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Platelet-Derived Growth Factor-BB and Thrombin Activate Phosphoinositide 3-Kinase and Protein Kinase B: Role in Mediating Airway Smooth Muscle Proliferation

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ABSTRACT

Proliferation of airway smooth muscle results from persistent inflammatory cytokine and growth factor stimulation and is a critical component of airway luminal narrowing in chronic asthma. Using primary cultures of bovine tracheal smooth muscle (BTSM) cells to examine the signaling basis of cell proliferation, platelet-derived growth factor (PDGF)-BB and thrombin (which act through distinct receptor types) were found to induce DNA synthesis in BTSM cells. Mitogen-induced DNA synthesis could be completely inhibited by LY294002, a selective phosphoinositide 3-kinase (PtdIns 3-kinase) inhibitor. Exposure of BTSM cells to PDGF-BB or thrombin resulted in rapid activation of PtdIns 3-kinase and accumulation of phosphoinositide-3,4,5-trisphosphate. Protein kinase B, a novel signaling protein kinase, was identified in BTSM cells and was activated by PDGF-BB and thrombin in a PtdIns 3-kinase-dependent manner; this may underlie mitogen-stimulated activation of $p70^{s6k}$. PD98059, a mitogen-activated protein kinase kinase 1 inhibitor, also partially inhibited PDGF-BB- and thrombin-stimulated DNA synthesis, indicating a modulatory role for mitogen-activated protein kinase in proliferation. GF109203X, Ro 31–8220, calphostin C, and chelerythrine (selective protein kinase C inhibitors) had no effect on PDGF-BB- or thrombin-stimulated DNA synthesis, suggesting that, despite abolishment of mitogen-stimulated protein kinase C activity, cell proliferation stimulated by PDGF-BB and thrombin is protein kinase C-independent. These data demonstrate that the PtdIns 3-kinase/protein kinase B pathway represents a key signaling route in airway smooth muscle proliferation, with the mitogen-activated protein kinase kinase 1/mitogen-activated protein kinase cascade providing a complementary signal required for the full mitogenic response.

Hypertrophy and hyperplasia of airway smooth muscle are major components of the structural changes that result in airway luminal narrowing in chronic asthma. This response is thought to occur as a consequence of inflammation and the subsequent release of cytokines and mitogens. Because airway resistance relates to the fourth power of the luminal radius, minor increases in the cell size and the number of airway smooth muscle cells in the bronchial walls of asthmatic patients have pronounced effects on airway resistance. In addition, mathematical modeling has demonstrated that an increase in smooth muscle mass is the most important component in reducing airflow in patients with chronic asthma (James *et al.*, 1989). Although a number of candidate pathways have been proposed, the signaling events that mediate airway smooth muscle proliferation have not been fully elucidated. Activation of the serine/threonine protein kinase p70 ribosomal S6 kinase family ($p70^{s6k}$) has been shown to be involved in protein and DNA synthesis in these cells (Scott *et al.*, 1996) and may explain in part the mitogenic effects of certain growth factors. In addition, growth factor-induced activation of PtdIns 3-kinase, which results in phosphorylation of phosphoinositide-4,5-bisphosphate to PtdIns$(3,4,5)P_3$, has been proposed to play an important role in DNA synthe-

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ABBREVIATIONS: PtdIns 3-kinase, phosphoinositide 3-kinase; BTSM, bovine tracheal smooth muscle; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MAP kinase, mitogen-activated protein kinase; MEK1 or -2, mitogen-activated protein kinase kinase 1 or 2; PDK-1, 3-phosphoinositide-dependent protein kinase-1; PtdIns$(3,4,5)P_3$, phosphoinositide-3,4,5-trisphosphate; ERK, extracellular signal-regulated kinase; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle medium; PDGF, platelet-derived growth factor; EGF, epidermal growth factor.

sis. Furthermore, because wortmannin, a PtdIns 3-kinase inhibitor, inhibits $p70^{s6k}$ activation by growth factors, PtdIns 3-kinase has been proposed to lie upstream of $p70^{s6k}$ in the proliferative pathway (Scott *et al.*, 1996).

More recently, protein kinase B has been identified as a novel signaling protein kinase that is activated in response to insulin and growth factors (Marte and Downward, 1997). Overexpression of a constitutively active form of protein kinase B was found to activate $p70^{s6k}$, and this response was sensitive to inhibition by rapamycin (Burgering and Coffer, 1995). Because protein kinase B activity can be inhibited by wortmannin, this again suggests that protein kinase B operates upstream of $p70^{s6k}$ and downstream of PtdIns 3-kinase.

Another pathway shown to be associated with growth in a variety of cell types is the MAP kinase cascade; activation of this pathway through Ras/Raf-1 stimulates phosphorylation of substrates such as $p90^{s6k}$ and c-*jun* and initiates protein synthesis (Malarkey *et al.*, 1995b). The MAP kinases, also termed ERKs, are 42- and 44-kDa serine/threonine kinases that are activated after phosphorylation by the dual-specificity protein kinase MEK1 (MAP or ERK kinase). Activation of MEK1, and a second isoform termed MEK2, occurs after phosphorylation on Ser218 and Ser222 by Raf-1 (Zheng and Guan, 1994).

A role for protein kinase C in airway smooth muscle proliferation has also been proposed, on the basis that selective inhibitors of this enzyme family could attenuate mitogen-induced proliferation of rabbit tracheal smooth muscle cells (Hirst *et al.*, 1995). Recently, a number of protein kinase C isoforms, namely protein kinase C δ, ϵ, η, and ζ, have been demonstrated to be activated by 3-phosphorylated phosphoinositides generated by the action of PtdIns 3-kinase (Nakanishi *et al.*, 1993; Toker *et al.*, 1994). A study in canine airway smooth muscle has identified the presence of protein kinase C βI, βII, δ, ϵ, θ, and ζ isoforms (Donnelly *et al.*, 1995). Most protein kinase C inhibitors do not, however, show substantial selectivity among protein kinase C isoforms; therefore, the possibility that these novel isoforms represent downstream targets of PtdIns 3-kinase remains to be addressed.

In this study, we have investigated the role of PtdIns 3-kinase in mitogen-induced BTSM cell proliferation and demonstrated that PDGF-BB and thrombin activate PtdIns 3-kinase, stimulate PtdIns(3,4,5)P_3 accumulation, and activate protein kinase B. We have also shown that the degree of activation of PtdIns 3-kinase achieved by PDGF-BB and thrombin correlates closely with their mitogenic efficacy and that 10-min exposure to these stimuli is sufficient to commit cells to DNA synthesis. Finally, inhibitors of PtdIns 3-kinase, in contrast to inhibitors of protein kinase C or MEK1, cause complete inhibition of proliferation. These data suggest that agonist-stimulated activation of the PtdIns 3-kinase/PtdIns(3,4,5)P_3/protein kinase B pathway represents a key route for initiating cell division in airway smooth muscle cells.

Materials and Methods

Cell culture. Bovine trachealis was obtained from the local abattoir. Small strips of trachealis muscle, dissected free of epithelium and connective tissue, were washed in DMEM containing penicillin/streptomycin (5 units/ml and 5 μg/ml, respectively) and amphotericin B (2.5 μg/ml) and were incubated in this medium with collagenase type IV (1 mg/ml) for 1 hr at 37°, with intermittent shaking. After addition of fetal calf serum, the muscle digest was filtered through gauze, and isolated tracheal smooth muscle cells were collected by centrifugation at 250 $\times$ *g* for 4 min. Cells were plated and cultured in supplemented DMEM containing fetal calf serum (10%, v/v). Cells from passages 3–9 were used for all experiments. Cells were made quiescent in DMEM containing fetal calf serum (0.5%, v/v) for 48 hr before experiments. The identity of the tracheal smooth muscle cells was confirmed by immunocytochemistry using a smooth muscle-specific, anti-α-actin, mouse monoclonal antibody (data not shown).

PCR amplification of PDGF-α and -β receptor mRNA. Total RNA was isolated by lysis of adherent cells with Trizol reagent and extraction, according to the instructions provided by the manufacturer (Life Technologies, Paisley, UK). A total of 2 μg of RNA was reverse-transcribed in 50 mM Tris·HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 10 units of RNase inhibitor, 0.1 mM deoxynucleotide mixture, 50 μg/ml $oligo(dT)_{11-18}$, with 200 units of murine leukemia virus reverse transcriptase, at 35° for 60 min. After first-strand cDNA synthesis, PCR amplifications were carried out in 25-μl volumes containing 10 mM Tris·HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 1 μM primers, 200 μM deoxynucleotide mixture, and 1 unit of *Thermus aquaticus* DNA polymerase and were performed as follows: 94° for 30 sec, 55° for 60 sec, and 72° for 30 sec for 35 cycles and then 72° for 5 min. The PDGF-α primers were 5′-AATAAGATCAAGAGTGGGTACAGG-3′ and 5′-TATGCCGATGTCGTCCATCATGTC-3′, PDGF-β primers were 5′-TGACCACCCAGCCATCCTTTC-3′ and 5′-GAGGAGGTGTTGACTTCATTC-3′, and glyceraldehyde-3-phosphate dehydrogenase primers were 5′-CCACCCATGGCAAATTCCATGGCA-3′ and 5′-TCTAGACGGCAGGTCAGGTCCACC-3′. The amplified products were subjected to electrophoresis on 1.5% agarose gels in 45 mM Tris-borate, 1 mM EDTA, were identified with a UV transilluminator after staining with ethidium bromide (0.5 μg/ml), and were photographed using a Polaroid DS-5 system.

[^{3}H]Thymidine incorporation. Confluent and quiescent cells were washed twice in serum-free DMEM, inhibitors and mitogens were added as indicated, and cells were incubated for an additional 24 hr. [^{3}H]Thymidine (0.1 μCi/ml) was added for the final 4 hr of the incubation. Cells were washed twice with phosphate-buffered saline, twice with trichloroacetic acid (5%, w/v), and twice with ethanol and were finally solubilized with NaOH (0.3 M). [^{3}H]Thymidine incorporation was determined by liquid scintillation counting.

Cell stimulation, immunoprecipitation, and PtdIns 3-kinase activity assay. Cells were incubated in serum-free DMEM before the addition of inhibitors and agonists, as detailed in the figure legends. Reactions were terminated by rapid aspiration of the medium, followed by two washes with phosphate-buffered saline and the addition of ice-cold lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10%, v/v, glycerol, 1%, v/v, Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 200 μM Na_3VO_4, 10 mM sodium pyrophosphate, 100 mM NaF). Insoluble material was removed from cell lysates by centrifugation, and PtdIns 3-kinase was immunoprecipitated using anti-p85 or anti-phosphotyrosine antibody complexed to Pansorbin (Calbiochem, Nottingham, UK) (Scott *et al.*, 1996). PtdIns 3-kinase activity in immunoprecipitates was assayed as previously described (Carter and Downes, 1993). Immunoprecipitates were suspended in 50 μl of assay buffer (100 mM HEPES, pH 7.4, 200 mM NaCl, 1 mM EGTA) and mixed with 20 μl of sonicated phosphatidylinositol and phosphatidylserine (3:1, 0.2 mg/ml). Reactions were initiated by the addition of [γ-^{32}P]ATP (10 μCi) and were incubated for 30 min at 30°. Reactions were terminated by the addition of 750 μl of chloroform/methanol/HCl (40:80:1, v/v/v) and phase partitioning achieved with the subsequent addition of 250 μl of chloroform and 250 μl of HCl (0.1 M). Phospholipids were washed with chloroform/methanol/HCl (47:48:3, v/v/v). ^{32}P-labeled phosphoinositide 3-phosphate was then quantified by thin layer chromatography using a solvent system

containing chloroform/methanol/ammonia/water (20:15:3:5, v/v/v/v); ^{32}P incorporation was determined by liquid scintillation counting.

PtdIns(3,4,5)P_3 accumulation. Cells grown to confluence in 100-mm cell culture dishes were made quiescent, washed twice with phosphate-free HEPES-buffered saline (20 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $MgSO_4$, 1 mM $CaCl_2$, 5 mM KCl), and labeled in the same buffer containing 2 mCi/ml [^{32}P]orthophosphate, for 3 hr at 37°. Cells were then washed twice with HEPES-buffered saline before addition of agonists, as detailed in the figure legends. Reactions were terminated by the addition of trichloroacetic acid (0.5 M), and lipids were extracted with chloroform/methanol/0.1 M HCl (4:8:3, v/v/v) in the presence of Folch fraction I phosphoinositides (0.5 mg/extraction). Dried lipids were deacylated with monomethylamine at 53° for 30 min, with intermittent shaking, and the resulting deacylated lipids were analyzed by strong anion exchange high performance liquid chromatography using a two-step $(NH_4)H_2PO_4$ (0.5 M)/water gradient, essentially as described by Carter and Downes (1993). Deacylated lipids were identified by comparison of retention times with those of ^3H-labeled phosphoinositide standards and relevant nucleotides; ^{32}P incorporation was determined by liquid scintillation counting.

Assay of protein kinase Bα activity. BTSM cells were lysed in buffer containing 50 mM Tris·HCl, pH 7.5, 0.1% (w/v) Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 10 mM sodium glycerophosphate, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 0.1% (v/v) 2-mercaptoethanol, 0.27 M sucrose, 1 μM microcystin-LR, and protease inhibitors (protease inhibitor cocktail; Boehringer Mannheim, Lewes, UK). Insoluble material was removed from cell lysates by centrifugation, and protein kinase Bα was immunoprecipitated by incubation at 4° with 2 μg of anti-protein kinase Bα antibody conjugated to 5 μl of Protein G-Sepharose. Immunoprecipitates were washed three times with 1 ml of 50 mM Tris·HCl buffer, pH 7.5, containing 0.1 mM EGTA, 0.1 mM EDTA, 0.1% (v/v) 2-mercaptoethanol, and 0.5 M NaCl and twice in the same buffer without NaCl. Kinase activity was assayed by incubation with peptide substrate (GRPRTSSFAEG, termed 'Crosstide') and [γ-^{32}P]ATP (0.5 μCi) for 10 min at 30° in a shaking water bath; the reaction was terminated by transferring 40 μl of the assay mixture to phosphocellulose P81 paper. The phosphocellulose paper, which binds the peptide substrate but not ATP, was washed five times with orthophosphoric acid (0.5%, v/v) and once with acetone and dried; ^{32}P incorporation was determined by liquid scintillation counting.

Assay of MAP kinase activity. After pretreatment with inhibitors and addition of mitogens as described in the figure legends, BTSM cells were lysed under conditions identical to those used for protein kinase B activity measurements; insoluble material was removed by centrifugation, and activity was immunoprecipitated using anti-p42 MAP kinase antibodies. After conjugation with Protein G-Sepharose, enzymatic activity was assayed using a specific MAP kinase substrate peptide derived from the EGF receptor (5 μg/incubation) and [γ-^{32}P]ATP (1 μCi), in a buffer containing 75 mM HEPES, pH 7.4, and 1.2 mM $MgCl_2$, for 30 min at 37°. Reactions were terminated and phosphorylated peptide was isolated as detailed for assays of protein kinase B activity.

Assay of cytosolic protein kinase C activity. BTSM cells were lysed in buffer containing 20 mM Tris·HCl, pH 7.4, 2 mM EDTA, 0.5 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 0.02% (v/v) Triton X-100, and protease inhibitors (protease inhibitor cocktail; Boehringer Mannheim), after pretreatment with inhibitors and mitogens. After centrifugation at 30,000 $\times$ *g* for 30 min at 4°, supernatants were removed, Nonidet P-40 (1%, v/v, final concentration) was added, and the mixture was shaken for 30 min at 4° to form the cytosolic fraction. Protein kinase C activity in the cytosolic fraction was partially purified on DE52 diethylaminoethyl cellulose matrix, with activity being eluted with lysis buffer containing 120 mM NaCl. Enzymatic activity was assayed in a buffer containing 10 mM $MgCl_2$, 1.2 mM $CaCl_2$, phospholipids (96 μg/ml phosphatidylserine and 6.4 μg/ml diolein), 1 μCi of [γ-^{32}P]ATP, and 1 mg/ml histone IIIs as substrate. Reactions were carried out at 30° for 30 min and were terminated by addition of buffer containing 10% (w/v) trichloroacetic acid, 10 mM sodium pyrophosphate, and 1 mM ATP plus bovine serum albumin (500 μg/reaction, added before vortex-mixing). Samples were filtered through phosphocellulose P81 paper and washed four times with buffer containing 5% (w/v) trichloroacetic acid and 10 mM sodium pyrophosphate; ^{32}P incorporation was determined by liquid scintillation counting.

Materials. Antibody to the p85 regulatory subunit of PtdIns 3-kinase (monoclonal anti-amino-terminal SRC homology 3 domain of p85) was purchased from TCS (Botolph Claydon, UK), and antibodies to phosphotyrosine (PY20) and p42 MAP kinase (ERK2) were purchased from Insight Biotechnology (Wembley, UK). [γ-^{32}P]ATP, [^{32}P]orthophosphate, [^3H]thymidine, and enhanced chemiluminescence reagents were purchased from Amersham International (Amersham, UK), secondary antibodies were obtained from SAPU (Carluke, UK), and antibody to smooth muscle α-actin was purchased from DAKO (Ely, UK). Trizol reagent, murine leukemia virus reverse transcriptase, and *T. aquaticus* DNA polymerase were purchased from Life Technologies (Paisley, UK), and the deoxynucleotide mixture was purchased from Pharmacia (St. Albans, UK). Protein kinase Bα peptide substrate (Crosstide) and antibodies to protein kinase Bα were generous gifts from Dr. D. Alessi and Prof. P. Cohen (University of Dundee, UK). Folch fraction I phosphoinositides were obtained from Sigma (Poole, UK). Protease inhibitor cocktail tablets were purchased from Boehringer Mannheim. All other reagents were of the highest purity commercially available.

Results

PDGF receptor expression in primary cultures of BTSM cells. PDGF is a dimer composed of A chains and B chains connected by disulfide bonds, and it exists in three isoforms, namely PDGF-AA, PDGF-AB, and PDGF-BB. PDGF receptors are composed of two subunits, namely α and β, that form dimeric receptors as $\alpha\alpha$, $\alpha\beta$, or $\beta\beta$ forms; ligand binding studies have demonstrated that $\alpha\alpha$ receptors bind all three PDGF isoforms, $\alpha\beta$ receptors bind PDGF-AB and -BB, and $\beta\beta$ receptors bind only PDGF-BB (Seifert *et al.*, 1989). Using reverse transcription-PCR of RNA derived from primary cultures of BTSM cells, we demonstrated the presence of both α and β chains of the PDGF receptor (Fig. 1). In subsequent experiments, PDGF-BB was used as an agonist with activity at all three PDGF receptor subtypes.

Induction of BTSM cell proliferation by PDGF-BB and thrombin. Confluent primary cultures of BTSM cells that had been made quiescent for 48 hr in DMEM containing 0.5% fetal calf serum were used to assess cell proliferation by measurement of [^3H]thymidine incorporation after agonist treatment. PDGF-BB and thrombin were used as mitogens, to allow comparison of the effects of growth factor receptor stimulation (which involves autophosphorylation of specific tyrosine residues) and G protein-coupled receptor stimulation (where activation occurs through a 'tethered-ligand' mechanism of action) (Vu *et al.*, 1991). As demonstrated in Fig. 2a, PDGF-BB (20 ng/ml) induced a 51.3 $\pm$ 1.0-fold increase and thrombin (1 unit/ml) induced a 13.3 $\pm$ 0.3-fold increase in [^3H]thymidine incorporation, compared with control values, after a 24-hr exposure. The potential role of PtdIns 3-kinase in mediating this response was studied initially by using the selective inhibitor LY294002 (Vlahos *et al.*, 1994). PDGF-BB- and thrombin-induced [^3H]thymidine incorporation was inhibited to control levels, in a concentration-dependent manner, by LY294002 (IC_{50}, 2.4 and 2.2 μM, respectively) (Fig. 2b).

Time course of mitogen-induced proliferation. A 'washout' protocol was used to assess the minimal period of agonist stimulation required to induce [^{3}H]thymidine incorporation; at each time point, PDGF-BB or thrombin was added to quiescent BTSM cells for varying times, the mitogen-containing medium was removed, and the cells were washed extensively with DMEM containing 0.5% fetal calf serum (quiescent medium) and then incubated for an additional 20 hr in fresh quiescent medium before assessment of [^{3}H]thymidine incorporation. The data presented in Fig. 3 demonstrate a rapid time-dependent increase in [^{3}H]thymidine incorporation and indicate that a 10-min exposure to PDGF-BB or thrombin is sufficient to induce proliferation equivalent to that observed after a 24-hr exposure to agonist. These data suggest that these cells are fully committed to undergo cell division after only a brief period of agonist exposure; hence, a rapid signaling mechanism underlies the proliferative response induced by these mitogens.

Time course of PtdIns 3-kinase activation. Because mitogen-induced BTSM cell proliferation was sensitive to inhibition by PtdIns 3-kinase inhibitors and BTSM cells could be committed to transition into the S-phase of the cell cycle by a rapid signaling mechanism, we examined the time course of PtdIns 3-kinase activation. In anti-p85 immunoprecipitates, PDGF-BB (20 ng/ml) and thrombin (1 unit/ml) induced time-dependent activation of PtdIns 3-kinase, which was maximal by 5 min for thrombin and by 10 min for PDGF-BB, with increases of 9 ± 1.2- and 3.5 ± 0.7-fold over control values, respectively (Fig. 4a). PDGF-BB- and thrombin-induced activation of PtdIns 3-kinase was sensitive to inhibition by LY294002 over a concentration range similar to that used for inhibition of [^{3}H]thymidine incorporation (IC_{50}, 1.7 and 1.9 μM, respectively) (Fig. 4b).

Time course of PtdIns(3,4,5)P_3 generation. Using [^{32}P]orthophosphate-labeled BTSM cells, we examined the generation of PtdIns(3,4,5)P_3, the lipid product of PtdIns 3-kinase activation, after cell stimulation with PDGF-BB or thrombin. Phosphoinositides were extracted from control and agonist-stimulated cells, deacylated to generate glycero-derivatives of phosphoinositide lipids, and analyzed by high performance liquid chromatography using a SAX 5 column (Whatman, Maidstone, UK) and an $(NH_4)H_2PO_4$/water two-step gradient. As shown in Fig. 4c, PDGF-BB (20 ng/ml) induced rapid generation of glycero-PtdIns(3,4,5)P_3 over 10 min, achieving an 8.8 ± 1.5-fold increase over control levels. Thrombin (1 unit/ml) induced a 4.1 ± 1.7-fold increase in glycero-PtdIns(3,4,5)P_3 levels, with maximal stimulation being observed at 5 min (Fig. 5).

Activation of protein kinase Bα in BTSM cells. Mitogen-induced BTSM cell proliferation was previously shown to occur through activation of $p70^{s6k}$ in a PtdIns 3-kinase-dependent manner (Scott *et al.*, 1996). Recently, protein kinase Bα has been proposed to act as a signaling intermediate between PtdIns 3-kinase and the activation of $p70^{s6k}$ after stimulation of cells with growth factors (Marte and Downward, 1997). Protein kinase Bα was identified by Western blotting in BTSM cells (data not shown) and was found to be 4.5 ± 0.7- and 3.7 ± 0.2-fold activated by PDGF-BB and thrombin, respectively, by 10 min (Fig. 5a). In addition, protein kinase Bα activity was found to be inhibited after pretreatment of BTSM cells with the PtdIns 3-kinase inhibitor LY294002, confirming that protein kinase Bα lies downstream from PtdIns 3-kinase in the proliferative pathway (Fig. 5b).

Role of MAP kinase in BTSM cell proliferation. To investigate a potential role for the MAP kinase pathway in mitogen-induced proliferation, we used the specific MEK1 inhibitor PD98059. The mechanism of action of PD98059 involves interaction with and inhibition of the inactive form of MEK1, the upstream activator of MAP kinase (Dudley *et al.*, 1995). Pretreatment of BTSM cells with 100 μM PD98059 inhibited PDGF-BB- and thrombin-induced [^{3}H]thymidine incorporation by 79.4 ± 4.5 and 82.9 ± 4.5%, respectively (Fig. 6a); however, PD98059-mediated inhibition of PDGF-BB-stimulated MAP kinase activity in these cells was maximal at a concentration of 10 μM (Fig. 6b), suggesting that PD98059 may have nonspecific effects on DNA synthesis and cell integrity at concentrations above 10 μM. Of note, pretreatment of BTSM cells with a maximally effective concentration of LY294002 (10 μM) had no significant effect on MAP kinase activation induced by PDGF-BB or thrombin (97.9 ± 1.2 and 98.3 ± 1.8% of control values, respectively) (Fig. 6b, *inset*), suggesting that the MAP kinase pathway is not a downstream target of PtdIns 3-kinase. In addition, PD98059 (10 μM) had no significant effect on PtdIns 3-kinase activation induced by PDGF-BB or thrombin (95.0 ± 6.8 and 98.2 ± 4.4% of control values, respectively) (Fig. 4b). Taken together, these data suggest that the PtdIns 3-kinase pathway and MAP kinase pathway act in a parallel manner.

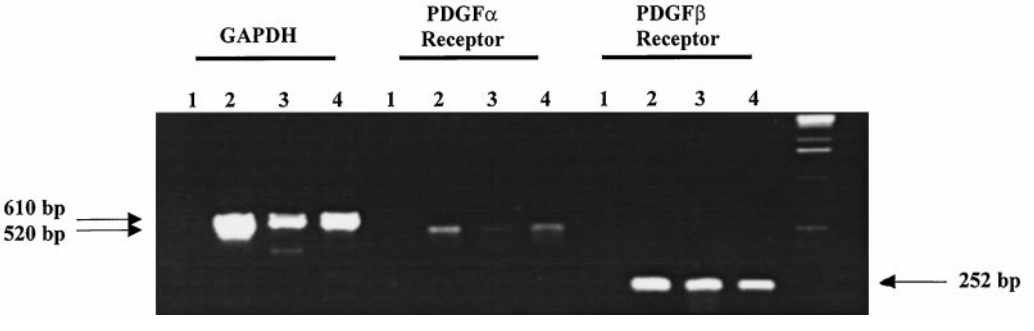

Fig. 1. Expression of PDGF-α and -β receptor subunits in BTSM cells. After reverse transcription-PCR with appropriate primers, amplified products were separated by electrophoresis on 1.5% (w/v) agarose gels, stained with ethidium bromide, and visualized using a UV transilluminator. Primers specific for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (610-base pair product) were used to amplify this 'housekeeping' gene product; the PDGF-α receptor (520-base pair product) and PDGF-β receptor (252-base pair product) were identified as indicated. Amplification was performed using cDNA originally derived from normal human articular chondrocytes (*lane 2*), LL24 fetal lung fibroblasts (*lane 3*), and primary cultures of BTSM cells (*lane 4*); negative controls containing no cDNA were included in each PCR amplification (*lane 1*).

Role of protein kinase C in BTSM cell proliferation. In a number of cell types, including airway smooth muscle cells, protein kinase C has been proposed to play a role in cell proliferation. Despite the identification of a number of isoforms of protein kinase C, with differing requirements for Ca^{2+} and phosphatidylserine, no inhibitors exist that are entirely selective for distinct protein kinase C isoforms, to enable the precise cellular roles for these isoforms to be established. Using purified baculovirus-expressed kinases, activation of certain protein kinase C isoforms has been demonstrated to occur in the presence of 3-phosphorylated phosphoinositides generated as a result of PtdIns 3-kinase activation. GF109203X, a bisindolylmaleimide that is reported to be a potent, selective, broad-spectrum, protein kinase C inhibitor whose action is mediated by competitive inhibition for ATP (Toullec *et al.*, 1991), was found to have no effect on either PDGF-BB- or thrombin-induced [^{3}H]thymidine incorporation, even at a maximally effective concentration of 1 μM (Fig. 7). Use of GF109203X at concentrations above 3 μM was found to induce cell detachment and necrosis (data not shown). Similar results were obtained with the protein kinase C inhibitor Ro 31–8220, which acts by a similar mechanism (data not shown). In addition, calphostin C (100 nM), an inhibitor of protein kinase C that acts at the regulatory domain, and chelerythrine (1 μM), which inhibits protein kinase C in a noncompetitive manner with respect to ATP, were both found to have no significant effect on PDGF-BB- and thrombin-induced [^{3}H]thymidine incorporation (97.3 ± 2.7 and 99.3 ± 1.4% of PDGF-BB control values and 98.1 ± 2.5 and 97.6 ± 3.4% of thrombin control values, respectively). Both PDGF-BB and thrombin were found to activate protein kinase C, as assessed in cytosolic extracts of BTSM cells using histone IIIs as substrate, and this activity was completely inhibited by GF109203X (1 μM) (Fig. 7b). These data suggest that protein kinase C does not play a major role in mediating or modulating mitogen-induced cell proliferation in primary cultures of BTSM cells.

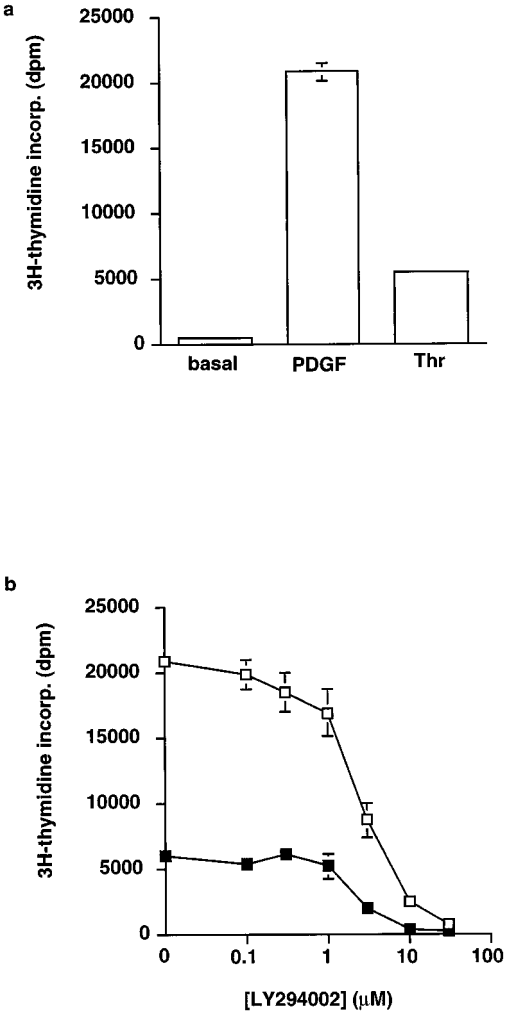

Fig. 2. DNA synthesis induced by PDGF-BB and thrombin in BTSM cells. a, [^{3}H]Thymidine incorporation was assessed in quiescent BTSM cells after exposure to PDGF-BB (20 ng/ml) (*PDGF*) or thrombin (1 unit/ml) (*Thr*) for 24 hr, as detailed in Materials and Methods. b, BTSM cells were treated with LY294002 for 20 min before addition of PDGF-BB (20 ng/ml) (□) or thrombin (1 unit/ml) (■). [^{3}H]Thymidine incorporation was assessed after 24 hr, as detailed in Materials and Methods; results are expressed as mean ± standard error of incorporation from an experiment that was performed in triplicate and was representative of three other experiments with similar results.

Discussion

In this study, we have demonstrated that PDGF-BB and thrombin are potent mitogens for primary cultures of BTSM cells and that a relatively short exposure to these agonists (<10 min) is sufficient to initiate a full mitogenic response. PDGF-BB and thrombin were used as agonists because both are known to act through a distinct receptor type; this would possibly allow us to determine whether a common key signaling pathway operates to mediate cell proliferation.

The ability of LY294002 to completely abolish PDGF-BB- and thrombin-induced [^{3}H]thymidine incorporation by BTSM cells suggested that PtdIns 3-kinase is a key regulator of this response. This finding was supported by the demonstration that these agonists caused rapid activation of PtdIns 3-kinase and generation of the second messenger PtdIns(3,4,5)P_3, the extent of which correlated closely with the magnitude of [^{3}H]thymidine incorporation. Importantly, this is one of the first direct observations that stimulation of a G protein-coupled receptor can activate a p85/p110 PtdIns 3-kinase in a nonhematopoietic cell type. Other studies examining G protein-coupled receptors demonstrated activa-

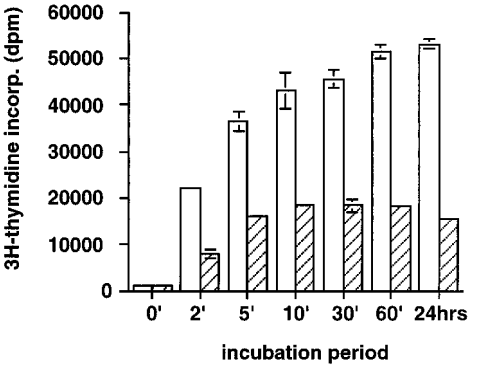

Fig. 3. Time course of mitogen-induced DNA synthesis in BTSM cells. BTSM cells were exposed to PDGF-BB (20 ng/ml) (□) or thrombin (1 unit/ml) (▨) for the times indicated and were then removed by washing the cells with quiescent DMEM containing 0.5% (v/v) fetal calf serum; [^{3}H]thymidine incorporation was assessed after an additional 24 hr. Results are expressed as mean ± standard error of incorporation from an experiment that was performed in triplicate and was representative of four other experiments with similar results.

tion of a novel $\beta\gamma$-sensitive form of PtdIns 3-kinase; in particular, thrombin was shown to activate this form of PtdIns 3-kinase in platelets, as was formyl-methionyl-leucyl-phenylalanine in neutrophils (Zhang *et al.*, 1995; Stephens *et al.*, 1997). A recent study by Belham *et al.* (1997) showed that thrombin-induced activation of $p70^{s6k}$ in pulmonary artery fibroblasts was inhibited by wortmannin but PtdIns 3-kinase activity was undetectable in antiphosphotyrosine immunoprecipitates. These observations suggested the involvement of a PtdIns 3-kinase isoform that was distinct from the activity found in antiphosphotyrosine immunoprecipitates from PDGF-treated cells. Wilson *et al.* (1996) demonstrated that lysophosphatidic acid activated $p70^{s6k}$ via a G protein-coupled receptor in Rat-1 fibroblasts and this activation could be abrogated by pretreatment with wortmannin; therefore, it was speculated that a $\beta\gamma$-sensitive form of PtdIns 3-kinase might mediate this activity.

A previous study with BTSM cells demonstrated that cell growth was mediated through activation of $p70^{s6k}$ in a PtdIns 3-kinase-dependent manner (Scott *et al.*, 1996). In addition, that study showed that rapamycin, a selective inhibitor of $p70^{s6k}$ activation, could completely inhibit BTSM cell proliferation, thus supporting the proposal that activation of $p70^{s6k}$ is a key step in the growth pathway.

Protein kinase B was originally identified as a product of the v-*akt* oncogene, from the acutely transforming retrovirus AKT-8 found in rodent T cell lymphoma cells (Bellacosa *et al.*, 1991). Reports that protein kinase B lies downstream from PtdIns 3-kinase and can associate with 3-phosphorylated phosphoinositides (James *et al.*, 1996) led to the characterization of two phosphorylation sites on protein kinase B, namely Thr308 and Ser473, which must both be phosphorylated for full activation (Alessi *et al.*, 1997a).

Burgering and Coffer (1995) reported that a constitutively active form of protein kinase B induced $p70^{s6k}$ activation but had no effect on MAP kinase. In addition, because both protein kinase B and $p70^{s6k}$ are known to lie downstream from PtdIns 3-kinase, this suggests that $p70^{s6k}$ activation may be mediated by protein kinase B. We demonstrated in this study that protein kinase B is activated in BTSM cells after stimulation with PDGF-BB and thrombin, both of which were shown to be potent mitogens and were able to induce rapid activation of PtdIns 3-kinase. More recently, PDK-1 was demonstrated to directly phosphorylate and activate $p70^{s6k}$ both *in vivo* and *in vitro* (Alessi *et al.*, 1997b). Phosphorylation of $p70^{s6k}$ *in vitro* was found to be independent of PtdIns(3,4,5)P_3, which contrasts with the phosphoinositide-dependent phosphorylation of protein kinase B by PDK-1. These observations suggest that PDK-1 is involved in a cooperative role to phosphorylate and mediate $p70^{s6k}$ activation.

Activation of the MAP kinase cascade is known to be involved in mitogenesis. Using PD98059, a specific MEK1 inhibitor, a role for the MAP kinase pathway in BTSM cell proliferation was investigated. At concentrations of PD98059 that completely inhibit MAP kinase activity, DNA synthesis was only partially inhibited. This suggests that MAP kinase is necessary, but not sufficient, for a full mitogenic response in these cells. These data are in accord with those reported by Malarkey *et al.* (1995a), who showed marked differences in the mitogenic potency of PDGF-BB and endothelin-1 in BTSM cells despite identical degrees of MAP kinase activa-

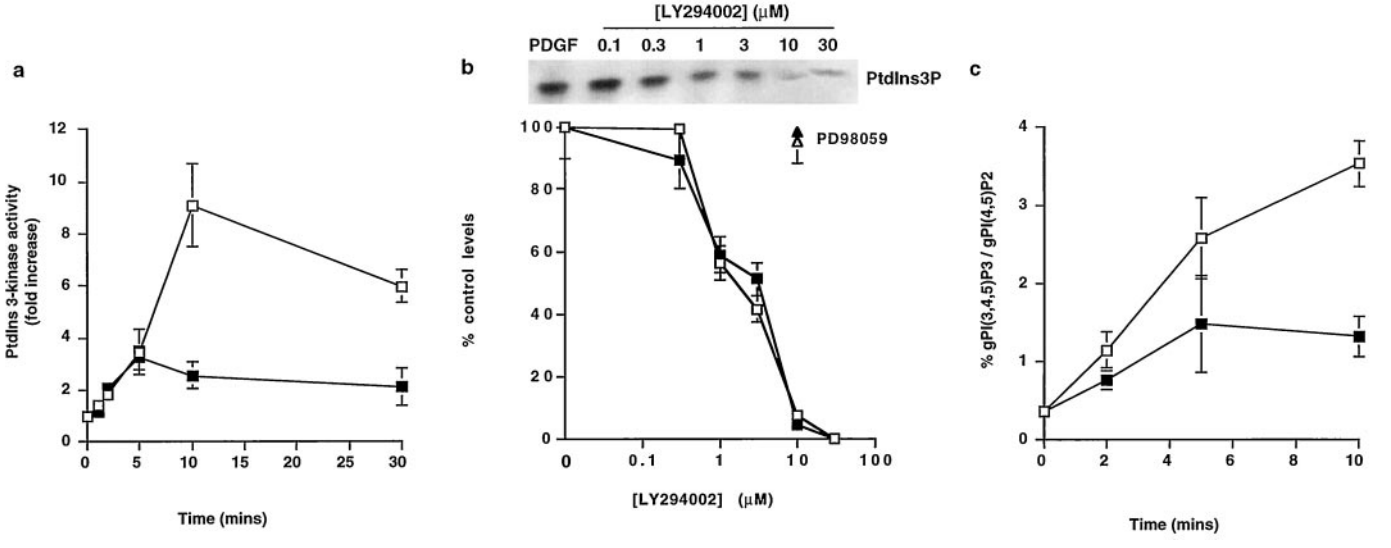

Fig. 4. PtdIns 3-kinase activation and PtdIns(3,4,5)P_3generation in mitogen-stimulated BTSM cells. a, BTSM cells were stimulated with PDGF-BB (20 ng/ml) (□) or thrombin (1 unit/ml) (■) for the times indicated. Cells were lysed and PtdIns 3-kinase was immunoprecipitated using an anti-p85 antibody; PtdIns 3-kinase activity was assayed using phosphoinositide as substrate. Results are expressed as mean ± standard error of the fold increase from three independent experiments performed in duplicate. b, BTSM cells were pretreated with LY294002 at varying concentrations, as indicated, for 20 min before addition of PDGF-BB (20 ng/ml) (□) or thrombin (1 unit/ml) (■) for 10 min. Alternatively, BTSM cells were pretreated with PD98059 (10 μM, 30 min) before addition of PDGF-BB (20 ng/ml) (△) or thrombin (1 unit/ml) (▲) for 10 min; these experiments were carried out in the absence of LY294002. Cells were assayed for PtdIns 3-kinase activity as described above; results are expressed as mean percentage of control values (PDGF-BB- or thrombin-treated cells, as appropriate) from a single experiment that was performed in triplicate and was representative of two others with similar results. An autoradiograph of phosphoinositide-3-phosphate (*PtdIns3P*), separated by thin layer chromatography, from a representative experiment is also shown (*top*). c, BTSM cells were labeled with [^{32}P]orthophosphate as detailed in Materials and Methods and were stimulated with PDGF-BB (20 ng/ml) (□) or thrombin (1 unit/ml) (■) for varying times, as indicated. Phosphoinositide lipids were extracted, deacylated, and analyzed by strong anion exchange high performance liquid chromatography. Results are expressed as mean ± standard error of the percentage of deacylated PtdIns(3,4,5)P_3/deacylated phosphoinositide-4,5-bisphosphate [*gPI(3,4,5)P3 / gPI(4,5)P2*] from three individual experiments; mean deacylated phosphoinositide-4,5-bisphosphate values were 386,809 cpm.

tion. In contrast, a report by Karpova *et al.* (1997) concluded that activation of MEK1 and the MAP kinase pathway was essential for PDGF-induced DNA synthesis. However, in this study even supramaximal concentrations of PD98059 did not attenuate PDGF-induced [^{3}H]thymidine synthesis to control levels; the pathway mediating this resistant component has not yet been elucidated. Mitogen-induced MAP kinase activation was unaffected by pretreatment with a PtdIns 3-kinase inhibitor, and PD98059 did not inhibit PtdIns 3-kinase activity; this suggests that in BTSM cells the MAP kinase and PtdIns 3-kinase pathways act in a parallel manner to induce proliferation. However, it is evident from our study that PtdIns 3-kinase is the principal regulatory pathway, with MAP kinase activation being required for a full mitogenic response. Interactions with PtdIns 3-kinase seem to vary among cell types and within cell lines, depending on the strength of the mitogenic signal. Duckworth and Cantley (1997) demonstrated that PDGF-induced MAP kinase activity was inhibited by wortmannin at low PDGF concentrations but wortmannin had no effect at maximal concentrations of PDGF, suggesting that PtdIns 3-kinase activates MAP kinase when small numbers of PDGF receptors are activated but a parallel pathway activates MAP kinase when most PDGF receptors are activated. Our study has focused on cell proliferation and its underlying signaling events induced by maximally effective concentrations of mitogens; the possibility remains, however, that a more complex interaction be-

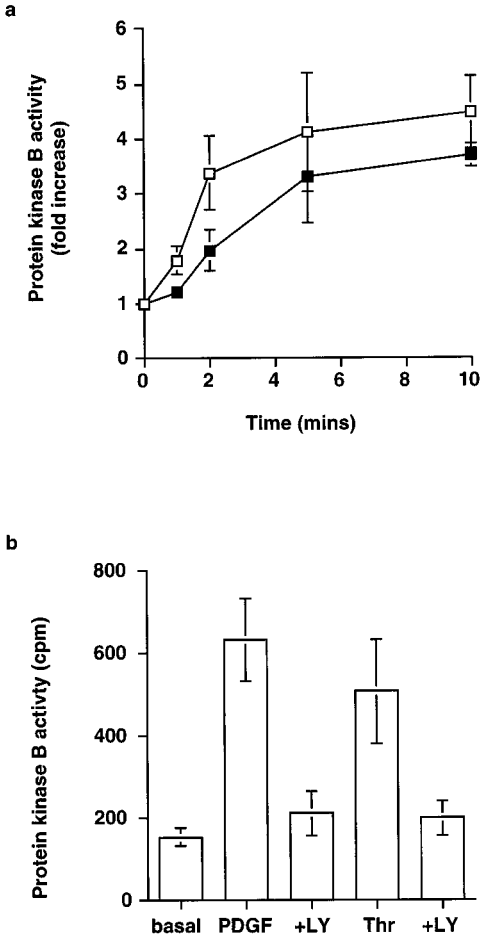

Fig. 5. Time course of protein kinase B activation in mitogen-stimulated BTSM cells. a, BTSM cells were stimulated with PDGF-BB (20 ng/ml) (□) or thrombin (1 unit/ml) (■) for varying times, as indicated. Cells were lysed, protein kinase B was immunoprecipitated, and activity was assayed using Crosstide as the substrate, as detailed in Materials and Methods. Results are expressed as the mean ± standard error of the fold increase above control values (untreated cells) from four experiments, each performed in duplicate. b, BTSM cells were pretreated with LY294002 (10 μM) (*+LY*) for 20 min before addition of PDGF-BB (20 ng/ml) (*PDGF*) or thrombin (1 unit/ml) (*Thr*) as indicated. Protein kinase B activity was assessed in immunoprecipitates as detailed above; results are expressed as mean ± standard error of activity from a single experiment that was performed in triplicate and was representative of two others with similar results.

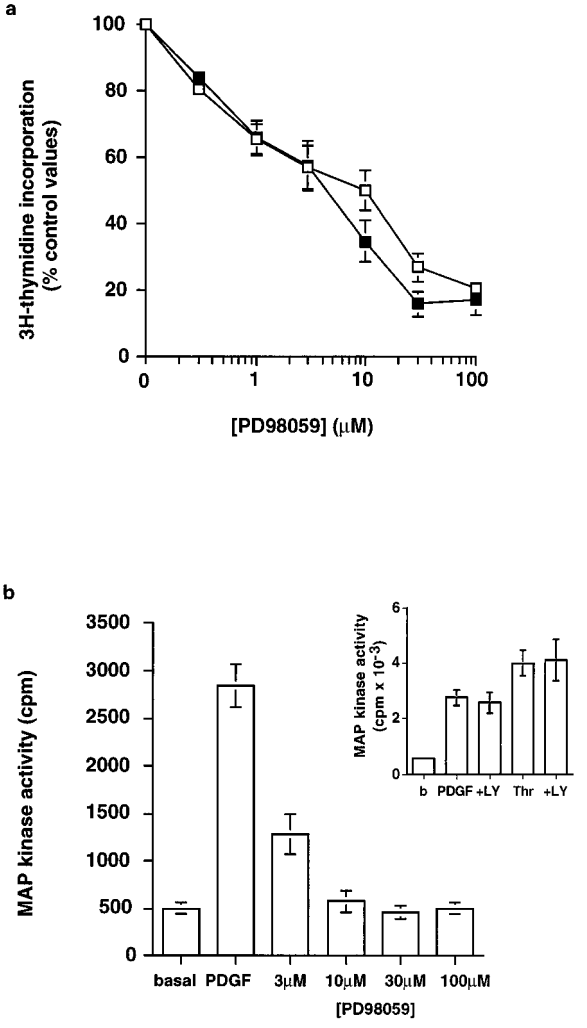

Fig. 6. Role of MAP kinase in mediating DNA synthesis in BTSM cells. a, BTSM cells were incubated with varying concentrations of PD98059 for 30 min before the addition of PDGF-BB (20 ng/ml) (□) or thrombin (1 unit/ml) (■), as indicated. [^{3}H]Thymidine incorporation was assessed after 24 hr, as detailed in Materials and Methods; results are expressed as mean ± standard error of the incorporation from five independent experiments, each performed in triplicate. b, BTSM cells were stimulated with PDGF-BB (20 ng/ml, 10 min) (*PDGF*) after preincubation with PD98059 at varying concentrations, as indicated. Cells were lysed, MAP kinase was immunoprecipitated using an anti-p42 MAP kinase antibody, and enzyme activity was assayed using an EGF receptor fragment as the substrate, as detailed in Materials and Methods. Results are expressed as mean ± standard error of the activity from a single experiment that was performed in triplicate and was representative of two others with similar results. *Inset*, BTSM cells were incubated with LY294002 (10 μM) (*+LY*) for 20 min before addition of PDGF-BB (20 ng/ml) (*PDGF*) or thrombin (1 unit/ml) (*Thr*), as indicated, and assessment of MAP kinase activity; results are expressed as mean ± standard error from three experiments performed in triplicate. *b*, basal.

tween signaling pathways may occur at submaximal mitogen concentrations.

We have demonstrated that DNA synthesis is unaffected by a maximally effective concentration of GF109203X, suggesting that mitogen-induced proliferation of BTSM cells is independent of protein kinase C. Similar results were also obtained with calphostin C and chelerythrine, which have different mechanisms of action, either targeting the regulatory domain of protein kinase C or acting as a noncompetitive inhibitor for ATP. Furthermore, it has been shown that pretreatment of BTSM cells with phorbol-12-myristate-13-acetate to down-regulate protein kinase C does not inhibit PDGF-induced [^{3}H]thymidine incorporation (Scott *et al.*, 1996), and it remains unlikely that protein kinase C is significantly involved in DNA synthesis in this tissue. Recently, preliminary data were presented indicating that transfection

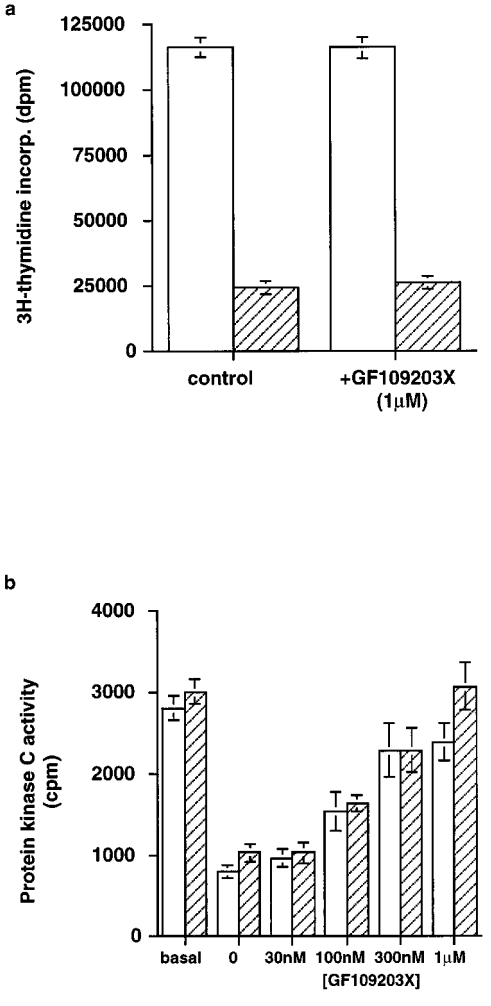

Fig. 7. Role of protein kinase C in mediating DNA synthesis in BTSM cells. a, BTSM cells were incubated with GF109203X (1 μM), as indicated, for 5 min before the addition of PDGF-BB (20 ng/ml) (□) or thrombin (1 unit/ml) (▨) as indicated. [^{3}H]Thymidine incorporation was assessed after 24 hr, as detailed in Materials and Methods; results are expressed as mean ± standard error of the incorporation from four independent experiments, each performed in triplicate. b, BTSM cells were stimulated with PDGF-BB (20 ng/ml) (□) or thrombin (1 unit/ml) (▨) after preincubation with GF109203X at the concentrations indicated. Cells were lysed and cytosolic protein kinase C activity was assayed as detailed in Materials and Methods. Results are expressed as mean activity from a single experiment that was performed in triplicate and was representative of two others with similar results.

of human airway smooth muscle cells with a dominant negative protein kinase C ζ inhibited PDGF-induced proliferation by approximately 80% (Black *et al.*, 1998). Hirst *et al.* (1995) also reported inhibition of serum-stimulated proliferation of rabbit tracheal smooth muscle cells by protein kinase C inhibitors. Donnelly *et al.* (1995) demonstrated the presence of conventional protein kinase C isoforms βI and βII, novel isoforms δ, ϵ, and θ, and the atypical isoform ζ in canine airway smooth muscle but did not address whether selective activation occurs after spasmogenic or mitogenic stimulation. The differences in results in these studies may reflect differential expression of protein kinase C isoforms or may indicate that there is differential regulation of these isoforms among species.

The ability of thrombin, acting through a G protein-coupled receptor, to activate PtdIns 3-kinase has been observed in only a limited number of cell types. In neutrophils, a novel form of PtdIns 3-kinase that is composed of a p101 regulatory subunit and a p120 catalytic subunit and is sensitive to activation by $\beta\gamma$ subunits derived from heterotrimeric G proteins has been identified (Stephens *et al.*, 1997). However, the finding that thrombin induced activation of PtdIns 3-kinase in anti-p85 immunoprecipitates suggests an alternative mechanism of activation. Such an effect could be explained by *trans*-activation of a growth factor receptor, as has been described by Daub *et al.* (1996) for Rat-1 fibroblasts transfected with EGF receptors. Agonists such as endothelin-1, lysophosphatidic acid, and thrombin induced tyrosine phosphorylation of EGF receptors in these cells in a manner similar to that observed after stimulation with EGF. Therefore, thrombin may act through tyrosine kinases to induce phosphorylation of growth factor receptors in BTSM cells and thus induce activation of a p85/p110 form of PtdIns 3-kinase. Chen *et al.* (1994) showed that thrombin, acting through its G protein-coupled receptor, induces activation of *src* and *fyn* in a lung fibroblast cell line, suggesting a possible mechanism of activation to initiate proliferation.

In conclusion, this study has demonstrated that BTSM cell DNA synthesis induced by two distinct mitogens is initiated by the rapid activation of PtdIns 3-kinase. The MAP kinase pathway contributes significantly to the proliferative response and is necessary for a full mitogenic response. This pathway, however, does not seem to be the key regulatory pathway that drives growth but seems to act in a parallel manner. Because proliferation in BTSM cells is known to be dependent on PtdIns 3-kinase-mediated p70^{s6k} activation (Scott *et al.*, 1996), the precise role of mitogen-induced protein kinase B activation in this response remains to be determined. The predominant involvement of receptor-mediated PtdIns 3-kinase activation in airway smooth muscle proliferation and the major consequences of this cellular response *in vivo* make this pathway an attractive therapeutic goal that could be targeted by the inhalation route.

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