Phosphatidylinositol 3-kinase mediates mitogen-induced human airway smooth muscle cell proliferation

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1Pulmonary and Critical Care Division, Department of Medicine, University of Pennsylvania Medical Center, Philadelphia 19104-4283; 2Department of Pharmacology, Jefferson Cancer Institute, Thomas Jefferson University, Philadelphia, Pennsylvania 19107; 3Department of Physiology and Pharmacology, Royal College, University of Strathclyde, Glasgow G1 1XW; and 4Rayne Laboratory, Respiratory Medicine Unit, Department of Medicine (Royal Infirmary of Edinburgh), University of Edinburgh Medical School, Edinburgh EH8 9AG, United Kingdom

Krymskaya, Vera P., Raymond B. Penn, Michael J. Orsini, Pamela H. Scott, Robin J. Plevin, Trevor R. Walker, Andrew J. Eszterhas, Yassine Amrani, Edwin R. Chilvers, and Reynold A. Panettieri, J r. Phosphatidylinositol 3-kinase mediates mitogen-induced human airway smooth muscle cell proliferation. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L65–L78, 1999.—Hypertrophy and hyperplasia of airway smooth muscle (ASM) are important pathological features that contribute to airflow obstruction in chronic severe asthma. Despite considerable research effort, the cellular mechanisms that modulate ASM growth remain unknown. Recent evidence suggests that mitogen-induced activation of phosphoinositide (PI)-specific phospholipase C (PLC) and PI-dependent calcium mobilization are neither sufficient nor necessary to stimulate human ASM proliferation. In this study, we identify phosphatidylinositol (PtdIns) 3-kinase as a key regulator of human ASM proliferation. Pretreatment of human ASM with the PtdIns 3-kinase inhibitors wortmannin and LY-294002 significantly reduced thrombin- and epidermal growth factor stimulation, class IA PtdIns 3-kinase complex activity. Collectively, these data suggest that activation of phosphoinositide-specific phospholipase C (PI-PLC) enzymes divided into three main classes (48). Each class exhibits distinct substrate specificity. Class IA PtdIns 3-kinase is a cytosolic heterodimer composed of a 110-kDa (p110α, β, or δ) catalytic subunit and an 85-kDa (p85) adaptor protein. In response to growth factor stimulation, class IA PtdIns 3-kinases complex with activated receptor protein tyrosine kinases (through SH2 domains of the p85 subunit) or tyrosine-

Numerous contractile agonists and growth factors have been shown to induce proliferation of airway smooth muscle (ASM) cells in culture and thus may play an important role in stimulating the smooth muscle hypertrophy and hyperplasia associated with several disease states (32, 34). Although numerous studies have identified agonists associated with acute bronchoconstriction in asthmatic patients as potent effectors of human ASM (HASM) cell proliferation, the mechanism by which spasmogens induce human ASM growth is not known (reviewed in Ref. 32). Because contractile responses are mediated primarily by increases in intracellular phosphoinositide (PI) turnover and calcium, these elements represent potentially important regulators of HASM growth. However, the inability of some contractile agonists to induce HASM growth suggests that increased PI turnover and calcium flux per se are not sufficient to induce HASM proliferation and that activation of other signaling pathways is likely required (34).

PIs play a key role in signal transduction. To date, two PI pathways have been characterized. In the canonical PI pathway, activation of one of a family of PI-specific phospholipase C (PLC; PI-PLC) enzymes hydrolyzes phosphatidylinositol (PtdIns) 4,5-bisphosphate [PtdIns(4,5)P2] to inositol 1,4,5-trisphosphate [Ins(1,4,5)P3] and diacylglycerol. In the 3-PI pathway, activation of PtdIns 3-kinase phosphorylates PIs at the D3 position of the inositol ring, leading to the formation of PtdIns 3-phosphate (PtdIns3P), PtdIns 3,4-bisphosphate [PtdIns(3,4)P2], and PtdIns 3,4,5-trisphosphate [PtdIns(3,4,5)P3]. PtdIns(3,4,5)P3 plays a significant role as a second messenger in signaling induced by a wide array of extracellular stimuli and appears to be the immediate and dominant PtdIns 3-kinase product in vivo (43, 46).

PtdIns 3-kinases are a family of structurally diverse enzymes divided into three main classes (48). Each class exhibits distinct substrate specificity. Class IA PtdIns 3-kinase is a cytosolic heterodimer composed of a 110-kDa (p110α, β, or δ) catalytic subunit and an 85-kDa (p85) adaptor protein. In response to growth factor stimulation, class IA PtdIns 3-kinases complex with activated receptor protein tyrosine kinases (through SH2 domains of the p85 subunit) or tyrosine-

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phosphorylated proteins. The class IB PtdIns 3-kinase-γ does not have an adaptor protein but instead is activated by Gβγ subunits of G proteins (44). Class II PtdIns 3-kinases contain a COOH-terminal C2 domain that appears involved in the regulation of this class by phospholipid and calcium (11). The recently identified class III PtdIns 3-kinases are structurally related to the product of the Saccharomyces cerevisiae gene Vps34 (51).

D3-PIs are not substrates for any known PLC (43, 46) and are not components of the classic or conventional PI turnover pathway. Auger et al. (3) were the first to report that platelet-derived growth factor (PDGF) activated PtdIns 3-kinase in human vascular smooth muscle (VSM) cells; however, the role of PtdIns 3-kinase in modulating cell growth was not clarified. Several studies have since demonstrated that PtdIns 3-kinase and its lipid product(s) can regulate mitogenesis in a mitogen- and cell-specific manner (e.g., Ref. 8). In human umbilical vein VSM cells, PtdIns 3-kinase appears to be required for PDGF-induced focal adhesion kinase activation (40). Focal adhesion kinase activation is an important integration site for a number of extracellular signals including integrin-receptor family members, G protein-coupled receptors, and both receptor and non-receptor tyrosine kinases (40). Conversely, PtdIns 3-kinase is not required for the mitogenic signaling of thrombin in bovine VSM (52). These studies suggested that the role of PtdIns 3-kinase in regulating smooth muscle growth remains largely unknown, and no study to date has examined the role of PtdIns 3-kinase in mediating HASM cell proliferation.

An essential step in the pathway by which growth factors trigger cellular proliferation is the biogenesis of translational components regulating protein synthesis required for cell cycle progression in G0 phase. The 70/85-kDa S6 protein kinases, referred to collectively as pp7056k, are the principal kinases that exert translational control of mRNA transcripts and are required for growth factor-induced G0 progression (19, 24, 28). The pp7056k signaling pathway appears to be distinct from those that activate c-Ras, c-Raf, mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase kinase, MAPK, and ribosomal S6 kinase (5). In some cell types, pp7056k appears to be an essential downstream effector of PtdIns 3-kinase (9). In response to mitogen stimuli, pp7056k is phosphorylated and activated by PI-dependent kinase 1, a multifunctional effector of PtdIns 3-kinase (2, 38). Mitogen-induced activation of pp7056k is also necessary for expression of early-immediate genes such as c-fos, and pp7056k activation is required for the duration of the G0 phase of the cell cycle in rat embryo fibroblasts (24). Whether activation of pp7056k is necessary to stimulate growth factor- or spasmogen-induced smooth muscle cell proliferation remains unexplored.

In this study, we examined the relationship between mitogen-induced activation of PtdIns 3-kinase and ASM cell mitogenesis. We show that epidermal growth factor (EGF) and thrombin induce PtdIns 3-kinase activation and that inhibition of PtdIns 3-kinase abolishes mitogen-stimulated ASM cell growth. In addition, we demonstrate that transient expression of constitutively active PtdIns 3-kinase (p110α) activates pp7056k. Moreover, expression of a dominant-negative PtdIns 3-kinase (Δp85) blocks EGF- and thrombin-induced pp7056k activity, suggesting that PtdIns 3-kinase is required for pp7056k activation by EGF and thrombin. These data suggest that mitogenic signals elicited by contractile agonists and growth factors share common signaling pathways that converge to activate PtdIns 3-kinase.

METHODS

ASM cell culture. Human tracheae were obtained from lung transplant donors in accordance with procedures approved by the University of Pennsylvania (Philadelphia, PA) Committee on Studies Involving Human Beings. A segment of trachea just proximal to the carina was removed under sterile conditions, and the trachealis muscle was isolated. With this technique, 0.5 g of wet tissue was obtained, minced, centrifuged, and resuspended in 10 ml of buffer containing 0.2 mM CaCl2, 640 U/ml of collagenase, 1 mg/ml of soybean trypsin inhibitor, and 10 U/ml of elastase. Enzymatic dissociation of the tissue was performed for 90 min in a shaking water bath at 37°C. The cell suspension was filtered through 105-µm Nitex mesh, and the filtrate was washed with equal volumes of cold Ham’s F-12 medium supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT). Aliquots of the cell suspension were plated at a density of 1.0 104 cells/cm2. The cells were cultured in Ham’s F-12 medium supplemented with 10% FBS, 100 U/ml of penicillin, 1 mg/ml of streptomycin, and 2.5 µg/ml of amphotericin B, and this was replaced every 72 h. Cell counts were obtained from triplicate wells with 0.5% trypsin in a 1 mM EDTA solution.

HASM cells in subculture during the second through fifth cell passages were used because during these cell passages, the cells retain native contractile protein expression as demonstrated by immunocytochemical staining for smooth muscle actin and myosin (33). These cells retain functional cell-excitation coupling systems as determined by fura 2 measurements of agonist-induced changes in cytosolic calcium (29, 33).

All experiments were performed with a minimum of three different cell lines. Each ASM cell line was established with tracheal tissue from a single human donor.

Cytosolic calcium measurements. Cultured HASM cells were grown to near confluence on 22-mm glass coverslips and loaded with fura 2-AM (2.5 µM in medium 199 supplemented with 1 mg/ml of BSA for 30 min at 37°C). After being loaded with fura 2, the cells were washed with a HEPES-buffered saline solution as previously described (13) and placed in a thermostatically controlled cell chamber on a Nikon inverted microscope (Diaphot). The cells were imaged with a ×40 (oil) fluorescence objective lens. Excitation energy was switched between 340- and 380-nm wavelengths from a 75-W xenon lamp source via a fura 2 dichroic mirror (Chroma Technology, Brattleboro, VT). Emitted light was passed through a 510-nm filter and diverted to an image-intensified charge-coupled device camera (Hamamatsu, Hamamatsu City, Japan) attached to the video side port of the microscope. Images were digitized with a video analog-to-digital board (Maatrox). Image analysis of individual cursor-defined regions corresponding to individual cells was accomplished with the Image-1AT/fluor program (Universal Imaging, West Chester, PA). The 340- to 380-nm ratio was converted to an estimate of cytosolic calcium with previously described calibration meth-
iods (13, 29, 30). Calibration measurements were made with ionomycin (10 µM) and added calcium (total calcium = 12 mM) to measure the ratio of the 340-nm signal to the 380-nm signal under saturating conditions of calcium (Rmax) or with the addition of a stoichiometric excess of EGF to achieve the ratio of the 340-nm signal to the 380-nm signal in the absence of calcium (Rmin). Values used for the calibration equation were: Rmin = 0.3, Rmax = 5.0, dissociation constant = 224 mM, and the ratio of the 380-nm fluorescence at high calcium to that at low calcium = 5. Additions of plasmogen-free bovine α-thrombin (0.01 U/ml) were made by direct bath additions. Fura 2, ionomycin, bradykinin, and α-thrombin (1,000 U/mg protein) were obtained from Calbiochem (La Jolla, CA). All other reagents were obtained from Sigma (St. Louis, MO).

Accumulation of total [3H]inositol phosphates. [3H]inositol phosphate formation in cultured cells was determined as previously reported (6, 53), with minor modifications. The medium was aspirated from near-confluent cell monolayers in 24-well plates and replaced with 300 µl of ionomycin-free Dulbecco’s modified Eagle’s medium (DMEM) containing [methyl-3H]inositol (10 20 Ci/mmol; DuPont-New England Nuclear, Boston, MA) at a concentration of 2 µCi/ml. The cells were then incubated for 24 h, the medium was removed, and the cells were washed twice with ionomycin-free DMEM containing 10 mM LiCl and then incubated with this medium for 30 min at 37°C. Agonists were added in a volume of 10 µl, and the final incubation volume was 300 µl. Using this labeling protocol, Daykin et al. (10) have shown that membrane inositol phospholipids achieve isotopic equilibrium and that over the time course of these experiments, no significant depletion of labeled membrane inositol phospholipids occurs. The reactions were stopped by removing the medium from each well and adding 1 ml of methanol-0.12 M HCl (1:1 vol/vol) previously stored at 20°C. The samples were then stored at −20°C overnight. An 800-µl aliquot of each sample was then neutralized to pH 7 with 4.2 ml of buffer containing 25 mM Tris-0.5 M NaOH-H2O (0.238:0.025:0.737 vol/vol). Total [3H]inositol phosphates were finally separated from free [methyl-3H]inositol by anion-exchange chromatography (53).

Statistical analysis of the data was performed with paired or unpaired t-tests as appropriate. Reported values are means ± SE from at least four separate experiments.

[3H]thymidine and cell proliferation assays. Proliferation studies were performed on near-confluent, growth-arrested HASM cells. The cells were growth arrested by incubating the cultures on day 10 in serum-free medium consisting of Ham’s F-12 medium with 5 ng/ml of insulin and 5 ng/ml of transferrin (36). Near-confluent, growth-arrested cells were used because cells can be synchronized in the G0/G1 phase of the cell cycle and, at this baseline, minimally incorporate [3H]methionine and [3H]thymidine (33, 36). After 24 h in serum-free medium, the cells were then stimulated with either 10 ng/ml of EGF (Sigma), 0.1% FBS, or plasmogen-free bovine α-thrombin (1,000 U/mg protein; Calbiochem). In some experiments, monolayers were treated with wortmannin or LY-294002. The reactions were stopped by removing the medium from near-confluent cell monolayers in 24-well plates and replaced with 300 µl of inositol-free Dulbecco’s modified Eagle’s medium (DMEM) containing 10 mM LiCl and then incubated with this medium for 30 min at 37°C. Agonists were added in a volume of 10 µl, and the final incubation volume was 300 µl. Using this labeling protocol, Daykin et al. (10) have shown that membrane inositol phospholipids achieve isotopic equilibrium and that over the time course of these experiments, no significant depletion of labeled membrane inositol phospholipids occurs. The reactions were stopped by removing the medium from each well and adding 1 ml of methanol-0.12 M HCl (1:1 vol/vol) previously stored at 20°C. The samples were then stored at −20°C overnight. An 800-µl aliquot of each sample was then neutralized to pH 7 with 4.2 ml of buffer containing 25 mM Tris-0.5 M NaOH-H2O (0.238:0.025:0.737 vol/vol). Total [3H]inositol phosphates were finally separated from free [methyl-3H]inositol by anion-exchange chromatography (53).

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The [3H]thymidine and cell count data are means ± SE from a minimum of four wells from at least four separate experiments; significance was assessed by one-way analysis of variance (Bonferroni-Dunn test). The proliferation assays were performed with a minimum of three different ASM cell lines.

Transient transfection. HASM cells were transiently transfected with DEAE-dextran and a replication-defective adenovirus, Ad5-GPT, as previously described (37). The plasmid directing expression of Myc-tagged p110* was a generous gift from Dr. A. Klippel (Chiron, Emeryville, CA). The p110* plasmid is an activated p110 mutant generated by covalent attachment of the inter-Src homology region 2 of the p85 regulatory subunit to the NH2 terminus of the p110 catalytic subunit (16). Ap85 was a generous gift from Dr. M. Kasuga (Kobe University School of Medicine, Kobe, Japan). The Δp85 plasmid is a mutant of p85 that lacks a binding site for p110(14). All assays were performed 72 h after transfection. The cells were starved overnight in Ham’s F-12 medium containing 0.1% BSA before agonist stimulation. Transient transfection of Myc-tagged p110* and Ap85 plasmids were verified by immunoprecipitation and immunoblotting with anti-Myc Tag (3 µg/ml) or anti-PI 3-kinase p85 antibodies (Upstate Biotechnology, Lake Placid, NY), respectively.

Preparation of cell lysates and immunoprecipitation. Confluent HASM cells were growth arrested in serum-free Ham’s F-12 medium supplemented with 0.1% BSA for 48 h. The cells were incubated with growth factors, LY-294002, and wortmannin at 37°C for the times indicated. The cells were then washed twice with ice-cold wash-buffer (137 mM NaCl, 20 mM Tris-HCl, 1 mM MgCl2, 1 mM CaCl2, and 0.2 mM vanadate, pH 7.5) and were lysed in lysis buffer [wash buffer plus 10% (vol/vol) glycerol, 1% (vol/vol) Nonidet P-40 (NP-40), 1 mM phenylmethylsulfonfluoride (PMSF), 10 µg/ml of aprotinin, and 10 µg/ml of leupeptin] (23). The lysates were centrifuged at 13,200 g for 10 min. The supernatants were incubated with anti-phosphotyrosine (anti-P-tyr; 5 µg/ml; UBI). Protein A-Sepharose (60 µl; Pharmacia Biotech, Uppsala, Sweden) was then added to lysates for 2 h at 4°C. The immunoprecipitates were washed three times in PBS containing 1% NP-40, two times in 0.1 M Tris-HCl (pH 7.5)-0.5 M LiCl, and two times in 10 mM Tris-HCl-100 mM NaCl-1 mM EDTA, pH 7.5. All solutions contained 0.2 mM vanadate.

PtdIns 3-kinase activity assay. PtdIns 3-kinase activity assays were performed as previously described (23). Sonicated PtdIns in Tris-HCl-EGTA (0.2 mg/ml final concentration) was added to the immunoprecipitates, and the phosphorylation reactions were started by the addition of MgCl2, ATP, and [γ-32P]ATP (30 µCi/sample) at a final concentration of 4 mM MgCl2 and 50 mM ATP) for 10 min at room temperature. Reactions were stopped by the addition of 20 µl of 6 N HCl and extracted with 160 µl of chloroform-methanol (2:1). Lipids were separated on oxalate-coated thin-layer chromatography (TLC) plates (Silica Gel 60, Merck, Darmstadt, Germany) with a solvent system of chloroform-methanol-water-ammonium hydroxide (60:40:11.3:2). They were then detected by autoradiography. The position of phosphatidylinositol 3-monophosphate (PIP)-[32P]PIP was determined by the position of standard PIP subsequently separated on TLC in parallel and developed in iodine vapor. Identification of proteins by immunoblot assay. Immunoprecipitated proteins were subjected to 8% SDS-PAGE and Western blot assays as previously described (23). The blots were exposed to anti-PI 3-kinase p85 antibody (1:1,000 dilution).
tion in some cell lines, we investigated whether PtdIns 3-kinase activation is required for the induction of cell proliferation. Because PtdIns 3-kinase inhibition abolishes EGF- and thrombin-induced ASM cell growth. Wortmannin (Wort; 100 nM) inhibited thrombin- and EGF-induced DNA synthesis by 93 ± 9.2 and 67 ± 7.8%, respectively (Fig. 1A). This effect of wortmannin was not observed if wortmannin was added 17 h after the cells were stimulated with mitogens (Fig. 1C and D). Pretreatment of HASM cells with 30 µM LY-294002 before stimulation with thrombin or EGF completely inhibited DNA synthesis (Fig. 1B). The effects of LY-294002 (30 µM) and wortmannin (100 nM) on the basal levels of [3H]thymidine incorporation were not different from those of diluent alone. These data suggest that Ptdlns 3-kinase modulates early mitogenic signaling events involved in agonist-induced ASM cell growth. Because incorporation of [3H]thymidine correlates with DNA synthesis and may occur without cell division, we investigated whether wortmannin inhibited thrombin- and EGF-stimulated increases in cell number. After 36 h of agonist stimulation, thrombin stimulated a 17.6 ± 2.0% increase in cell number compared with that induced by diluent alone (Fig. 1E). EGF and 10% serum increased cell number by 22.3 ± 2.1 and 27 ± 2.5%, respectively. Pretreatment of cells with 100 nM wortmannin inhibited thrombin- and EGF-induced cell proliferation by 85 ± 5.2 and 61 ± 6%, respectively (Fig. 1D). Cell counts from monolayers treated with wortmannin alone were comparable to those of diluent-treated cells. Trypsin blue staining of cells treated with

**RESULTS**

Ptdlns 3-kinase inhibition abolishes EGF- and thrombin-induced ASM cell growth. Because Ptdlns 3-kinase activation is required for the induction of cell proliferation in some cell lines, we investigated whether Ptdlns 3-kinase mediated ASM cell growth induced by EGF or thrombin. Confluent ASM cells were growth arrested for 24 h, pretreated with the Ptdlns 3-kinase inhibitors wortmannin or LY-294002, and then stimulated with 1 U/ml of thrombin or 10 ng/ml of EGF. DNA synthesis was subsequently measured by assessment of [3H]thymidine incorporation. Wortmannin and LY-294002 abrogated thrombin- and EGF-induced DNA synthesis in a concentration-dependent manner (IC_{50} ~ 10 nM and ~3 µM, respectively; Fig. 1, A and B, respectively). Wortmannin (100 nM) inhibited thrombin- and EGF-induced DNA synthesis by 93 ± 9.2 and 67 ± 7.8%, respectively (Fig. 1A). This effect of wortmannin was not observed if wortmannin was added 17 h after the cells were stimulated with mitogens (Fig. 1C and D). Pretreatment of HASM cells with 30 µM LY-294002 before stimulation with thrombin or EGF completely inhibited DNA synthesis (Fig. 1B). The effects of LY-294002 (30 µM) and wortmannin (100 nM) on the basal levels of [3H]thymidine incorporation were not different from those of diluent alone. These data suggest that Ptdlns 3-kinase modulates early mitogenic signaling events involved in agonist-induced ASM cell growth.

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100 nM wortmannin alone revealed 99.9% viability, suggesting that wortmannin is not toxic to ASM cells. Collectively, these data suggest that PtdIns 3-kinase activation is important in modulating ASM cell proliferation induced by growth factors and contractile agonists.

Mitogens activate PtdIns 3-kinase in ASM cells. Although wortmannin and LY-294002 inhibited EGF-
and thrombin-induced myocyte proliferation, studies were also performed to confirm that mitogens directly activate PtdIns 3-kinase in HASM cells. Confluent, growth-arrested cells were stimulated with 10 ng/ml of EGF for 1, 3, 5, and 10 min or 1 U/ml of thrombin for 1, 5, 15, and 30 min. PtdIns 3-kinase activities were detected in anti-PTyr immunoprecipitates from cell lysates. Immunoprecipitates were incubated with PtdIns and [γ-32P]ATP, and in vitro phosphorylation of PtdIns was examined by TLC and autoradiography as described in METHODS. EGF maximally increased PtdIns 3-kinase activity at 1 min (Fig. 2A). Interestingly, maximal PtdIns 3-kinase activation by thrombin was observed at 15 min (Fig. 2B). Bradykinin, a contractile agonist that is not a HASM cell mitogen (34), did not activate PtdIns 3-kinase (data not shown).

In other studies, experiments were performed to address whether EGF also increased the amount of PtdIns 3-kinase associated with tyrosine-phosphorylated proteins in EGF-stimulated ASM cells. Immunoblot analysis with an anti-p85 subunit of PtdIns 3-kinase antibody was performed on anti-PTyr immunoprecipitates from EGF- and diluent-treated cells. EGF treatment markedly increased association of PtdIns 3-kinase with tyrosine-phosphorylated proteins when compared with that obtained from cells treated with diluent alone (Fig. 2C). Immunoblot testing of whole cell lysate confirmed the presence of PtdIns 3-kinase in HASM cells. Cell lysates from Jurkat T cells, which contain high levels of PtdIns 3-kinase, were used as positive controls. Negative controls included isotype-matched nonimmune IgG for immunoprecipitations in the PtdIns 3-kinase activity assays and immunoblots to support the specificity of protein-protein interactions (data not shown).

To establish the effectiveness of PtdIns 3-kinase inhibitors in HASM cells, the cells were pretreated for wortmannin or LY-294002 and then stimulated with EGF or thrombin. Wortmannin (100 nM) pretreatment inhibited EGF-induced activation of PtdIns 3-kinase (Fig. 2D). Similarly, LY-294002 also decreased activity of the enzyme and, in a concentration-dependent manner, abolished PtdIns 3-kinase activation by EGF at 30 µM (Fig. 2E). Pretreatment of cells with wortmannin (1 100 nM) before thrombin stimulation also induced concentration-dependent inhibition of PtdIns 3-kinase activation (Fig. 2F). In separate experiments, pretreatment of cells with U-73122, a PLC inhibitor, had no effect on EGF- or thrombin-induced PtdIns 3-kinase activation (data not shown).

PtdIns 3-kinase inhibition has no effect on mitogen-induced calcium transients or PI turnover. To address the specificity of wortmannin for inhibition of PtdIns 3-kinase and to determine whether mitogen-induced calcium mobilization is necessary to induce ASM cell growth, we examined the effects of wortmannin on mitogen-evoked calcium transients and PI turnover. Monolayers were pretreated with wortmannin (100 nM) and then stimulated with 1 U/ml of thrombin. Cytosolic calcium measurements in fura 2-loaded ASM cells were performed as described in METHODS. As shown in Fig. 3A, pretreatment with wortmannin did not affect thrombin-induced calcium transients. In parallel experiments, the effects of wortmannin and LY-294002 on total PI turnover were studied. ASM cell monolayers were pretreated with 100 nM wortmannin for 10 min and stimulated with either 1 U/ml of thrombin, 10 ng/ml of EGF, or diluent. PI turnover was measured after 30 min of agonist stimulation as described in METHODS. As shown in Fig. 3B, thrombin and EGF increased [3H]PtdIns 1,4,5-trisphosphate formation in ASM cells by 2.59 ± 0.06- and 3.09 ± 0.07-fold, respectively. Wortmannin pretreatment did not alter either thrombin- or EGF-induced PI turnover. Similarly, pretreatment of HASM cells with 30 µM LY-294002 for 30 min had no effect on thrombin-induced PI turnover (data not shown). In addition, pretreatment of cells overnight with 100 nM wortmannin had no effect on thrombin- and EGF-evoked calcium transients (data not shown).

These data suggest that the effects of wortmannin or LY-294002 on cell growth are not due to nonspecific cytotoxicity and that mitogen-induced calcium mobilization and PI turnover are not sufficient to stimulate ASM cell proliferation.

PtdIns 3-kinase inhibition does not significantly alter p42/p44 MAPK activation. Because a previous study (15) in non-HASM cells has asserted a requirement for p42/p44 MAPK activation in mitogen-induced proliferation, we examined the effects of LY-294002 and wortmannin on EGF- and thrombin-induced p42/p44 MAPK activation. Growth-arrested HASM cells were pretreated with 30 µM LY-294002 or 100 nM wortmannin before EGF (10 ng/ml) or thrombin (1 U/ml) stimulation for 0–180 min, and immunoblot analysis of p42/p44 MAPK phosphorylation was subsequently performed. As depicted in Fig. 4, no significant effects of either LY-294002 or wortmannin pretreatment were observed.
in basal and agent-stimulated p42/p44 MAPK phosphorylation. These data suggest that the effects of PtdIns 3-kinase, LY-294002, and wortmannin on mitogenesis are primarily mediated through pathways distinct from that of p42/p44 MAPK.

Treatment of HASM with wortmannin or LY-294002 does not stimulate cAMP accumulation. Numerous studies (see Ref. 32) have demonstrated that stimulation of the cAMP-dependent PKA can inhibit mitogenic signaling that includes the Raf-dependent p42/p44 MAPK and PI 3-kinase pathways in multiple cell types. We therefore investigated whether treatment with wortmannin or LY-294002 could induce cAMP accumulation (possibly either directly or secondarily through exocytic release of an activating agent) in HASM and thereby activate PKA. Treatment of HASM with 100 nM wortmannin or 30 µM LY-294002 did produce cAMP accumulation identical to that of (vehicle-stimulated) basal levels (data not shown). Thus the antimitogenic effects of wortmannin and LY-294002 do not appear to be mediated by nonspecific activation of PKA.

Activation of pp70S6k in ASM cells is mediated by stimulation of PtdIns 3-kinase. To further assess mechanisms by which mitogens induce HASM cell proliferation, we investigated the relationship between PtdIns 3-kinase and pp70S6k activation in modulating HASM mitogenesis. Growth-arrested ASM cells were treated with fetal calf serum, thrombin, or bradykinin for 15 min and lysed, pp70S6k was immunoprecipitated, and activity was assessed as described in METHODS. As shown in Fig. 5A, thrombin and serum stimulated pp70S6k activity in ASM cells by 5.4 ± 1.1- and 5.9 ± 0.9-fold, respectively. Conversely, bradykinin, a contractile agonist that evokes levels of intracellular calcium concentration and PI turnover comparable to those induced by thrombin in HASM cells (34) did not activate pp70S6k. In other experiments, the specificity of the in vitro activity assay for pp70S6k was studied. ASM cell monolayers were treated with EGF and rapamycin, a pp70S6k inhibitor, EGF alone, or diluent alone for 15 min. EGF induced a 9.6 ± 0.5-fold increase in pp70S6k activity as shown in Fig. 5B. Pretreatment of cells with rapamycin completely inhibited EGF-induced pp70S6k.

Fig. 3. Effects of PI3K on cytosolic calcium mobilization and total PI turnover in mitogen-treated ASM cells. A: PI3K inhibition did not modulate agonist-induced calcium transients. Monolayers were pretreated for 10 min with 100 nM Wort and then stimulated with 1 U/ml of Thr. Cytosolic calcium measurements in fura 2-loaded ASM cells were performed as described in METHODS. Each tracing represents a cytosolic calcium transient from a single cell. Experiment is representative of 5 experiments. B: inhibition of PI3K did not modulate agonist-induced PI turnover in human ASM cells. Confluent, growth-arrested ASM cells were pretreated with 100 nM Wort for 10 min and then stimulated with either 1 U/ml of Thr, 10 ng/ml of EGF, or Cont. PI turnover was then measured after 30 min of agonist stimulation as described in METHODS. [3H]inositol 1–4-phosphate formation was compared with that obtained from cells treated with either Thr, EGF, 100 nM Wort, or Cont. Data are means ± SE from 3 experiments, each containing 3 replicates/condition. *P < 0.01 compared with Cont by ANOVA.
activation. The effect of rapamycin on EGF-induced pp70S6k activation was shown to be both time and concentration dependent (data not shown). Rapamycin also inhibited EGF- and thrombin-induced DNA synthesis in a concentration-dependent manner (IC50, 0.2 µM for EGF and 0.4 µM for thrombin), and 5 µM rapamycin blocked EGF- and thrombin-induced DNA synthesis by 99.1 ± 0.2 and 98.6 ± 2.5%, respectively (Fig. 5).

In separate experiments, the role of PtdIns 3-kinase in modulating mitogen-induced pp70S6k activation was examined. Pretreatment of the cells with wortmannin inhibited thrombin- and EGF-induced pp70S6k activation by 99.8 ± 0.92 and 99.8 ± 1.01%, respectively (Fig. 5D). Another PtdIns 3-kinase inhibitor, LY-294002, blocked thrombin- and EGF-induced pp70S6k activation by 96.4 ± 0.9 and 96.3 ± 1.4%, respectively (Fig. 5E). To further address whether PtdIns 3-kinase activation is sufficient to stimulate pp70S6k activation in HASM cells, transient transfection with p110* or Dp85 was performed. Expression was examined with an in vitro PtdIns 3-kinase activity assay and immunoblotting. Immunoprecipitation followed by immunoblotting revealed expression of anti-Myc-tagged p110* in HASM cells (Fig. 6A, top). Measured PtdIns 3-kinase activity in anti-Myc Tag immunoprecipitates demonstrated robust intrinsic PtdIns 3-kinase activity of expressed protein (Fig. 6A, bottom). As shown in Fig. 6B, expression of p110* activates pp70S6k in the absence of mitogen stimulation. Stimulation with EGF or thrombin had no additive effect on pp70S6k activation in cells expressing p110*. Expression of Dp85 blocked pp70S6k activation by EGF and thrombin. These results suggest that EGF and thrombin induce activation of pp70S6k in HASM cells and that mitogen-induced activation of pp70S6k is PtdIns 3-kinase dependent.

**DISCUSSION**

The principal finding of this study is that PtdIns 3-kinase activation is necessary for mitogen-induced HASM proliferation. α-Thrombin and EGF, both potent ASM cell mitogens, evoked comparable increases in cytosolic calcium, PI hydrolysis, and DNA synthesis and activated PtdIns 3-kinase, p42/p44 MAPK, and pp70S6k. The PtdIns 3-kinase inhibitors wortmannin and LY-294002 both abrogated mitogen-induced stimulation of PtdIns 3-kinase and ASM proliferation in a time- and dose-dependent manner. The specificity of these compounds was suggested by experiments demonstrating their failure to alter PI turnover and calcium-, cAMP-, and MAPK-regulated signaling. Last, a role for pp70S6k in PtdIns 3-kinase-mediated mitogenesis was suggested by effective inhibition of mitogen-activated pp70S6k activity by wortmannin and LY-294002 and by expression of Dp85.

PtdIns 3-kinase and its lipid products are pivotal signaling events in regulating various cellular functions including mitogenesis (8, 20, 22). PtdIns 3-kinase is required for DNA synthesis induced by some, but not all, growth factors. Microinjection of 3T3 fibroblasts with a neutralizing antibody to the p110 catalytic subunit of PtdIns 3-kinase completely inhibited PDGF- and EGF-induced mitogenesis (39). Stimulation of a PDGF-receptor mutant incapable of binding PtdIns 3-kinase could not induce DNA synthesis in a human hepatocyte cell line HepG2, and mitogenic signaling was restored when PtdIns 3-kinase binding sites were added back to the mutant (47). Microinjection of a monoclonal antibody against the NH2-terminal SH2 domain of the p85 regulatory subunit of PtdIns 3-kinase induced stimulation of DNA synthesis in Chinese...
Fig. 5. Growth factor-induced stimulation of pp70S6k is mediated by PI3K activation. A: growth-arrested ASM cells were treated for 10 min with 1 U/ml of Thr, 5 µM bradykinin (BK), 10% fetal bovine serum (FBS), or Cont. Monolayers were washed and lysed, and pp70S6k activity was measured as described in METHODS. Data are means ± SE from 3 separate experiments. *P < 0.05 vs. Cont by ANOVA. B: rapamycin (R) abolished EGF-induced pp70S6k activity. Confluent, growth-arrested cells were treated with 10 ng/ml of EGF, 50 nM R, R-EGF, or Cont. Monolayers were washed and lysed, and pp70S6k activity was measured. Data are means ± SE from 3 separate experiments. *P < 0.001 vs. Cont by ANOVA. C: R inhibited EGF- and Thr-stimulated DNA synthesis. Confluent, growth-arrested cells were treated with 10 ng/ml of EGF or 1 U/ml of Thr alone or in combination with 0.05, 0.5, or 5 µM R. Data are means ± SE from 4 experiments; each time point represents 6 replicates/experiment. D and E: PI3K inhibition abolished pp70S6k activation. ASM cells were pretreated for 10 min with 100 nM Wort (D) or 10 µM LY-294002 (E) and then stimulated with either 1 U/ml of Thr, 10 ng/ml of EGF, or Cont. Levels of pp70S6k activation in cells treated with Wort or LY-294002 and mitogen were compared with those treated with Thr or EGF alone. Data are means ± SE from 2–3 replicates from a representative experiment. *P < 0.001 vs. Cont by ANOVA.
hamster ovary cells, an effect blocked by coinjection of antibodies against pp70S6k or Ras or inhibition of MAPK (26). Expression of an inducible form of PtdIns 3-kinase in rat embryo fibroblasts demonstrated that activation of PtdIns 3-kinase is sufficient for cell cycle progression into the S phase (21). Other studies (4, 41, 42, 52) suggested a role for PtdIns 3-kinase in ASM and VSM cell proliferation. Wortmannin- and LY-294002-mediated inhibition of DNA synthesis was demonstrated in bovine ASM and porcine and rat VSM cells stimulated with PDGF, basic fibroblast growth factor, angiotensin II, and serum. Stimulation of \( \alpha_1 \)-adrenergic receptors with norepinephrine activated mitogenes, Ras, MAPK, and PtdIns 3-kinase in human VSM cells in a wortmannin-sensitive manner (17). In rat thoracic aorta VSM cells, wortmannin completely blocked angiotensin II-induced Ras activation but had no effect on MAPK activation and protein synthesis (45). These data suggest that in both ASM and VAM cells PtdIns 3-kinase is involved in mitogenic signaling induced by numerous agents.

The role of PtdIns 3-kinase activation in modulating cell proliferation is cell-type specific. In some cell types, bombesin and lysophosphatidic acid, which induce cell proliferation by activating receptors coupled to G proteins, stimulate cell growth in the absence of PtdIns 3-kinase activation (8, 39). Our data, however, suggest that thrombin, which induces HASM cell growth by activating a receptor presumably coupled to both G1 and Gq proteins (34), requires PtdIns 3-kinase activation to mediate its growth effects (35). Bradykinin, a contractile agonist that potently mediates bronchoconstriction in asthmatic patients, stimulates similar increases in PI turnover and cytosolic calcium comparable to those stimulated by either thrombin or EGF (34). However, bradykinin is not a HASM cell mitogen (34) and does not activate PtdIns 3-kinase. In bovine ASM, the mitogenic effects of PDGF or endothelin-1 have been attributed to their ability to stimulate PtdIns 3-kinase or pp70S6k (42). Taken together, these studies suggest that mitogens may activate different intracellular signaling pathways in a cell-specific manner. Our data demonstrate a role for PtdIns 3-kinase in mediating human smooth muscle cell proliferation induced by both growth factors and contractile agonists.

PIs are generated from two separate and distinct pathways. Activation of PI-PLC hydrolyzes PtdIns(4,5)P2 to Ins(1,4,5)P3 and diacylglycerol. Activation of PtdIns 3-kinase, which involves protein tyrosine kinase-mediated recruitment, phosphorylates PIs at the D3 position of the inositol ring and leads to the formation of PtdIns3P, PtdIns3(4)P2, and PtdIns3(4,5)P3 (43, 46). Recently, these phospholipids have been recognized as a new class of second messengers. Based on a number of studies (43, 46), PtdIns3(4,5)P3 appears to be the critical signaling 3-phospholipid. This assumption is supported by the time course of accumulation and the subsequent metabolism of the individual 3-PIs after agonist stimulation (12, 18). The 3-PIs are not substrates for any known PLC (8, 43, 49) and are not
components of the canonical PI turnover pathway. Moreover, their rapid accumulation on growth factor stimulation suggests that the lipids themselves act as second messengers mediating PtdIns 3-kinase mitogenic signals (8, 46). The recent finding that PtdIns 3-kinase, through its products (3-PIs), exerts control of the novel and atypical isoforms of PKC may have important implications in understanding the mechanisms that induce smooth muscle cell proliferation, insomuch as myocyte growth is thought to be PKC dependent (1, 25, 27, 31).

In our studies, numerous approaches were used to investigate the role of PtdIns 3-kinase in mediating ASM cell proliferation. We demonstrate that EGF and thrombin, which transduce their signals by activating distinct pathways associated with receptor tyrosine kinase and heterotrimeric G protein activation, respectively, both induce PtdIns 3-kinase activity in HASM cells. Ligand-induced PtdIns 3-kinase activity was similar to that observed when p110α was expressed in ASM. Wortmannin, a fungal metabolite that binds covalently to the 110-kDa subunit of PtdIns 3-kinase, and LY-294002, another PtdIns 3-kinase inhibitor, the site of action of which differs from that of wortmannin (54), both inhibited EGF-stimulated PtdIns 3-kinase activity. Nanomolar concentrations of wortmannin have been shown to inhibit PtdIns 3-kinase in numerous mammalian cell types (7, 20, 50). At these concentrations, wortmannin does not affect other PKs and lipid kinases, which include PtdIns 4-kinase (IC50 value of >100 μM), PI-PLC (IC50 value of 100 μM), or myosin light chain kinase (IC50 value of 1 μM) (7, 54). Our data suggest that wortmannin inhibited ASM cell proliferation with an IC50 value of 10 ± 3 nM. At this concentration, it is likely that the effects of wortmannin on ASM growth are specific for PtdIns 3-kinase. In addition, because wortmannin had no effect on agonist-induced calcium transients, this suggests that wortmannin does not inhibit PI-PLC activation in ASM cells at these concentrations. To further characterize the specificity of these inhibitors in ASM cells, we examined whether LY-294002 modulated EGF- or thrombin-induced ASM cell growth. LY-294002 also inhibited mitogen-induced DNA synthesis in ASM cells at concentrations that abolished PtdIns 3-kinase activation.

The specificity of wortmannin and LY-294002 for PtdIns 3-kinase in HASM cells was further supported by experiments examining their effects on p42/p44 MAPK activation and cAMP accumulation. p42/p44 MAPK activation, shown to be required for proliferation in numerous cell types including non-HASM cells, was not affected by maximal concentrations of either wortmannin or LY-294002. This finding also suggests that PtdIns 3-kinase activation by mitogens in HASM cells is of little consequence to p42/p44 MAPK activation (i.e., PtdIns 3-kinase does not regulate p42/p44 MAPK activity). cAMP accumulation and consequent activation of PKA has the potential to inhibit several promitogenic pathways in numerous cell types. We observed no significant stimulation of cAMP production by either wortmannin or LY-294002, suggesting that the inhibition of PtdIns 3-kinase activity or HASM cell proliferation by wortmannin or LY-294002 is not mediated by nonspecific activation of PKA.

In numerous cell types, PtdIns 3-kinase has been shown to be an important mediator of pp70S6k activation in response to serum and growth factors (9). pp70S6k, a critical enzyme for mitogen-induced cell cycle progression through the G1 phase and translational control of mRNA transcripts that contain a polyypiridine tract at their transcriptional start site (19, 24, 28), is phosphorylated and activated by PI-dependent kinase 1, a multifunctional effector of PtdIns 3-kinase (2, 38). In T cells, interleukin-2 activates p70S6k, this suggests that wortmannin inhibited ASM cell proliferation by stimulating PtdIns 3-kinase, which, in turn, activates pp70S6k. Inhibition of pp70S6k with rapamycin, an immunosuppressant, did not alter interleukin-2 activation of PtdIns 3-kinase but inhibited T-cell growth (9, 28). In another study (24), microinjection of neutralizing antibodies to pp70S6k completely inhibited serum-induced DNA synthesis in an embryonic fibroblast cell line. These data suggest that in some cell types activation of pp70S6k is necessary for cell progression from the G1 to the S phase of the cell cycle and that pp70S6k appears to be an essential downstream effector of PtdIns 3-kinase. The kinetics of pp70S6k activation suggest that this kinase may also be an attractive target for therapeutic interventions. Mitogen-induced activation of pp70S6k is essential for expression of early-immediate genes such as c-fos, and pp70S6k expression is required for the duration of the G1 phase of the cell cycle (24). Whether activation of pp70S6k is necessary to stimulate growth factor- or contractile agonist-induced smooth muscle cell proliferation remains unknown. Our data, however, suggest that EGF and thrombin significantly stimulate pp70S6k, and wortmannin, LY-294002, and rapamycin completely block this activation. Bradykinin, a contractile agonist that does not induce ASM cell growth, had no effect on pp70S6k activation. Moreover, overexpression of Δp85 abolished EGF and thrombin pp70S6k activation. Transient expression of p110α activates pp70S6k in the absence of stimulation with mitogens. Thus EGF and thrombin induce activation of pp70S6k in HASM cells and mitogen-induced activation of pp70S6k appears PtdIns 3-kinase dependent.

In summary, results from the present study demonstrate a requirement for PtdIns 3-kinase and pp70S6k activation in both EGF- and thrombin-stimulated HASM cell proliferation. Future studies are needed to identify features by which those signaling intermediates are regulated as well as the effect of PtdIns 3-kinase and pp70S6k activity on downstream signaling elements involved in HASM mitogenesis.

We thank Dr. A. Klippel for the constitutively active phosphatidylinositol 3-kinase (p110α) expression vector and Dr. M. Kasuga for the dominant-negative phosphatidylinositol 3-kinase (Δp85) expression vector. We also thank Mary McNichol for expert assistance in preparing this manuscript.

This work was supported by National Heart, Lung, and Blood Institute Grants HL-02647, HL-55301, and HL-58506; a Career Investigator Award from the American Lung Association; the National Aeronautics and Space Administration; The National Asthma...
REFERENCES


