~ 50% confluence in 35-mm dishes) were infected with empty vector or adenoviral vectors containing cDNA for wild type hPLD1 or mPLD2 or mutant forms of hPLD1 or mPLD2 (50 pfu/cell; 1.5×10^5 cells/dish) in complete BEBM for 12, 24, and 48 h. Cell lysates were prepared as described under "Experimental Procedures" and subjected to SDS-PAGE and Western blotting with internal plus N-terminal PLD1 and PLD2 antibodies. *B*, HBEpCs (~50% confluence in 35-mm dishes) were infected as described above for 24 h and subsequently labeled with $^{32}P_i$ for 18 h. Cells were challenged with BEBM or BEBM plus 1 μM LPA 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 lipid extracts by thin layer chromatography. Values are the means ± S.D. of triplicate determinations from three independent experiments and normalized to 10^6 dpm in total lipid extracts. *, $p < 0.05$ compared with vehicle control/vector; **, $p < 0.05$ compared with LPA treatment in vector-infected cells; ***, $p > 0.05$ compared with LPA treatment in vector-infected cells; ****, $p < 0.05$ compared with vector-infected cells.

sensitive manner with the possible involvement of Gβγ subunits (10). However, in several mammalian cells, LPA-induced stimulation of phospholipase C was PTx-insensitive, suggesting LPA-R coupling to G_q (50). A role for the $G_{12/13}$ family of heterotrimeric G proteins in LPA-induced actin stress fiber and focal adhesions has been described in quiescent Swiss 3T3 fibroblasts (13). Our present results demonstrate that LPA-induced transactivation of PDGF-Rβ and ERK1/2 stimulation is PTx-sensitive, suggesting involvement of G_i protein-dependent signaling responses in HBEpCs (Figs. 3 and 7). The LPA-R-mediated ERK1/2 activation appears to be secondary to transactivation and phosphorylation of PDGF-Rβ because the PDGF-R tyrosine kinase inhibitor AG 1296 (51, 52) partially blocked LPA-induced ERK stimulation (Fig. 2). There are several reports demonstrating transactivation of EGF-R by LPA (31, 50, 53–57). Tyrphostin AG 1478, an inhibitor of EGF-R kinase, blocked LPA-induced EGF-R phosphorylation, suggesting transactivation of EGF-R by LPA in HBEpCs (results not shown). Furthermore, pretreatment of HBEpCs with AG 1296 plus AG 1478 almost completely attenuated LPA-mediated phosphorylation of ERK1/2 (results not shown) compared with the partial block with AG 1296 alone (Fig. 2). These results suggest that in HBEpCs, transactivation of both EGF-R and PDGF-R by LPA plays a major role in the activation of ERK1/2. Further studies are required to evaluate the mechanisms for transactivation of the EGF receptor with respect to ERK signaling.

LPA-R-induced EGF-R transactivation involves the matrix metalloproteinase-dependent proteolytic processing of membrane-anchored proligands of EGF-R (31). However, in our experiments, LPA-induced transactivation of PDGF-R was not attenuated by the nonspecific matrix metalloproteinase inhibitor GM 6001 in HBEpCs (results not shown). Furthermore, inhibitors of protein kinase C such as bisindolylmalemide and Gö 6738 failed to block LPA-induced PDGF-R phosphorylation, suggesting that protein kinase C is not involved. LPA-induced transactivation of PDGF-Rβ was attenuated by butan-1-ol, but not butan-3-ol in HBEpCs (Fig. 9), demonstrating a role for

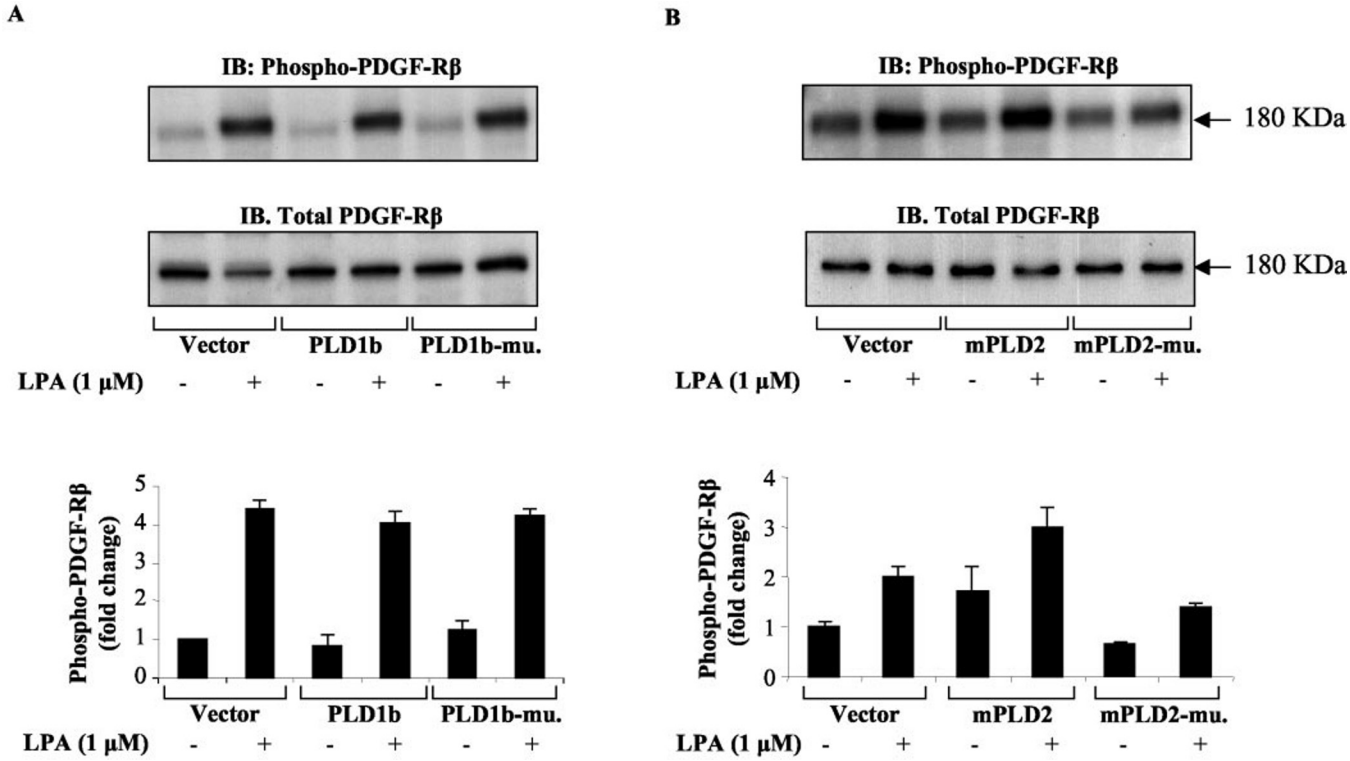

FIG. 11. **Effects of overexpression of wild type and mutants of hPLD1 and mPLD2 on LPA-induced PDGF-Rβ phosphorylation.** *A*, HBEpCs (~50% confluence in 35-mm dishes) were infected with adenoviral vectors containing cDNA for wild type hPLD1 or mutant hPLD1 (50 pfu/cell, 1.5×10^5 cells) for 48 h. Cells were challenged with BEBM or BEBM plus 1 μM LPA containing 0.1% BSA for 5 min. *B*, HBEpCs were infected with vector or wild type mPLD2 or mutant mPLD2 for 48 h before challenging with 1 μM LPA for 5 min as described above. Cell lysates were subjected to SDS-PAGE and Western blotting (*IB*) with phospho-specific PDGF-Rβ (tyrosine 716) and PDGF-Rβ antibodies. Values are the means ± S.D. of three independent experiments in triplicate. -Fold increases in PDGF-Rβ phosphorylation were normalized to total PDGF-Rβ in the cell lysates.

PLD in transactivation of PDGF-R. Using formation of [^{32}P]PBt in the presence of butan-1-ol as an index of PLD activation, we demonstrated that LPA but not PDGF-BB activated PLD in HBEpCs (Table I). Additionally, infecting cells with adenoviral constructs of wild type hPLD1 and mPLD2 demonstrated that LPA rapidly and transiently activated both PLD1 and PLD2 in HBEpCs.

Earlier, we showed that S1P activated both PLD1 and PLD2 in Beas-2B bronchial epithelial cells (21). In the present work, the participation PLD2, but not PLD1, in LPA-mediated transactivation of PDGF-R was established by the demonstration that overexpression of mPLD2-K758R, but not the hPLD1-K898R mutant, blocked LPA-induced phosphorylation of PDGF-Rβ. The two PLD mutants had no effect on PDGF-BB-induced phosphorylation of PDGF-R, confirming that PLD was not activated downstream of PDGF binding to PDGF-R in HBEpCs. These results provide the first direct evidence for activation of both PLD1 and PLD2 in HBEpCs and for the participation of PLD2 but not PLD1 in LPA-induced transactivation of PDGF-R. Our results (Fig. 12*A*) show that wild type and catalytically inactive mutant of mPLD2 coimmunoprecipitated with PDGF-Rβ to a greater extent compared with PLD1 before and after stimulation of HBEpCs with LPA. Also, mutation of mPLD2 at a single amino acid residue at 758 from Lys to Arg had no appreciable effect on its interaction with the PDGF-Rβ (Fig. 12*A*). However, this mutation at the catalytic site of mPLD2 almost completely reversed LPA-induced transactivation of PDGF-Rβ. Earlier studies using human embryonic kidney fibroblasts transfected with wild type PLD1 or PLD2 demonstrated that EGF stimulated PLD1 activity to a greater extent than PLD2, but only PLD2 was associated with EGF-R (58). Furthermore, PLD2 was tyrosine phosphorylated upon EGF-R activation by EGF (59). However, in HBEpCs, PLD was not activated by PDGF (Table I) or EGF (results not shown), and it is unclear whether LPA enhanced tyrosine phosphorylation of PLD2. Studies with rat 3Y1 fibroblasts, overexpressing the EGF-R established a role for both PLD1 and PLD2 in EGF-induced receptor degradation suggesting that PLD signaling is involved in receptor endocytosis (60).

Our work, therefore, established a novel mechanism by which PLD2 participates in the transactivation of PDGF-Rβ. We have not yet elucidated the mechanisms for activation other than to establish that PLD2 interacts physically with PDGF-Rβ and that the formation of PA is required. We have also not investigated the details of how PLD2 is activated by exogenous LPA, but this involves a receptor, probably LPA-1, that is coupled to $G\alpha_i$. Src kinase plays a central role in GPCR signaling by transactivating the EGF-R or vascular EGF-R (Flk-1/KDR) (61). For example, adrenergic receptor-mediated EGF-R phosphorylation and S1P-induced Flk-1/KDR transactivation (32, 62) were sensitive to Src kinase inhibitors PP1/PP2 (63). Our work with endothelial cells established that Src can be upstream of activation of PLD1 and PLD2 by diperoxovanadate (64). Therefore, such an LPA-induced activation of Src in HBEpCs could mediate the PLD2-induced activation of PDGF-Rβ. It is also significant that the PDGF-Rβ can be tethered to GPCRs, including S1P-1, thus providing an integrative signaling pathway (33). Our present results suggest that PLD2 could be a component of such a complex.

In summary, we demonstrate for the first time that LPA mediates transactivation of PDGF-R in HBEpCs through PLD and PA production. Although LPA activates both PLD1 and PLD2, PLD2, but not PLD1, regulates PDGF-R phosphorylation and activation. Also, LPA increases PLD2 association with

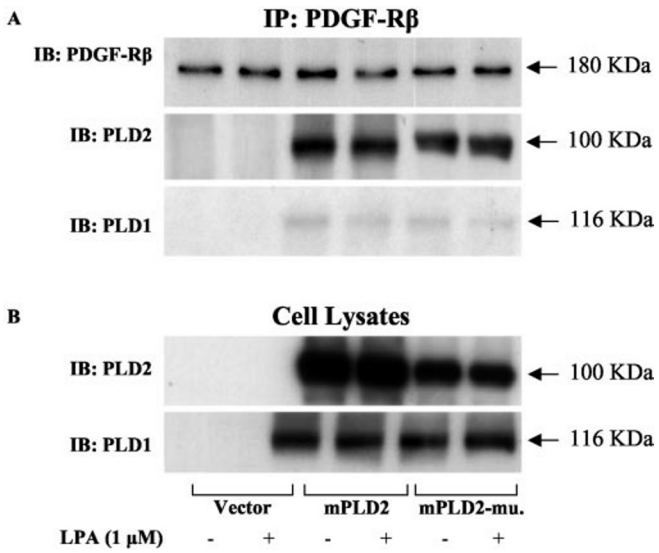

FIG. 12. **Effects of overexpression of wild type and mutant mPLD2 on coimmunoprecipitation of PDGF-Rβ with PLD2 after LPA treatment.** *A*, HBEpCs (passage 3, ~50% confluence in 35-mm dishes) were infected with adenoviral vectors containing cDNA for wild type or mutant mPLD2 (50 pfu/cell, 1.5×10^5 cells) for 24 h. Cells were then transfected with wild type PDGF-Rβ plasmid with FuGENE 6 for 5 h. Then the medium was replaced with fresh BEBM and cells cultured for an additional 24 h. Cells were challenged with BEBM or BEBM plus 1 μM LPA in 0.1% BSA for 5 min. Cell lysates (1 mg of protein/ml) were subjected to immunoprecipitation (*IP*) under nondenaturing conditions with PDGF-Rβ antibody for 18 h in the presence of Sepharose A/G (20 μl/mg protein). The PDGF-Rβ immunoprecipitates were subjected to SDS-PAGE and Western blotting (*IB*) with PDGF-Rβ and internal plus N-terminal PLD2 antibodies. *B*, cell lysates (20 μg of protein) were subjected to SDS-PAGE and Western blotting with internal plus N-terminal PLD2 antibody.

PDGF-Rβ. Our results, therefore, demonstrate that PLD2 is activated upstream from PDGF-Rβ activation. PDGF signaling is involved in cell proliferation and tumorigenesis as well as lung development. Our work establishes that LPA through stimulating PLD2 activity plays an important role in activating the PDGF-Rβ and therefore ERK. LPA-induced transactivation of the PDGF-R could, therefore, be involved in modulating airway remodeling and lung function.

Acknowledgments—We thank Drs. Henry Sarau and Kristen Belmonte from Glaxo Smith Kline for providing the LPA-1, LPA-2, and LPA-3 receptor antibodies. We appreciate the technical assistance of Tonya Watkins and Dong Hong He. We also thank Dr. Evegnia Berdyshev for a critical reading of the manuscript.

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