A regulatory role for cAMP in phosphatidylinositol 3-kinase/p70 ribosomal S6 kinase-mediated DNA synthesis in platelet-derived-growth-factor-stimulated bovine airway smooth-muscle cells

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In bovine airway smooth-muscle cells platelet-derived growth factor (PDGF) and endothelin (Et-1) stimulate sustained and comparable activation of mitogen-activated protein kinase (MAP kinase) but display very different mitogenic efficacies, with PDGF inducing 50 times more DNA synthesis than Et-1. To examine additional signalling pathways which may be involved in this response, we investigated the role of phosphatidylinositol 3-kinase (PtdIns 3-kinase)/p70 ribosomal protein S6 kinase (p70^{S6k}) in mediating PDGF- and Et-1-induced mitogenesis, and whether inhibition of this pathway may underly the ability of cAMP to inhibit cell proliferation. PDGF stimulated an increase in PtdIns 3-kinase activity and a sustained 15-fold increase in p70^{S6k} activity that was abolished by both wortmannin and rapamycin. Et-1, however, stimulated only a 2-fold increase in p70^{S6k} activity

that was rapamycin-sensitive but wortmannin-insensitive. DNA synthesis stimulated by PDGF (50-fold) and Et-1 (2-fold) followed a similar pattern of inhibition. Pretreatment with phorbol ester did not affect p70^{S6k} activation in response to PDGF. Raising intracellular cAMP levels using forskolin, however, resulted in a marked time-dependent inhibition of p70^{S6k} activity, a decrease in the tyrosine phosphorylation of the PtdIns 3-kinase p85 subunit and reduced PtdIns 3-kinase activity. Forskolin also inhibited PDGF-stimulated DNA synthesis. These results suggest that PtdIns 3-kinase-dependent activation of p70^{S6k} may determine mitogenic efficacy of agonists that generate comparable MAP kinase signals. Negative regulation of PtdIns 3-kinase by cAMP may play an important role in the inhibition of airway smooth-muscle cell proliferation.

INTRODUCTION

Chronic asthma is characterized by gross structural changes in airway walls including marked hypertrophy and hyperplasia of airway smooth-muscle cells [1]. This accelerated growth and division possibly results from altered mitogenic responses to autocrine and/or paracrine peptide factors such as platelet-derived growth factor (PDGF) and endothelin (Et-1). An understanding of the mechanisms regulating this hyperactive cell division may lead to the development of novel anti-asthmatic therapies.

The mechanisms that control airway smooth-muscle cell growth have not been characterized fully, however, a number of intracellular signalling pathways are believed to play a role in the regulation of cell growth and division in response to stimulation of growth factors and G-protein-coupled receptors. Of particular importance is the activation of a series of kinase cascades involving multiple serine/threonine- and tyrosine-phosphorylation events [2]. One such pathway is the mitogen-activated protein kinase (MAP kinase) cascade [3,4]. MAP kinase stimulates the phosphorylation of several intracellular substrates believed to play a role in initiating mitogenesis, such as cytosolic phospholipase A₂, p90^{S6k} and c-Jun [5–7], and also stimulates glucose transport [8]. Thus MAP kinase may play a crucial role in regulating the entry of cells into the growth cycle [9–11]. In a number of cell types it has been proposed that the kinetics of

MAP kinase activation may dictate the relative efficacies of both growth factor and G-protein-coupled receptor agonists as mitogens [12,13].

Activation of the serine/threonine kinase p70 ribosomal protein S6 kinase family (p70^{S6k}) has also been shown to be involved in the regulation of both protein and DNA synthesis [14–15]. The S6 kinase family consists of two known isoforms, cytosolic p70^{S6k} and nuclear p85^{S6k}, referred to collectively as p70^{S6k} [16]. Injection of antibodies directed against both isoforms prevents the progression of cells from G₁- into S-phase [16]. A number of growth factors have been shown to activate p70^{S6k} and this may underlie their ability to act as mitogens. Activation of PtdIns 3-kinase resulting in the phosphorylation of PtdIns(4,5)P₂ on the D-3 position forming PtdIns(3,4,5)P₃ is also a feature of growth factor stimulation [17] and has been proposed to play an important role in the regulation of glucose transport and DNA synthesis [18,19]. The physiological roles of the D-3 phosphorylated lipids are unknown. Based on the ability of the PtdIns 3-kinase inhibitor wortmannin to inhibit p70^{S6k} activation, it has been suggested that PtdIns 3-kinase lies upstream of p70^{S6k} in the growth-factor-activated kinase cascade [20,21]. Information is limited, however, on the ability of growth factors to activate the PtdIns 3-kinase/p70^{S6k} pathway in smooth-muscle cell systems and the possible role of this pathway in cell division.

In a previous study we have shown that PDGF and Et-1 stimulate a comparable and sustained activation of MAP kinase.

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; Et-1, endothelin-1; MAP kinase, mitogen-activated protein kinase; p70^{S6k}, p70 ribosomal protein S6 kinase; PDGF, platelet-derived growth factor; PKC, protein kinase C; PtdIns 3-kinase, phosphatidylinositol 3-kinase; PMA, phorbol 12-myristate 13-acetate; IBMX, isobutylmethylxanthine; EGF, epidermal growth factor.

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However, their efficacies as mitogens are markedly different, with PDGF causing a 50-fold greater increase in [^3H]thymidine incorporation than Et-1, indicating a role for an additional pathway in the regulation of DNA synthesis [22]. In this study we provide clear evidence for a role of PtdIns 3-kinase-dependent activation of p70^{s6k} in the regulation of airway smooth-muscle cell proliferation in response to PDGF. This suggests that sustained activation of MAP kinase alone is not responsible for agonist-induced DNA synthesis in airway smooth-muscle cells which appears to be dependent on the concurrent activation of the PtdIns 3-kinase/p70^{s6k} pathway. Furthermore there is a clear indication that p70^{s6k} activity is negatively regulated by cAMP and that this site of inhibition is at or above the level of tyrosine phosphorylation of the p85 regulatory subunit of PtdIns 3-kinase. This finding may explain the ability of cAMP-raising agents to potentially inhibit growth-factor-stimulated DNA synthesis in airway smooth-muscle cells.

MATERIALS AND METHODS

Materials

Antibodies to phosphotyrosine, p85 α -subunit of PtdIns 3-kinase and MAP kinase were obtained from Affiniti Research Products Ltd. (Exeter, Devon, U.K.). The antibody to p70/85^{s6k} was purchased from Santa Cruz (Santa Cruz, CA, U.S.A.). Rapamycin was a gift from Professor Philip Cohen (Department of Biochemistry, University of Dundee, Dundee, Scotland, U.K.). S40 and protein kinase A inhibitor peptides were purchased from UBI. Secondary antibodies, ECL detection reagents, the BIOTRAKTM MAP kinase assay kit and [γ - ^{32}P]ATP (3000 Ci/mmol) were purchased from Amersham International (Amersham, Bucks., U.K.). Pansorbin was from Calbiochem (Nottingham, U.K.). [^3H]Thymidine (38 Ci/mmol) was from New England Nuclear. All general reagents were supplied by Sigma or BDH and were of the highest commercial purity available.

Cell culture

Bovine trachea were obtained from the local abattoir. Small strips of the trachealis muscle, dissected free of epithelium and connective tissue, were washed in Dulbecco's modified Eagle's medium (DMEM) containing 50 $\mu\text{g}/\text{ml}$ gentamicin and 2.5 $\mu\text{g}/\text{ml}$ amphotericin and incubated in the above medium containing 1 mg/ml collagenase type IV for 60 min at 37 °C with intermittent shaking. After the addition of foetal calf serum, the solution was filtered through gauze and the tracheal smooth-muscle cells collected by centrifugation at 250 g for 6 min. Cells were plated out and cultured in DMEM containing 10% foetal calf serum, 2 mM L-glutamine, 50 $\mu\text{g}/\text{ml}$ gentamicin and 2.5 $\mu\text{g}/\text{ml}$ amphotericin. Cells from passages 3–10 were used for all experiments. Cells were quiesced in serum-free medium containing 10 ng/ml insulin for 48 h before experimentation.

Cell stimulation and immunoblotting

Cells were incubated in serum-free DMEM for 30 min then stimulated with agonist or vehicle for the time periods indicated. The reactions were terminated by rapid aspiration of the buffer followed by three washes of the cell monolayer in ice-cold 20 mM Hepes buffer, pH 7.4, containing 150 mM NaCl, 50 mM NaF, 10 mM Na₄PO₇, 4 mM EDTA, 2 mM EGTA and 2 mM Na₃VO₄. Samples were then subjected to immunoprecipitation and assayed as outlined below.

Assay of PtdIns 3-kinase activity

After serum starvation and agonist stimulation, cells (10 cm culture dishes) were harvested in lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ aprotinin, 1 mM PMSF, 200 μM Na₃VO₄, 10 mM sodium pyrophosphate and 100 mM NaF). Insoluble material was removed from cell lysates by centrifugation, and PtdIns 3-kinase activity was measured essentially as described [23] in immunoprecipitates obtained using anti-phosphotyrosine antibody complexed to Pansorbin. Immunoprecipitates were suspended in 50 μl of assay buffer (100 mM Hepes, pH 7.4, 200 mM NaCl and 1 mM EGTA) and mixed with 20 μl of sonicated phosphatidylinositol and phosphatidylserine (both at 0.2 mg/ml). [γ - ^{32}P]ATP (50 μM ; 10 μCi) was added to start the reaction and the mixture was incubated for 30 min at 30 °C. Reactions were terminated with 600 μl of chloroform/methanol/conc. HCl (3:4:8, by vol.) and the phospholipids extracted twice in 500 μl of chloroform/methanol/conc. HCl/0.1 M HCl (2:1:2:2.5, by vol.). ^{32}P -labelled PtdIns-3-*P* was resolved by TLC in a solvent system containing methanol/chloroform/ammonia/water (11:7:2:2.5, by vol.), detected by autoradiography, and ^{32}P incorporation was quantified by liquid-scintillation counting of the corresponding TLC spots.

Assay of p70^{s6k} activity

Cells stimulated and washed as outlined above were solubilized in a 50 mM Tris/HCl buffer (pH 8.0) containing 120 mM NaCl, 20 mM NaF, 5 mM EGTA, 1 mM EDTA, 10 mM sodium pyrophosphate, 10 mM *p*-nitrophenyl phosphate, 1 mM benzamide, 0.1 mM PMSF and 1% (v/v) Nonidet P40 for 60 min at 4 °C (lysis buffer) [24]. Lysates were centrifuged at 1000 g for 2 min and equal quantities of the supernatant (500 μl , 150–200 μg protein) incubated with 2 μg of a polyclonal anti-p70/85^{s6k} antibody. After 90 min, the lysates were further incubated with 25 μl of Pansorbin. The immunoprecipitates were washed twice in lysis buffer and twice in the same buffer without detergents. For immunoblotting, Laemmli sample buffer was added to the precipitates, then the samples were boiled for 5 min and the supernatants retained for Western blotting. For *in vitro* kinase assay, immunoprecipitates were washed twice in 25 mM Hepes, pH 7.4, containing 20 mM β -glycerophosphate, 20 mM MgCl₂, 3 mM EGTA, 0.2 mM Na₃VO₄ and 2 mM dithiothreitol, and then incubated in the same buffer containing 100 μM S40 substrate peptide [25], 10 μM protein kinase A inhibitor peptide and [γ - ^{32}P]ATP (10 μM ; 2.5 μCi) in a volume of 30 μl for 20 min at 30 °C. The reaction was terminated by the addition of 10 μl of 300 mM H₃PO₄. The phosphorylated peptide was separated from other products by ion-exchange chromatography on P81 ion-exchange paper using 75 mM H₃PO₄ and quantified by liquid-scintillation counting.

MAP kinase activity

Bovine tracheal smooth-muscle cells grown in 75 cm² flasks were rendered quiescent as outlined above and incubated with agonists for the times given. The reactions were terminated by replacement of the buffer with ice-cold 150 mM NaCl containing 5 mM EDTA and 0.1 mM PMSF. The cells were scraped into 10 ml tubes and centrifuged for 5 min at 1000 g . The pellet was transferred to Eppendorf tubes and dispersed into 10 mM Tris/HCl buffer, pH 7.4, containing 150 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol, 1 mM Na₃VO₄, 1 mM PMSF,

10 μ g/ml leupeptin and 10 μ g/ml aprotinin by repeated aspiration through a 26G needle. The samples were centrifuged for 15 min at 25000 *g* and the supernatant was assayed for MAP kinase activity (BIOTRAK™ assay kit) by measuring the incorporation of [γ -³²P]ATP into a specific MAP kinase substrate peptide [the epidermal growth factor (EGF) receptor peptide] [26]. The reactions were carried out in 75 mM Hepes buffer, pH 7.4, containing 1.2 mM MgCl₂, 2 mM substrate peptide and [γ -³²P]ATP (0.12 mM; 1 μ Ci) in a volume of 30 μ l at 37 °C for 30 min. The phosphorylated peptide was separated by ion-exchange chromatography as for the p70^{s6k} assay.

[³H]Thymidine incorporation

Confluent and quiescent cells were washed twice in serum-free DMEM, agonists added and the cells incubated for a further 24 h. [³H]Thymidine (0.1 μ Ci/ml) was added for the final 4 h of the incubation. Cells were washed twice in PBS, three times in 5% trichloroacetic acid and twice with ethanol. Cells were solubilized in 0.3 M NaOH, and [³H]thymidine incorporation was assayed by liquid-scintillation counting.

Statistical analysis

Data are reported as means $\pm$ S.E.M. Statistical analysis was performed using Student's unpaired *t* test.

RESULTS

We first measured tyrosine phosphorylation of the p85 subunit of PtdIns 3-kinase and activity of the enzyme in response to PDGF. Figure 1 shows PtdIns 3-kinase activity measured in phosphotyrosine immunoprecipitates from PDGF-stimulated bovine airway smooth-muscle cells. Maximal recovery of tyrosine-

Figure 1 Wortmannin-sensitive PDGF-stimulated PtdIns 3-kinase activity in bovine airway smooth-muscle cells

Cells were stimulated with 20 ng/ml PDGF, and phosphotyrosine-containing proteins were immunoprecipitated from cell lysates with PY20 anti-phosphotyrosine antibody. In (A) immunoprecipitates were immunoblotted for p85 PtdIns 3-kinase subunit and in (B) and (C) immunoprecipitates were assayed for PtdIns 3-kinase activity as outlined in the Materials and methods section. In (C) cells were pretreated with 50 nM wortmannin (W) for 30 min before PDGF (P) stimulation (5 min). Each blot is a representative example from at least three independent experiments.

Figure 2 PDGF- and Et-1-induced activation of p70^{s6k} in bovine airway smooth-muscle cells

Cells were stimulated with 20 ng/ml PDGF (□) or 100 nM Et-1 (■) for the times indicated and p70^{s6k} protein was immunoprecipitated using a specific antibody. Cell immunoprecipitates were assayed for p70^{s6k} activity as outlined in the Materials and methods section. Results show a representative experiment; similar results were obtained in three separate experiments.

Table 1 Effect of rapamycin and wortmannin on Et-1- and PDGF-stimulated p70^{s6k} activity

Bovine airway smooth-muscle cells, pretreated for 15 min with 50 nM wortmannin or 30 nM rapamycin, were stimulated with Et-1 (100 nM) for 10 min or PDGF (20 ng/ml) for 15 min and assayed for p70^{s6k} activity as outlined in the Materials and methods section. Each value represents the mean $\pm$ S.E.M. of duplicate determinations from an independent experiment representative of three others.

Treatment	p70 ^{s6k} activity (pmol of P _i /min per mg of protein)
Control	0.034
PDGF	0.566 $\pm$ 0.004
PDGF + Wortmannin	0.040 $\pm$ 0.002
PDGF + Rapamycin	0.030 $\pm$ 0.000
Et-1	0.084 $\pm$ 0.004
Et-1 + Wortmannin	0.088 $\pm$ 0.008
Et-1 + Rapamycin	0.036 $\pm$ 0.001

phosphorylated p85 α occurred between 2 and 5 min of stimulation with PDGF (20 ng/ml) and maximal activity of PtdIns 3-kinase occurred between 5 and 10 min (Figures 1A and 1B). Et-1 failed to stimulate PtdIns 3-kinase phosphorylation or activity (results not shown). The PtdIns 3-kinase inhibitor wortmannin (50 nM) totally abolished PtdIns 3-kinase activity in response to PDGF stimulation (Figure 1C).

In bovine airway smooth-muscle cells p70^{s6k} activity was measured in response to PDGF and Et-1. PDGF (20 ng/ml) stimulated the activation of p70^{s6k} as assessed by *in vitro* kinase assay, reaching a maximum 15-fold activity over control at about 60 min (Figure 2). Activation of p70^{s6k} was time-dependent, reaching a plateau between 60 and 120 min (Figure 2). In contrast, Et-1 (100 nM) stimulated p70^{s6k} activity only 2-fold, with peak activity at 10 min. This activation was not sustained and returned to basal by 120 min. PDGF-stimulated p70^{s6k} activation was also concentration-dependent reaching a peak between 10 and 30 ng/ml (results not shown).

The effect of the PtdIns 3-kinase inhibitor wortmannin and the p70^{s6k} inhibitor rapamycin on PDGF- and Et-1-stimulated p70^{s6k} activity is shown in Table 1. Rapamycin (30 nM) inhibited both PDGF- and Et-1-stimulated p70^{s6k} activity by more than 95%

Figure 3 Time course of PDGF- and Et-1-stimulated *in vitro* MAP kinase activity in bovine airway smooth-muscle cells

Cells were incubated with 20 ng/ml PDGF (□) or 100 nM Et-1 (■) for the times indicated then assayed *in vitro* for MAP kinase activity as outlined in the Materials and methods section. Each value represents the mean $\pm$ S.E.M. of duplicate determinations from an experiment performed at least three times.

Table 2 Effect of rapamycin and wortmannin on PDGF- and Et-1-stimulated *in vitro* MAP kinase activity in bovine airway smooth-muscle cells

Cells were incubated with either 50 nM wortmannin or 30 nM rapamycin for 15 min before stimulation with Et-1 (100 nM) for 5 min or PDGF (20 ng/ml) for 10 min and then assayed *in vitro* for MAP kinase activity as outlined in the Materials and methods section. Each value represents the mean $\pm$ S.E.M. of duplicate determinations from an experiment performed at least three times.

Treatment	MAP kinase activity (pmol of P _i /min per mg of protein)
Control	3.16 $\pm$ 1.59
PDGF	19.6 $\pm$ 1.30
PDGF + Wortmannin	17.88 $\pm$ 2.19
PDGF + Rapamycin	16.97 $\pm$ 0.41
Et-1	22.77 $\pm$ 1.42
Et-1 + Wortmannin	21.36 $\pm$ 1.89
Et-1 + Rapamycin	22.09 $\pm$ 1.22

($P < 0.001$, $n = 3$). Wortmannin, however, was only effective against PDGF-stimulated activity, with an inhibition of more than 90% ($P < 0.001$, $n = 3$). Inhibition of PDGF-stimulated p70^{s6k} activity by both compounds was observed at both early (15 min, Table 1) and late (90 min) PDGF stimulation times (result not shown).

In order to check the specificity of the effects of wortmannin and rapamycin on activation of p70^{s6k}, MAP kinase activity was also measured. PDGF stimulated MAP kinase activity (Figure 3) up to the 90 min time period, reaching a peak of activity by 10 min stimulation. Et-1 stimulated MAP kinase activity in a similar manner to PDGF, reaching a peak of activity by 5 min and decreasing towards basal activity by 90 min (Figure 3). Pretreatment of the cells with either rapamycin or wortmannin had no effect on either the early or late phase of MAP kinase activation in these cells (Table 2). Similar results were obtained using both phosphotyrosine and MAP kinase immunoblotting (results not shown).

The effect of both rapamycin and wortmannin on PDGF- and Et-1-stimulated DNA synthesis was assessed using [³H]thymidine incorporation. PDGF stimulated an approximate 25-fold increase in [³H]thymidine incorporation over a 24 h period (Table

Table 3 Effect of wortmannin and rapamycin on mitogen-stimulated [³H]thymidine incorporation in bovine airway smooth-muscle cells

Cells were incubated with 50 nM wortmannin or 30 nM rapamycin for 20 min before addition of either 20 ng/ml PDGF or 100 nM Et-1. [³H]Thymidine incorporation was measured as outlined in the Materials and methods section. Each value represents the mean $\pm$ S.E.M. of triplicate determinations from a single experiment representative of at least three others.

Treatment	[³ H]Thymidine incorporation (d.p.m.)	Fold stimulation over control
Control	2042 $\pm$ 170	—
PDGF	50 627 $\pm$ 3538	25
PDGF + Wortmannin	6600 $\pm$ 745	3.2
PDGF + Rapamycin	3977 $\pm$ 130	1.9
Et-1	3416 $\pm$ 354	1.7
Et-1 + Wortmannin	2776 $\pm$ 158	1.4
Et-1 + Rapamycin	2028 $\pm$ 131	0

Table 4 Effect of PKC down-regulation on p70^{s6k} activity in bovine airway smooth-muscle cells

Cells were pretreated for 48 h with or without PMA (1 μ M) then stimulated with PDGF (20 ng/ml, 15 min) or Et-1 (100 nM, 5 min) and immunoprecipitated for p70^{s6k}. Immunoprecipitates were used to assay for p70^{s6k} activity as outlined in the Materials and methods section. The results are means $\pm$ S.E.M. of duplicate samples, from one experiment representative of three.

Treatment	p70 ^{s6k} activity (pmol of P _i /min per mg of protein)
Control	0.146 $\pm$ 0.013
PMA	0.133 $\pm$ 0.015
PDGF	1.390 $\pm$ 0.056
PDGF + PMA	1.160 $\pm$ 0.018
Et-1	0.487 $\pm$ 0.023
Et-1 + PMA	0.312 $\pm$ 0.011

3). In contrast, Et-1 stimulated a small (1.5–2-fold) increase in DNA synthesis comparable with previous studies [21]. PDGF stimulation of DNA synthesis was substantially (> 90%) reduced ($P < 0.001$, $n = 3$) by pretreatment of the cells with rapamycin or wortmannin (Table 3). These agents also inhibited DNA synthesis in response to other agonists such as EGF and the G-protein-coupled receptor agonist, thrombin (results not shown).

To explore the possibility that protein kinase C (PKC) may lie upstream of p70^{s6k}, activity of the enzyme was measured after PKC down-regulation. Tables 4 and 5 show the effect of a 48 h pretreatment with phorbol 12-myristate 13-acetate (PMA) on PDGF-stimulated p70^{s6k} activity and DNA synthesis respectively. PMA inhibited both PDGF- and Et-1-stimulated p70^{s6k} activity by only 10–15% (Table 4) whereas [³H]thymidine incorporation was enhanced by 20–30% (Table 5). Under these conditions, PMA pretreatment abolished PMA-stimulated [³H]thymidine incorporation in bovine airway smooth-muscle cells.

It has previously been demonstrated that raising intracellular cAMP levels inhibits interleukin-2-stimulated p70^{s6k} activity. The effects of raising cAMP were therefore investigated in bovine airway smooth-muscle cells. Immunoprecipitation of p70^{s6k} from PDGF-stimulated cells and subsequent Western blotting using a specific p70^{s6k} antibody demonstrates phosphorylation and a

Table 5 Effect of PKC down-regulation on DNA synthesis in bovine airway smooth-muscle cells

Cells were pretreated for 48 h with 1 μ M PMA (pre) then incubated for a further 24 h with either PMA (100 nM) or PDGF (20 ng/ml). [³H]Thymidine incorporation was determined as described in the Materials and methods section. The results are means $\pm$ S.E.M. of triplicate samples, from one experiment representative of three.

Treatment	[³ H]Thymidine incorporation (d.p.m.)
Control	2154 $\pm$ 119
PMA (pre)	2925 $\pm$ 219
PMA	8752 $\pm$ 163
PMA + PMA (pre)	3154 $\pm$ 246
PDGF	29157 $\pm$ 1468
PDGF + PMA (pre)	39338 $\pm$ 3393

Figure 4 Effect of forskolin pretreatment on PDGF-stimulated p70^{s6k} and PtdIns 3-kinase activity in bovine airway smooth-muscle cells

Cells were incubated with 10 μ M forskolin (F) plus 100 μ M isobutylmethylxanthine (IBMX) for 15 min before stimulation with PDGF (P; 20 ng/ml) for the times indicated. In (A) cells were assayed for p70^{s6k} as described in the Materials and methods section. In (B) phosphotyrosine immunoprecipitates were immunoblotted for p85 and in (C) phosphotyrosine immunoprecipitates were assayed for PtdIns 3-kinase activity. Each blot is representative of at least three others.

subsequent electrophoretic mobility shift of the p70 protein (Figure 4A). A further band at about p85 was also immunoprecipitated along with p70^{s6k}, but this was usually only identified after prolonged exposure. Pretreatment of airway smooth-muscle cells for 15 min with 10 μ M forskolin in the presence of IBMX (100 μ M) reduced PDGF-stimulated p70^{s6k} phosphorylation resulting in no shift of the p70^{s6k} protein bands (Figure 4A). This also coincided with the loss of activity of p70^{s6k} where pretreatment with forskolin abolished activity after both a 30 min stimulation with PDGF (control = 0.03 ± 0.00 , PDGF = 0.48 ± 0.01 , PDGF + forskolin = 0.04 ± 0.00 pmol of P_i/min per mg of pro-

Figure 5 Effect of forskolin post-treatment on PDGF-stimulated p70^{s6k} activity and DNA synthesis in bovine airway smooth-muscle cells

In (A) cells were treated with PDGF (20 ng/ml) for 30 min, then incubated with forskolin (10 μ M) in IBMX (100 μ M) for the indicated times ($\circ$). Cell immunoprecipitates were assayed for p70^{s6k} activity at these times as outlined in the Materials and methods section. p70^{s6k} activity was also measured in the absence of forskolin at the 120 min PDGF time point ($\square$). In (B) cells were stimulated with PDGF for a total period of 24 h. After the addition of the growth factor, forskolin (10 μ M) in IBMX (100 μ M) was added after the indicated times. [³H]Thymidine incorporation was measured as described in the Materials and methods section. Results are means $\pm$ S.E.M. of triplicate measurements from an experiment performed three times.

tein, $n = 3$) and 90 min PDGF time point (PDGF = 0.67 ± 0.03 , PDGF + forskolin = 0.03 ± 0.00 pmol of P_i/min per mg of protein, $n = 3$). This effect of forskolin was also observed with respect to the recovery of p85 in phosphotyrosine immunoprecipitates stimulated with PDGF (Figure 4B) and at the level of PtdIns 3-kinase activity (Figure 4C). PtdIns 3-kinase activity was significantly reduced by forskolin pretreatment: by more than 60% after 5 min PDGF post treatment and by more than 80% after 30 min ($P > 0.001$, $n = 3$). IBMX alone (concentrations up to 500 μ M) had no effect on p85 phosphorylation or activity of PtdIns 3-kinase (results not shown). Pretreatment of cells with forskolin did not affect total recovery of the p85 subunit of PtdIns 3-kinase as shown in p85 immunoprecipitates stimulated by PDGF (result not shown).

Post-treatment of bovine airway smooth-muscle cells with 10 μ M forskolin in the presence of IBMX (100 μ M) resulted in a time-dependent inactivation of p70^{s6k} (Figure 5A). The longer the post-treatment with forskolin, the greater the reduction in p70^{s6k} activity, with values at near control levels by 120 min. Addition of forskolin after PDGF stimulation also reduced the ability of PDGF (24 h, 20 ng/ml) to stimulate DNA synthesis (Figure 5B). Addition of forskolin up to 4 h after PDGF stimulation resulted in more than 95% abolition of [³H]thymidine

incorporation measured between 24 and 36 h post-PDGF addition ($P < 0.001$, $n = 3$). This ability of forskolin to inhibit PDGF-stimulated DNA synthesis was less marked when the forskolin was added 8–12 h after PDGF (Figure 5B).

DISCUSSION

In this study we sought to determine which intracellular signalling events regulate DNA synthesis in airway smooth-muscle cells. Previously, we have shown that although Et-1 and PDGF both induce a sustained activation of MAP kinase, the relative efficacies of these agents to stimulate [3 H]thymidine incorporation differs substantially [22]. In this study we demonstrate for the first time an obligatory role for a PtdIns 3-kinase/p70^{s6k} pathway in PDGF-mediated stimulation of DNA synthesis in airway smooth-muscle cells. PDGF stimulated a sustained activation of p70^{s6k} which was abolished by the macrolide, rapamycin. This compound binds to an intracellular receptor FKBP12 [27] forming an FKBP12–rapamycin complex that targets a rapamycin-associated protein (FRAP) [28]. The rapamycin–FKBP12 complex does not appear to interact directly with the kinase, but in yeast two related gene products, the targets of rapamycin genes (TOR1 and TOR2), which have sequence homology to mammalian PtdIns 3-kinase p110 catalytic subunit, have been shown to interact with the complex. It remains to be elucidated exactly how FKBP12–rapamycin and FRAP link the growth factor receptor to p70^{s6k} thus causing the inhibitory action on G₁-phase progression observed in mammalian cells [20]. Indeed we found that rapamycin also potently inhibited PDGF-stimulated DNA synthesis in airway smooth-muscle cells in response to PDGF and Et-1 as well as to EGF and thrombin (P. H. Scott and R. Plevin, unpublished work), indicating an obligatory role for this kinase pathway in response to a number of mitogens in this cell system.

Recent evidence has indicated a role for PtdIns 3-kinase in the regulation of p70^{s6k} in fibroblasts [20,29]. We found that wortmannin, which binds to the p110 catalytic subunit of PtdIns 3-kinase [30], inhibited both PDGF-stimulated PtdIns 3-kinase and p70^{s6k} activities in airway smooth-muscle cells. This correlated with the potent inhibitory effects of wortmannin on DNA synthesis, which occurred with an IC₅₀ value (10–20 nM) very similar to that reported for the inhibition of enzyme activity *in vitro* [20]. This suggests that, as in fibroblasts, PtdIns 3-kinase lies upstream of p70^{s6k} in PDGF-stimulated airway smooth-muscle cells. Et-1, which is a very weak mitogen in this system, failed to stimulate PtdIns 3-kinase in phosphotyrosine immunoprecipitates. It is possible, however, that a distinct isoform of PtdIns 3-kinase is being activated by Et-1 since a p110 PtdIns 3-kinase has recently been cloned that is activated by α - and $\beta\gamma$ -subunits of G-proteins [31]. The involvement of the low-molecular-mass G-protein Rho has also been implicated in the activation of PtdIns 3-kinase by the G-protein-coupled receptor agonists, thrombin and lysophosphatidic acid [32,33]. It is therefore possible that different isoforms of PtdIns 3-kinase are involved in Et-1 coupling to downstream events which are insensitive to wortmannin.

It has recently been proposed that the serine phosphorylation and activation of p70^{s6k} may involve PKC [34]. In smooth-muscle cells PDGF has been shown to activate PKC γ_2 and stimulate the formation of inositol phosphates [35]. Thus, activation of p70^{s6k} may involve a PKC-dependent pathway. Although conventional isoforms such as PKC ϵ and δ can activate PtdIns 3-kinase *in vitro* [36], we present evidence that PDGF-stimulated activation of p70^{s6k} is not mediated by con-

ventional PKC isoforms since pretreatment with PMA for 48 h failed to affect p70^{s6k} activation to any great extent. Consistent with this notion is the fact that Et-1, which induces very marked PKC activation in smooth-muscle cells, only stimulated a very small activation of p70^{s6k}. However, since one phorbol ester-insensitive isoform of PKC (PKC ζ) has been shown to be activated by PtdIns 3-kinase cellular products *in vitro* [37], and since PKC ζ expression is not affected by PMA pretreatment in airway smooth-muscle cells, we cannot discount the possibility that this isoform may be an upstream regulator of p70^{s6k}. Activity of the serine/threonine kinase protein kinase B (PKB, c-Akt), which has sequence homology to PKC, was recently shown to be mediated by PtdIns 3-kinase and appears to be involved in p70^{s6k} activation [38]. Since this kinase is also unaffected by phorbol ester treatment, PKB activation is a possible component of the growth-factor-stimulated PtdIns 3-kinase pathway in these cells.

It has recently been demonstrated that elevated cAMP-mediated inhibition of G₁ progression of interleukin-2-dependent T-cells correlated to inhibition of p70^{s6k} [34]. We also found that post-treatment of cells with forskolin, in the presence of IBMX, could abolish DNA synthesis when added within 8 h of PDGF stimulation. This may represent an inhibition of the cell cycle at G₁ through raised intracellular cAMP and subsequent inactivation of p70^{s6k} since our experiments also revealed that raising intracellular cAMP using forskolin strongly inhibited p70^{s6k} activity after forskolin post-treatment in response to PDGF. Previous reports have shown that IBMX alone partially inhibits p70^{s6k} activity and that other more potent phosphodiesterase inhibitors have a more pronounced effect [39]. Therefore it cannot be ruled out that p70^{s6k} phosphorylation and activation is, at least in part, being inhibited by IBMX. However, the site of inhibition by cAMP revealed by our experiments is different from that implicated in these studies. In our cells, PDGF-stimulated tyrosine phosphorylation of p85 was reduced after forskolin pretreatment, strongly implicating a site of inhibition at the level of the receptor–p85 interaction. Furthermore IBMX alone was not effective at inhibiting the phosphorylation and activation of PtdIns 3-kinase.

Activation of the PDGF receptor results in the association with adapter molecules which integrate signals from the receptor to downstream proteins, for example, GRB2 associates with p85 [40] and the *src* homology/collagen (Shc) adapter protein which has been shown to act as a docking protein for GRB2 [41] in PDGF-stimulated cells. Shc was also found to complex with a number of tyrosine-phosphorylated proteins including one of approximately 85 kDa. Preliminary results in our laboratory indicate that, although there is a low level of complex-formation between Shc and p85 and between GRB2 and p85 in our cells, this is not affected by raising intracellular cAMP. It would therefore appear that the effect of forskolin is at the level of p85 binding directly to the receptor or at an unknown adapter protein. Irrespective of the mechanism by which cAMP elicits its effect, this novel intervention of cAMP on growth-factor-stimulated PtdIns 3-kinase activation could adequately explain the potent inhibition of DNA synthesis in smooth-muscle cells in response to several classes of mitogen.

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