0026-895X/99/051079-08$3.00/0

MOLECULAR PHARMACOLOGY, **56:**1079–1086 (1999).

β2-Adrenergic Receptor Agonists and cAMP Arrest Human Cultured Airway Smooth Muscle Cells in the G_1 Phase of the Cell Cycle: Role of Proteasome Degradation of Cyclin D1

ALASTAIR G. STEWART, TRUDI HARRIS, DARREN J. FERNANDES, LESLIE C. SCHACHTE, VALENTINA KOUTSOUBOS, ELIZABETH GUIDA, CLAIRE E. RAVENHALL, PETER VADIVELOO, and JOHN W. WILSON

Department of Pharmacology, University of Melbourne, Parkville, Victoria, Australia (A.G.S., T.H., D.J.F., V.K., E.G., C.E.R.); Bernard O'Brien Institute of Microsurgery, St. Vincent's Hospital, Fitzroy, Victoria, Australia (L.C.S., P.V.); and Respiratory Medicine, Alfred Hospital, Prahran, Victoria, Australia (J.W.W.).

Received April 19, 1999; accepted July 30, 1999

This paper is available online at http://www.molpharm.org

ABSTRACT

Hyperplasia of airway smooth muscle (ASM) contributes to the airway hyperresponsiveness that characterizes asthma. We have investigated the relationship between cAMP-induced growth arrest of ASM cells and thrombin-stimulated, extracellular-regulated protein kinase (ERK) activity, cyclin D1, and the restriction protein retinoblastoma. The β_2-adrenergic receptor agonist albuterol (100 nM) inhibited DNA synthesis after incubation with ASM for periods as brief as 1 h when these coincided with the timing of the restriction point. Inhibition of thrombin-stimulated DNA synthesis by albuterol (1–100 nM), 8-bromo-cAMP (300 μM), or prostaglandin E_2 (1 μM) was accompanied by a reduction in cyclin D1 protein levels. The ERK kinase inhibitor PD98059 (3–30 μM) attenuated thrombin-stimulated ERK phosphorylation and activity and the increase in cyclin D1 protein levels, as did albuterol (1–100 nM) or 8-bromo-cAMP (300 μM). In contrast, neither albuterol (100 nM) nor PD98059 (30 μM) reduced cyclin D1 mRNA levels between 4 and 20 h after thrombin addition, which suggests that elevation of cAMP regulates cyclin D1 by a post transcriptional mechanism. The proteasome inhibitor MG132 (30 and 100 nM) and the calpain I inhibitor *N*-acetyl-Leu-Leu-leucinal (10 μM) attenuated the reduction in thrombin-stimulated cyclin D1 levels in ASM exposed to albuterol (100 nM), 8-bromo-cAMP (300 μM), or the phosphodiesterase inhibitor isobutylmethylxanthine (100 μM). Thus, the cAMP-induced arrest of ASM in the G_1 phase of the cell cycle is associated with a proteasomal degradation of cyclin D1 protein and a reduced protein retinoblastoma phosphorylation that prevents passage through the restriction point.

Asthma is a disease of chronic airway inflammation characterized by eosinophilic bronchitis, mast-cell activation, and T cell infiltration. In common with other chronic inflammatory diseases, the inflammation initiates tissue remodeling in the airways that has been documented in postmortem studies (Dunnill and Massarella, 1969) and by bronchial biopsy from living donors (Brewster et al., 1990). The remodeling involves: epithelial sloughing; marked infiltration of eosinophils and lymphocytes into the mucosa; activation of mast cells; enlargement of mucous glands, goblet cell metaplasia; deposition of wound-type collagen immediately below the true basement membrane of the epithelium and throughout the mucosa; and an increase in the number of myofibroblasts (Brewster et al., 1990). The volume of airway smooth muscle (ASM) increases because of hypertrophy and hyperplasia (Ebina et al., 1993). These airway wall structural changes explain much of the increase in airway responsiveness to inhaled bronchoconstrictors, because airway wall thickening amplifies increases in airways resistance caused by smooth muscle shortening (James et al., 1989).

Long-term changes in the asthmatic airway represent new targets for therapeutic intervention (Stewart et al., 1993). Consequently, there is interest in identifying the mechanisms for this airway wall remodeling response and the influence of existing antiasthma drugs on these processes. In cultured human ASM, β_2-adrenergic receptor agonists reduce the proliferative response to several mitogens, including thrombin and the thromboxane A_2 analog U46619 (Tomlinson et al., 1994). The action of β_2-adrenergic receptor agonists is partly dependent on cyclic AMP (cAMP), because: 1) it is accompanied by elevation of the levels of this second messenger in ASM (Tomlinson et al., 1995); 2) other agents

This work was supported in part by National Health Medical Research Council (Australia) and GlaxoWellcome (Uxbridge, UK).

ABBREVIATIONS: ASM, airway smooth muscle; cAMP, cyclic AMP; pRb, retinoblastoma protein; PGE_2, prostaglandin E_2; cdk, cyclin-dependent kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal regulated kinase; MEK1, extracellular signal regulated kinase kinase; DMEM, Dulbecco's modified Eagle's medium; PECAM-1, platelet endothelial cell adhesion molecule 1; SSC, standard saline citrate; DMSO, dimethyl sulfoxide; LLN, *N*-acetyl-Leu-Leu-norleucinal; IBMX, isobutylmethylxanthine; PI3K, phosphoinositol-3-kinase.

that elevate cAMP, such as vasoactive intestinal peptide (Maruno et al., 1995) and prostaglandin E_2 (PGE_2; Tomlinson et al., 1995), also inhibit ASM proliferation; 3) the membrane permeable analog of cAMP, 8-bromo-cAMP, mimics the response (Maruno et al., 1995; Tomlinson et al., 1995); and 4) inhibition of cAMP activation of protein kinase A attenuates the inhibition of proliferation (Maruno et al., 1995; Tomlinson et al., 1995). These actions of β-agonists seem to be independent of intracellular calcium levels (Tomlinson et al., 1995) or potassium channels (Gillzan and Stewart, 1997), which have been implicated in the intracellular signaling of cell-cycle progression in other cell types, but not in ASM (Noveral and Grunstein, 1994; Stewart et al., 1994).

The signals that enable passage of cells through the restriction point of the cell cycle, the point at which there is commitment to completing one round of DNA replication (Pardee, 1974), have now been identified (Herwig and Strauss, 1997). The under-phosphorylated form of retinoblastoma protein (pRb) binds to and represses the activity of the heterodimeric transcription factor complex, E2F. E2F controls expression of genes that must be expressed to allow cells to enter the S phase, including components of DNA polymerase. The phosphorylation of pRb reduces its affinity for and repression of the E2F complex, allowing expression of critical S-phase genes (Herwig and Strauss, 1997). Phosphorylation of pRb occurs through the activity of an activated complex of cyclin D1 and cyclin-dependent kinase 4 (cdk4). The activity of cdks is inhibited by $p27^{Kip1}$ and several other cdk inhibitors. In a number of cell types, agents that mimic cAMP suppress the synthesis of cyclin D1 (Cocks et al., 1992; Sewen et al., 1993). Inhibition of upstream events, such as *ras* activation of *raf*, which regulates activation of the mitogen-activated protein kinase (MAPK) family member extracellular regulated kinase (ERK), are considered to explain the regulatory effects of cAMP (Cook and McCormick, 1993). The prolonged activation of ERK is considered essential for passage from G_1 to S phase (Meloche et al., 1992) and for increased cyclin D1 levels (Lavoie et al., 1996). The proximal regulator of ERK, ERK kinase (MEK1), and ERK activity have been implicated in the progression of cultured bovine tracheal smooth muscle cells to cyclin D1 expression and S-phase (Ramakrishnan et al., 1998).

In this study, the point at which β_2-adrenergic agonists inhibit cell-cycle progression to S-phase has been investigated in cultured human ASM cells by with time course experiments. The effects of β_2-adrenergic agonists on the levels of cyclin D1 protein and mRNA have been examined to establish the potential role of these cell cycle regulatory proteins in the antiproliferative effects of the β_2-adrenergic receptor agonist albuterol and cAMP on ASM. ERK and MEK1 have been evaluated as targets for the regulatory effects of cAMP on cyclin D1 and pRb phosphorylation, with the MEK1 inhibitor PD98059 (Dudley et al., 1995). Our findings suggest that β_2-adrenergic receptor agonists and cAMP regulate cyclin D1 protein levels post-transcriptionally by an action on a proteasome-dependent degradation pathway.

Experimental Procedures

Cell Culture. ASM was dissected from macroscopically normal lung resection specimens obtained from lung transplant recipients and was provided by the Alfred Hospital (Melbourne, Australia). Cultures were prepared as described previously in detail (Tomlinson et al., 1995) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin-G, 2 μg/ml amphotericin B, and 0.25% BSA, w/v, which is referred to as serum-free DMEM. Cells were maintained in Falcon culture flasks and incubated (37°C; 5% CO_2) until monolayer confluence was reached. They were harvested weekly by 10-min exposure to 0.5% (w/v) trypsin, 1 mM EDTA in PBS and passaged at a 1:3 split ratio. Cells at passage numbers 3 to 15 were used for experiments.

Immunocytochemistry. Cells were subcultured into eight-well glass tissue culture chamber slides, grown to 100% confluence in DMEM (10% fetal calf serum), serum-deprived for 4 days, and then fixed and subjected to immunohistochemistry for α-actin, myosin, platelet endothelial cell adhesion molecule 1 (PECAM-1) and cytokeratin, as described previously (Vlahos and Stewart, 1999). The expression of smooth muscle α-actin and myosin was observed in all cultures used in this study. These cultures did not express detectable PECAM-1 staining and less than 5% of the cells were positive for cytokeratin. Paraffin-embedded sections of the airway adjacent to that used for generation of cultures stained positively for smooth muscle α-actin and myosin in bundles of ASM and blood vessels only. The antibody against PECAM-1 stained vascular endothelium, whereas that against cytokeratin stained only the epithelium, confirming the specificity of these antibodies for the target antigens.

DNA Synthesis. Cells were subcultured into 24-well plates at a 1:3 ratio at a density of approximately $1.5 \times 10^4/cm^2$ and allowed to grow to monolayer confluence over 72 to 96 h in an humidified atmosphere of 5% CO_2 in air at 37°C. The serum-containing media was replaced with serum-free DMEM for 24 h to produce growth arrest. In some experiments, the cells were pretreated with β_2-adrenergic receptor agonists or the cAMP analog 8-bromo-cAMP 30 min before the addition of mitogen, which was added to the appropriate wells in modified serum-free DMEM containing insulin (100 ng/ml), transferrin, (50 ng/ml), and selenium (1.5 pg/ml; specially formulated by CSL Ltd, Parkville, Australia) to provide progression factors that are essential for the mitogen activity. Mitogens and inhibitors were left in contact with cells from the time of addition until the end of the experiment unless indicated otherwise. Cells were incubated for 24 h (37°C; 5% CO_2) before being pulsed with [^{3}H]thymidine (1 μCi/ml for 4 h) to measure incorporation of radiolabeled material into newly synthesized DNA, according to our previous study (Stewart et al., 1997).

Western Analyses of Cell Cycle Regulatory Proteins. Experiments to determine the effects of β_2-adrenergic receptor agonists or cAMP analogs on cell cycle regulatory proteins were carried out on cells grown to confluence in six-well plates ($\sim$ 9 cm^2), using 25- or 75-cm^2 flasks according to the requirement for lysate protein for Western blotting. These cells were rendered quiescent as described for experiments on DNA synthesis and pretreatment and mitogen exposure were carried out under identical conditions. At the end of the incubation period (usually 20 h), the cells were washed three times in ice-cold PBS (1 ml/10 cm^2) and extracted into a lysis buffer (100 mM NaCl; 10 mM Tris; 2 mM EDTA; 0.5% sodium deoxycholate, w/v; 1% triton X-100, v/v; 1 mM PMSF; 10 mM $MgCl_2$; 100 IU/ml aprotonin; pH 7.5) an aliquot of which was removed for protein assay (Biorad reagent; Biorad, Sydney, Australia). The final concentration of Triton X100 in samples for assay of 0.05% was below the level at which the assay accuracy may be affected (0.1%, according to BioRad instruction booklet). Moreover, the coefficient of variation of duplicate protein assays was 6.8 $\pm$ 0.6% (n = 90 duplicate estimates in four different assays). The samples were resolved on polyacrylamide gel electrophoresis and Western-blotted for phospho-ERK, cyclin D1, and pRb according to methods described previously (Fernandes et al., 1999). To visualize the antigen, enhanced chemiluminescence reagent (Amersham, Cardiff, UK) was added for 1 min and the membrane was then apposed to X-ray film (Kodak X-omat AR; Eastman-Kodak, Rochester, NY) for variable periods of time (0.5–4 min)

before development. X-ray films were subject to densitometry with a Molecular Dynamics Personal Densitometer (Sunnyvale, CA), and the volumes were normalized to thrombin-induced levels of cyclin D1.

Assay of ERK Activity. ERK activity was determined by immunoprecipitation by using a specific anti-ERK antibody (goat polyclonal IgG; C-16, Santa Cruz, CA). Cells were seeded onto six-well plates as described previously, grown to confluence, then serum-starved for 24 h. The cells were incubated for 30 min with PD 98059 (30 μM) as indicated, and all cells were incubated with thrombin in serum-free DMEM for 5 min, 2 h, or 20 h. At the end of the stimulation period, the cell lysates were assayed for ERK activity as described previously in detail (Fernandes et al., 1999).

Northern Blot Analyses. Cells were seeded into 75-cm^2 flasks at a density of 1.5×10^4 cells/cm^2, grown to confluence, serum-starved for 24 h as described previously, and stimulated for between 4 and 20 h with thrombin. Total RNA was extracted with 1 ml of Trizol reagent (Gibco BRL, Melbourne, Australia) according to the manufacturer's instructions. The mRNA was isolated from 75 μg of total RNA by using Dynabeads oligonucleotides $(dT)_{25}$ (Dynal, Oslo, Norway) according to the manufacturer's instructions and was separated on a 1.2% formaldehyde denaturing gel and transferred to Immobilon-Ny$^+$ nylon membranes (Millipore, Bedford, MA) by using 20× standard saline citrate (SSC). Cyclin D1 mRNA was detected by Northern hybridization to a 440-base-pair human cyclin D1 cDNA probe (Xiong et al., 1991) labeled with [α-^{32}P]dCTP (Megaprime labeling kit; Amersham). The membranes were hybridized overnight at 65°C, washed twice with 2× SSC and 0.1% SDS at 55°C (30 min), once with 1× SSC and 0.1% SDS at 55°C (30 min), and exposed to autoradiography film (Hyperfilm MP; Amersham). The autoradiographs were quantitated by using a Molecular Dynamics Personal Densitometer. The membranes were also probed for Tubulin by using a 200-base-pair cDNA probe generated by reverse transcription-polymerase chain reaction (with 5′- CCTGGAACCCACAGTTATTGATGAAGAAGTTCG-3′ and 5′-AGAAGCCCTGGAGACCCGTGCACTGGTCAG-3′ primers) and hybridized as described above. To control for loading differences, cyclin D1 mRNA levels were normalized against the levels of tubulin mRNA.

Preparation of Reagents. PD 98059, initially dissolved in 100% DMSO (BDH, Dorset, UK) at 50 mM, was diluted 1 in 5 with DMSO to produce a solution of 10 mM. The final concentration of 30 μM PD 98059 in medium resulted in a final concentration of 0.3% DMSO. In experiments with PD 98059, a vehicle control incubation of 0.3% DMSO was used. Growth factors were prepared in BSA (0.25% w/v in PBS).

Statistical Analyses. When measuring [^{3}H]thymidine incorporation, each treatment in an individual experiment was carried out in quadruplicate. Each experiment was performed in at least three different cultures obtained from three different individual samples. Results are presented as grouped data from multiple cultures and are expressed as mean ± S.E. of n cultures. Fold increments were calculated by dividing the response of treated wells by that of the control wells on the same 24-well plate. The grouped percentage data were normalized by log transformation, before analysis by ANOVA, followed by post hoc tests when differences were detected. Differences were considered to be significant when $P < .05$. IC_{50} values were calculated from linear regression of log-concentration response data.

Materials. All chemicals used were of analytical grade or higher. The compounds used and their sources were as follows: Calpain I inhibitor (*N*-acetyl-Leu-Leu-methioninal), L-glutamine, essentially fatty-acid–free BSA fraction V, albuterol, and thrombin (bovine plasma) were from Sigma Chemical Co. (St Louis, MO); collagenase type CLS 1 and elastase were from Worthington Biochemical (Freehold, NJ); Dulbecco 'A' PBS was from Oxoid (Hampshire, UK); trypsin, versene, penicillin-G, streptomycin, and serum-free DMEM were from CSL (Melbourne, Australia); fetal calf serum and amphotericin B (Fungizone) were from Flow Laboratories (Stanmore, Australia); DMEM was from Flow Laboratories (Irving, UK). PD98059 and phospho-specific p42/p44 ERK kinase antibody kit (rabbit polyclonal IgG, 1:1000) were from New England Biolabs (Beverly, MA). MG132 (Carbenzoxyl-L-leucyl-L-leucyl-L-leucinal) was from Calbiochem-Novabiochem GmBH (Usztweg, Bad Seden, Germany). [6-^{3}H]thymidine (185 GBq/mmol, 5 Ci/mmol) and [γ-^{32}P]ATP (2 mCi/ml) were from Amersham; rabbit polyclonal IgG anti-pRb and goat polyclonal anti-ERK were from Santa Cruz Biotechnology (Santa Cruz, CA); Microscint-O scintillant was from Canberra-Packard (Canberra, Australia). Murine monoclonal anticyclin D1 was from Upstate Biotechnology (Lake Placid, NY). The antibodies used for immunocytochemistry were anti-smooth muscle α-actin (mouse monoclonal) (DAKO M851) and monoclonal mouse anti-PECAM-1 (DAKO-CD31, JC/70A) (DAKO M823) from the DAKO Corporation (Carpinteria, CA); sheep antimouse Ig HRP-conjugated and sheep antirabbit Ig HRP-conjugated were from Silenus (Hawthorn, Australia); murine monoclonal anticytokeratin (CY90) was from Sigma; and anti-smooth muscle myosin (rabbit polyclonal) was provided by Prof. M. Sparrow (University of Western Australia, Perth, Australia).

Results

Inhibition of Thrombin-Stimulated DNA Synthesis by β_2-Adrenergic Receptor Agonists, 8-Bromo-cAMP and PGE_2. Thrombin (0.3 U/ml) increases incorporation of [^{3}H]thymidine, measured in the last 4 h of a 28-h incubation, and increases cell number when the incubation period is extended beyond 48 h (Tomlinson et al., 1994; Tomlinson et al., 1995; Stewart et al., 1997). Albuterol, 8-bromo-cAMP, or PGE_2 continuously incubated with serum-deprived ASM from 30 min before exposure to thrombin (0.3 U/ml) and then throughout the 28-h incubation, inhibited [^{3}H]thymidine incorporation (Fig. 1) in a concentration-dependent manner. Concentrations of albuterol, 8-bromo-cAMP, or PGE_2, (100 nM, 300 μM, and 1 μM, respectively) were chosen for further studies.

Time Course of Action of Albuterol. In quiescent (G_0) ASM cells, thrombin stimulates DNA synthesis with a delay of 20 to 22 h, defining the duration of G_1. Albuterol inhibits S-phase entry when added as late as 18 h after the addition

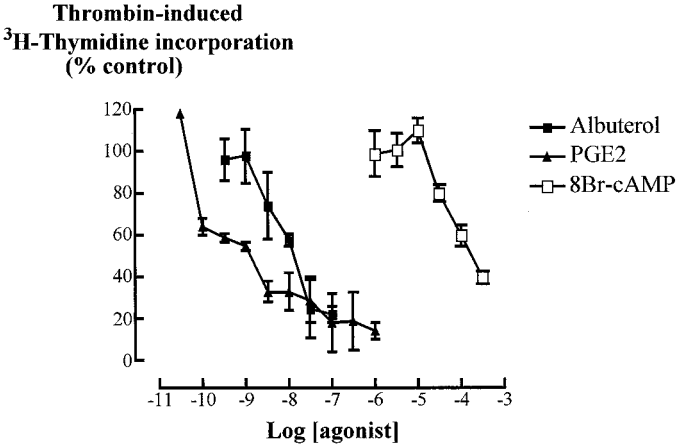

Fig. 1. Concentration-dependent inhibition of thrombin (0.3 U/ml)-induced [^{3}H]thymidine incorporation by albuterol, PGE_2, and 8-bromo-cAMP. Each data point represents the mean of data from at least three different cultures, each incubation in each culture having been carried out in quadruplicate. In this series of experiments, the mean and S.E.M. of the quadruplicate values for [^{3}H]thymidine incorporation (dpm) under basal conditions were 1,955 ± 237, 2,470 ± 191, and 3,729 ± 590, which, on exposure to thrombin, increased to 29,623 ± 2,170, 34,043 ± 2,170 and 37,178 ± 2,736, respectively.

of thrombin, consistent with an action at or near the restriction point (Stewart et al., 1997), which occurs in mid-to-late G_1 phase. Further evidence of an action at the restriction point of the cell cycle was sought by examining the inhibitory effect of albuterol (100 nM) exposures of varying durations, from 1 h to 8 h, commencing at different times from 4 h before thrombin until up to 26 h later (Fig. 2). Different durations of exposure to albuterol were achieved without medium exchange by adding the β_2-adrenergic receptor selective antagonist ICI118551 (1 μM at 1, 2, 4, or 8 h after addition of albuterol). ICI118551 completely blocks the effects of albuterol (100 nM) on [^{3}H]thymidine but had no direct effect on mitogen responses (Tomlinson et al., 1995; Stewart et al., 1997). Significant inhibition of [^{3}H]thymidine incorporation was observed with periods of exposure to albuterol of as short as 1 h when the exposure commenced between 7 and 15 h after the addition of thrombin. Longer periods of exposure to albuterol (2–8 h) had greater inhibitory effects but also showed time-dependence. The greatest activity was observed when addition occurred in a period spanning the presumed timing of the restriction point (5–15 h after thrombin stimulation). These findings directed our further studies toward examination of the levels of cyclin D1 and pRb that control passage of cells through the restriction point of the cell cycle.

Effects of Albuterol on Changes in Levels of Phosphorylated ERK, Cyclin D1, and pRb Levels and Phosphorylation in Response to Thrombin Stimulation. Cell lysates were prepared after a 20-h incubation in 0.3 U/ml thrombin to determine the effects of albuterol (1–100 nM, added 30 min before thrombin) on the stimulation of phospho-ERK, cyclin D1 levels, and the levels and phosphorylation of pRb (Fig. 3). Albuterol opposed the thrombin-induced increases in levels of phospho-ERK and cyclin D1 in a concentration-dependent manner. The potency of albuterol for inhibition of the increase in cyclin D1 levels (pIC_{50}, 8.12 ± 0.19; $n = 3$) was similar to that for inhibition of [^{3}H]thymidine incorporation (pIC_{50}, 7.83 ± 0.01; $n = 3$; $P > .05$, ANOVA). The repressor function of the restriction protein pRb is inhibited by phosphorylation by the active (cyclin D1-complexed) cdk4 (Herwig and Strauss, 1997). The levels of the restriction protein pRb were increased by thrombin (0.3 U/ml) and there was retardation of the pRb on polyacrylamide gel electrophoresis that was indicative of an increase in molecular weight because of phosphorylation (Fig. 3). Increases in pRb levels and phosphorylation induced by thrombin were inhibited in a concentration-dependent manner by albuterol (1–100 nM). The β_2-adrenergic receptor selective antagonist ICI118551 (1 μM) completely prevented the effects of albuterol (100 nM) on the changes in the levels of phospho-ERK, cyclin D1, and pRb (Fig. 3).

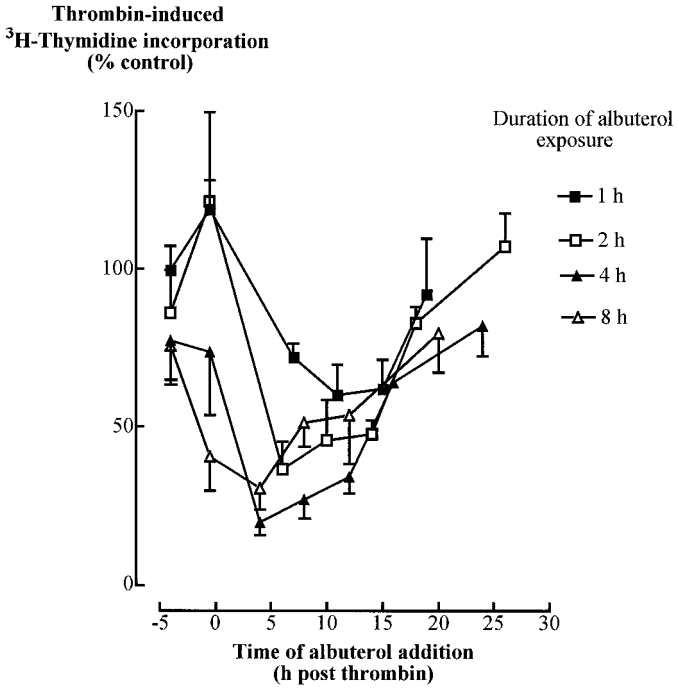

Fig. 2. Inhibition of thrombin (0.3 U/ml)-induced [^{3}H]thymidine incorporation by albuterol (100 nM) added at different times either before or after thrombin and for durations ranging from 1 to 8 h. Pulses of exposure to albuterol were achieved by adding the β_2-adrenergic receptor-selective antagonist, ICI118551 (1 μM) to terminate the receptor stimulation (see Fig. 3). Each data point represents the mean of data from at least three different cultures, each incubation in each culture having been carried out in quadruplicate. The basal levels of [^{3}H]thymidine incorporation (dpm) in the three cultures were 4,359 ± 780, 4,263 ± 314 and 7,006 ± 375, which, on exposure to thrombin, increased to 28,116 ± 1,460, 55,099 ± 1,845 and 62,686 ± 1,773, respectively. All time points between 5 and 16 h after thrombin were significant ($P < .05$; paired Student's t test) compared with thrombin response in the absence of albuterol.

Effects of 8-Bromo-cAMP and PGE_2 on Thrombin-Stimulated Changes in Cyclin D1 Levels. The effects of 8-bromo-cAMP (300 μM) and PGE_2 (1 μM) on thrombin-stimulated cyclin D1 levels were examined to investigate whether the effects of β_2-adrenergic receptor stimulation were observed with other agents that activate protein kinase A and inhibit DNA synthesis. Incubation of ASM cells in 8-bromo-cAMP or PGE_2 from 30 min before addition of thrombin attenuated the increase in cyclin D1 levels observed at 20 h after thrombin stimulation. The inhibitory effects of PGE_2 were significantly greater than those of 8-bromo-cAMP (Table 1).

Effect of the MEK1 Inhibitor PD98059 on ERK Activity, Cyclin D1 Levels, pRb Levels and Phosphorylation, and DNA Synthesis. Persistent activation of ERK activity seems to be required for cells to progress through G_1 of the cell cycle to S-phase, and ERK activity has been linked to cyclin D1 expression in bovine ASM (Ramakrishnan et al., 1998). Therefore, we investigated the effects of PD98059, a selective inhibitor of MEK1 (Dudley et al., 1995), on phos-

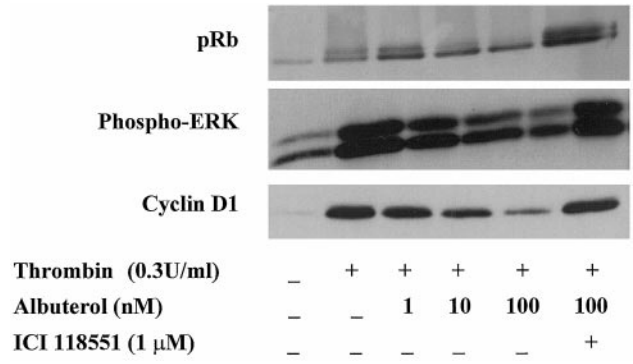

Fig. 3. Effect of albuterol (1–100 nM) on thrombin-induced increases in phosphorylation of ERK, cyclin D1 levels, and levels and phosphorylation of pRb. Albuterol was added 30 min before thrombin (0.3 U/ml) and left in the culture medium until the end of the 20-h experiment. The β_2-adrenergic receptor antagonist ICI118551 (1 μM) was added 30 min before the addition of albuterol. Data are presented as representative Western blots of at least three different experiments in three different cultures.

phorylation of ERK, cyclin D1, and pRb levels (Fig. 4). PD98059 (30 μM) inhibited thrombin-induced phosphorylation of ERK, increases in cyclin D1 levels, and increases in levels and phosphorylation of pRb, and in a previous study, has also been shown to inhibit [^{3}H]thymidine incorporation (Fernandes et al., 1999).

Effects of PD98059, Albuterol, and 8-Bromo-cAMP on ERK Activity. Our evidence (Fig. 4) and that in the literature implicating ERK activity as an upstream regulator of cyclin D1 protein levels (Cocks et al., 1992; Sewen et al., 1993; Ramakrishnan et al., 1998) and as a target for modulation by cAMP (Cook and McCormick, 1993), led to experiments examining the effects of albuterol on ERK activity in thrombin-stimulated cells. ERK activity was measured by an immunoprecipitation kinase assay in serum-deprived cells stimulated with thrombin (0.3 U/ml) for either 5 min, 30 min, or 8 h, at which time points the activity increased by 3.42, 1.45, and 1.77, respectively. Incubation with albuterol (100 nM) or 8-bromo-cAMP attenuated the increase in ERK activity at 30 min and 8 h after the addition of thrombin, but not at 5 min, whereas PD98059 inhibited ERK activity at all evaluated time points (Table 2).

Regulation of Cyclin D1 Protein but not mRNA Levels by Albuterol. Time course experiments were carried out to contrast the effects of albuterol on cyclin D1 mRNA (Fig. 5) and protein levels (Fig. 6). Cyclin D1 protein was increased above the basal level after 8 h exposure to thrombin and remained elevated until at least 20 h. At each of the time points investigated, albuterol (100 nM) inhibited the thrombin-stimulated increase in cyclin D1 protein level. In contrast, cyclin D1 mRNA levels, which were increased by thrombin as early as 4 h (1.38 $\pm$ 0.07; $n = 9$) and remained elevated at 8 h (1.45 $\pm$ 0.10; $n = 4$) and at 20 h (1.94 $\pm$ 0.27; $n = 4$), were not affected by albuterol or by PD98059 (Fig. 5).

TABLE 1

Effect of 8-bromo-cAMP and PGE_2 on levels of cyclin D1 after stimulation with thrombin (0.3 U/ml) for 20 hours ($n = 3$)

Inhibitor	Mitogen	Cyclin D1 protein[a] Mean $\pm$ S.E.M.
		20 $\pm$ 12
	Thrombin	100
8-bromo-cAMP (300 μM)		18 $\pm$ 4
8-bromo-cAMP (300 μM)	Thrombin	51 $\pm$ 7[b,c]
PGE_2 (1 μM)		5 $\pm$ 2
PGE_2 (1 μM)	Thrombin	8 $\pm$ 1[b]

[a] Densitometry-determined levels have been normalized to the level of cyclin D1 in thrombin-stimulated cells (100%) in individual experiments.
[b] $P < 0.05$ vs corresponding levels in absence of inhibitor.
[c] $P < 0.05$ vs corresponding levels in absence of thrombin.

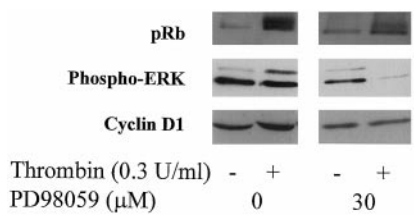

Fig. 4. Effect of the MEK1 inhibitor PD98059 (30 μM) on thrombin-induced increases in phosphorylation of ERK, cyclin D1 levels, and levels and phosphorylation of pRb. PD98059 was added 30 min before thrombin (0.3 U/ml) and left in the culture medium until the end of the 20-h experiment. Data are presented as representative Western blots of at least three different experiments in three different cultures.

Effects of the Proteasome Inhibitor MG132 and the Calpain I Inhibitors on Cyclin D1 Protein Levels. The lack of regulation of cyclin D1 mRNA levels by albuterol or PD98059 (Fig. 5) suggested that post-transcriptional mechanisms may underlie the ability of cAMP to reduce the level of cyclin D1 protein. Recent evidence supports a role for proteasome degradation in the control of cyclin D1 in mouse fibroblasts (Diehl et al., 1997). We investigated whether inhibition of the proteasome pathway with MG132 (30 and 100 nM) or inhibition of calpain I with *N*-acetyl-Leu-Leu-norleucinal (LLN; 3 and 10 μM) (Diehl et al., 1997) would interfere with the regulatory effects of cAMP and albuterol on cyclin D1 protein levels. The reduction by isobutylmethylxanthine (IBMX; 100 μM) or 8-bromo-cAMP (300 μM) of thrombin-stimulated cyclin D1 protein levels (measured 18 h after thrombin stimulation) was reversed by MG132 (100 nM) addition at 15 h after thrombin stimulation (Fig. 7). Late addition of MG132 (30 nM) alone had no effect on thrombin-stimulated increases in cyclin D1 protein levels (124 $\pm$ 14% of

TABLE 2

Effect of albuterol, 8-bromo-cAMP, and PD98059 on thrombin-stimulated ERK activity measured by an immunoprecipitation kinase assay ($n = 6$)

Incubation	Thrombin-Stimulated ERK Activity (% thrombin response)[a]		
	Albuterol	8-bromo-cAMP Mean $\pm$ S.E.M.	PD98059
	100 nM	*300 μM*	*30 μM*
5 min	102 $\pm$ 28	110 $\pm$ 21	35 $\pm$ 9[b]
30 min	49 $\pm$ 1[b]	15 $\pm$ 6[b]	32 $\pm$ 22[b]
8 h	36 $\pm$ 17[b]	45 $\pm$ 9[b]	19 $\pm$ 11[b]

[a] Data have been corrected for activity detected in the absence of anti-ERK antibody and normalized to the activity observed with thrombin in each of three separate experiments in two different cultures. The specific basal ERK activity varied between 36 and 201 $\times$ 10^3 arbitrary volume units (Molecular Dynamics PhosphorImager) in these three experiments.
[b] $P < 0.05$, paired Student's *t*-test compared with thrombin-stimulated activity in the absence of inhibitor (100%).

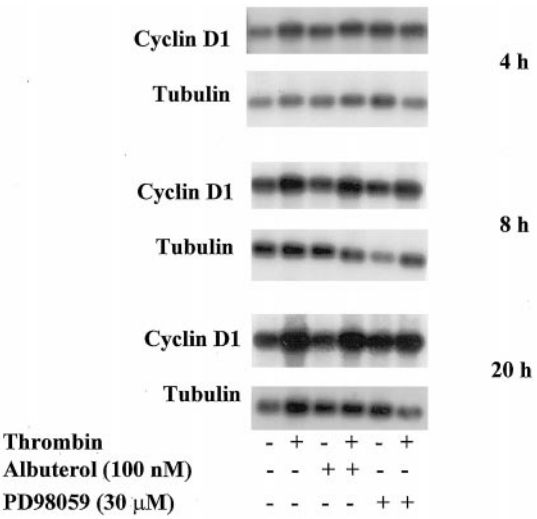

Fig. 5. Time-course investigation of the increase in cyclin D1 expression by Northern blotting of total RNA extracts at 4, 8, and 20 h after exposure to thrombin (0.3 U/ml) in the absence or presence of either albuterol (100 nM) or PD98059 (30 μM) added 30 min before thrombin. The levels of the housekeeping gene, tubulin, were unchanged by thrombin, which increased cyclin D1 by 4 h, an effect that was maintained until 20 h. Neither albuterol nor PD 98059 influenced the level of cyclin D1 expression. Data are from a single experiment and are representative of at least four experiments in different cultures each of the same design.

thrombin-stimulated level, $n = 4$). The decrease in cyclin D1 protein levels induced by albuterol (100 nM) was significantly reduced ($P < .05$, Dunnett's test) by the late addition of MG132 (Table 3). In a separate series of experiments examining albuterol responses, incubation with MG132 (30 nM) from 60 min before thrombin and then throughout the 18-h period reduced the inhibition ($P < .05$, Dunnett's Test) of thrombin-stimulated increases in cyclin D1 protein levels by albuterol (100 nM) from 48 ± 11% ($n = 6$) to the nonsignificant level of 18 ± 12% ($n = 6$). The continuous incubation of the cells with MG132 (30 nM) had no significant effect on the thrombin-stimulated cyclin D1 levels (80 ± 10% of thrombin-stimulated level; $n = 5$). Similarly, the basal levels of cyclin D1 (20 ± 6% of thrombin-stimulated level) were unchanged (35 ± 9% of thrombin-stimulated level) by continuous incubation with MG132 (30 nM).

Discussion

Elevation of cAMP levels attenuates proliferative responses of cultured airway smooth muscle cells of various species (Tomlinson et al., 1994, 1995; Maruno et al., 1995; Young et al., 1995; Schramm et al., 1996). We now show that albuterol inhibits the passage of cells through the restriction point by decreasing levels of cyclin D1, thereby decreasing cdk-mediated phosphorylation of pRb. Albuterol-mediated reduction in ERK activity was associated with a decrease in the levels of cyclin D1 protein, but not mRNA. This post-transcriptional regulation of cyclin D1 by albuterol was inhibited by the proteasome inhibitor MG132 (Rock et al., 1994) and by the calpain I inhibitor LLN (Diehl et al., 1997), suggesting that the β_2-adrenergic receptor agonist/cAMP pathway may accelerate cyclin D1 protein degradation. However, our findings do not exclude the possibility that cAMP has an additional or alternative action to alter translation of cyclin D1 mRNA.

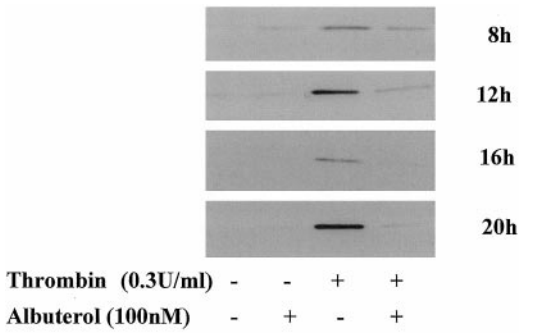

Fig. 6. Time course of albuterol (100 nM, 30 min before thrombin) regulation of thrombin (0.3 U/ml)-stimulated cyclin D1 protein levels. Western blot is representative of three different experiments carried out in three different cultures.

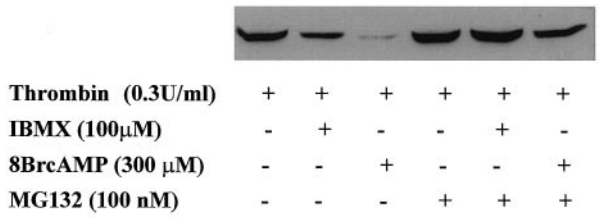

Fig. 7. Effect of the proteasome inhibitor MG132 (100 nM) on the ability of IBMX (100 μM) or 8-bromo-cAMP (300 μM) to reduce thrombin (0.3 U/ml)-stimulated cyclin D1 protein levels is exemplified in this Western blot that is representative of four experiments in four different cultures. Thrombin was added at time zero; IBMX and 8-bromo-cAMP at 0.5 h; MG132 at 15 h and lysates were prepared at 18 h.

Albuterol inhibited S-phase entry in ASM when present in the culture medium throughout the period of exposure to thrombin (Tomlinson et al., 1995). The magnitude of this cAMP-dependent G_1 arrest was less against receptor tyrosine kinase-type mitogens compared with those activating G protein-coupled receptors (Tomlinson et al., 1995). The inhibitory activity was maintained when albuterol was added up to, but not later than, 18 h after thrombin stimulation (Stewart et al., 1997). Because S-phase commences 20 to 22 h after thrombin addition, this placed the loss of activity of albuterol at a point coinciding with the restriction point in mid-to-late G_1. The restriction point is defined as the time in G_1 when cells continue to progress through the cycle to S-phase in a mitogen-independent manner (Pardee, 1974). This point in the cell cycle is considered to relate to the conversion of pRb from an under-phosphorylated state to a hyperphosphorylated state by active cyclin D1/cdk4 complexes (Herwig and Strauss, 1997). The exposure of ASM to albuterol for between 1 and 8 h commencing at different points of G_1 phase indicated that the inhibitory effect on DNA synthesis was observed even with short pulses of exposure (1 h) when these overlapped with the window of timing of the restriction point. The magnitude of inhibition was dependent on the duration of exposure, but greatest inhibition was consistently obtained between 5 to 15 h after the addition of thrombin. Thus, albuterol targeted discrete biochemical events critical for passage of cells through the restriction point, consistent with its ability to reduce the level and phosphorylation of pRb.

Cyclin D1 is one of two rate-limiting cyclins in the passage of cells through G_1 phase of the cell cycle (the other is cyclin E; Resnitzky and Reed, 1995). Cyclin D1 levels are low in quiescent (G_0) cells, rise throughout G_1, and remain elevated for the remainder of the cell cycle (Matsushime et al., 1991). Cdk4 phosphorylates pRb, leading to derepression of the transcriptional activity of the heterodimeric E2F transcription factor, allowing passage through the restriction point by enabling the expression of proteins that are essential for entry into S-phase. Cyclin E lies downstream of cyclin D1 in the signaling cascade and is considered to play a role in the passage through the G_1/S boundary (Resnitzky and Reed, 1995).

Concentrations of albuterol that inhibited DNA synthesis

TABLE 3
Effects of late (15 h) addition of the proteasome inhibitor MG132 and the calpain I inhibitor LLN on albuterol-induced suppression of cyclin D1 protein levels ($n = 3$–4)

Treatment	Albuterol Inhibition	Thrombin-Induced Cyclin D1 Protein Level[a]
		Mean ± SEM %
		100
	100 nM, 30 min before-thrombin	28 ± 6[b]
LLN (10 μM)	100 nM, 30 min before-thrombin	61 ± 11[b,c]
MG132 (30 nM)	100 nM, 30 min before-thrombin	62 ± 9[b,c]
	100 nM, 16 h after-thrombin	36 ± 11[b]
LLN (10 μM)	100 nM, 16 h after-thrombin	97 ± 21[b,c]
MG132 (30 nM)	100 nM, 16 h after-thrombin	73 ± 13[b,c]

[a] Densitometry-determined levels have been normalized to the level of cyclin D1 in thrombin-stimulated cells (100%) in individual experiments.
[b] $P < 0.05$ vs corresponding levels in absence of inhibitor.
[c] $P < 0.05$ vs corresponding levels in absence of thrombin.

reduced cyclin D1 levels via activation of a β_2-adrenergic receptor. The membrane-permeant and metabolically stable analog of cAMP, 8-bromo-cAMP, and PGE_2, an established stimulant of cAMP in ASM (Tomlinson et al., 1995), also opposed thrombin-induced increases in the levels of cyclin D1 protein at concentrations that reduced DNA synthesis. These findings are consistent with data showing 8-bromo-cAMP–induced suppression of cyclin D1 protein expression in CCL39 cells (L'Allemain et al., 1991).

The concurrent inhibition by PD98059 (Dudley et al., 1995) of thrombin-stimulated, ERK activity, cyclin D1 levels, pRb phosphorylation, and DNA synthesis is consistent with an important role for ERK in the control of both cyclin D1 levels and ASM cell cycle progression. The reduction in pRb levels and phosphorylation in the presence of PD98059 suggests that this compound prevents ASM passage through the restriction point. The suppression of thrombin-stimulated cyclin D1 levels by PD98059 suggests that MEK1 and ERK activity maintains elevated cyclin D1 levels.

ERK activation persisting through early G_1, is required for mitogenic activity (Meloche et al., 1992), but the duration of this persistence has not been clearly defined. Our findings indicate that the β_2-adrenergic receptor agonists albuterol or 8-bromo-cAMP suppress ERK activity between 30 min and 8 h after addition of thrombin, but do not reduce the peak of ERK activity at 5 min. These observations are consistent with an inhibitory action of cAMP on or upstream of ERK and with those in bovine tracheal smooth muscle cultures, showing that histamine-stimulated increases in the levels of cAMP inhibit *raf*-dependent ERK activation (Hershenson et al., 1995). In the rat1 fibroblast cell line, cAMP regulates the *ras*/*raf*/ERK signaling cascade by protein kinase A-mediated phosphorylation of *raf,* preventing its binding to and activation by *ras* (Cook and McCormick, 1993). This action of cAMP is evident in fibroblast cell lines at 5 min after exposure to stimulus. The cAMP-insensitive component of the early ERK activation in thrombin-stimulated ASM may be explained by the involvement of alternative *ras*/*raf* independent pathways that lead to ERK activation, including protein kinase C. There is evidence for a role of cAMP-insensitive ERK-activation pathways in platelet-derived growth factor-stimulated bovine ASM (Hershenson et al., 1995).

The steps linking ERK activity with the increased levels of cyclin D1 protein have not been fully elucidated, but several studies suggest that cyclin D1 reporter gene activity is increased by an ERK-dependent signaling pathway (Lavoie et al., 1996; Ramakrishnan et al., 1998). In Chinese hamster embryo fibroblasts, sustained activation of ERK is required for ongoing expression of cyclin D1 protein and mRNA (Weber et al., 1997). Superficially, our findings are compatible with ERK being an upstream regulator of cyclin D1 expression, because cyclin D1 protein levels were decreased by the MEK1 inhibitor PD98059 and there was parallel inhibition of ERK and cyclin D1 protein by cAMP elevation. However, a time-course study examining cyclin D1 expression as early as 4 h after exposure to thrombin did not find any evidence for inhibition of the elevated cyclin D1 mRNA levels with either PD98059 or the β_2-adrenergic receptor agonist. We have examined the effects of a number of agents on thrombin-stimulated cyclin D1 expression and found that of PD98059, rapamycin, albuterol, IBMX, 8-bromo-cAMP, and dexamethasone, only dexamethasone reduces cyclin D1 mRNA levels (Fernandes et al., 1999). A recent investigation points to a key role for nuclear factor-κB in the transcriptional regulation of cyclin D1 (Hinz et al., 1999). As glucocorticoids are well established and powerful regulators of the activation of nuclear factor-κB, examination of the role of this transcription factor in human ASM cell cycle is warranted.

Although reduced translation of cyclin D1 mRNA could potentially explain the cAMP-associated decrease in cyclin D1 protein levels, the recently reported involvement of the proteasome degradation pathway in cyclin D1 stability (Diehl et al., 1997, 1998) was considered more likely to be involved. Addition of the proteolysis inhibitors, MG132 or LLN to ASM late in G_1 at 15 h, attenuated the reduction in cyclin D1 protein levels (measured at 18 h) caused by continuous incubation in albuterol, the phosphodiesterase inhibitor, IMBX or 8-bromo-cAMP. These observations suggest that ongoing elevation of cAMP acutely regulates the level of cyclin D1 with a measurable effect within 3 h of interruption of this control mechanism. In some experiments, MG132 and LLN addition was delayed until 3 h before the harvest of cell lysates to reduce the impact of confounding influences of changes in the rate of degradation of other proteins by the proteasome pathway, such as the regulatory unit of protein kinase A. The latter studies also provided evidence that the albuterol/cAMP-induced decline in cyclin D1 levels, but not those under basal conditions or after incubation with thrombin alone, was opposed by MG132. Dibutyryl cAMP-induced increases in proteasomal degradation of the transcription factor GATA-6 (Nakagawa et al., 1997) support the inference that proteasome degradation is cAMP-sensitive. Furthermore, our studies with Clontech Atlas Array membranes (Palo Alto, CA) have identified that albuterol up-regulates ubiquitin ligase mRNA (E.G. and A.G.S., unpublished observations).

In NIH3T3 fibroblasts, phosphorylation of cyclin D1 by glycogen synthase kinase-3β (GSK-3β) triggers its rapid proteasome-dependent proteolysis (Diehl et al., 1998). The influence of cAMP on activation of GSK-3β in ASM is not known. However, in 3T3 fibroblasts, it seems that a *ras*-dependent PI3K pathway rather than the *ras*/*raf*/ERK pathway inhibits the activation of GSK-3β (Diehl et al., 1998). Interestingly, GSK-3β activity is inhibited by isoproterenol acting through β_3-adrenergic receptors independently of cAMP in L6 myotubes (Moule et al., 1997). However, β_3-adrenergic receptors are not implicated in the effects of albuterol on the ASM DNA synthesis or cyclin D1, because these are blocked by ICI118551 and propranolol (Tomlinson et al., 1994, 1995).

Although ERK activity is required for S-phase entry in ASM (Ramakrishnan et al., 1998), it is unlikely to be sufficient (Malarkey et al., 1995). In bovine tracheal smooth muscle, the phosphoinositol-3-kinase (PI3K)/p70 ribosomal S6 kinase pathway is also required for S-phase entry and is inhibited by elevated levels of cAMP (Scott et al., 1996; Walker et al., 1998). Inhibitors of PI3K (wortmannin) and p70 ribosomal S6 kinase activation (rapamycin) reduce platelet-derived growth factor-induced DNA synthesis (Scott et al., 1996) and also reduce thrombin-induced DNA synthesis in cultured human ASM (A.G.S. and T.H., unpublished observations). The PI3K pathway therefore represents an additional site for the inhibitory actions of cAMP in ASM, because PI3K is linked to inhibition of the activation of glycogen synthase kinase-3β and acceleration of cyclin D1

protein proteasome-dependent degradation (Diehl et al., 1998).

Our observations indicate that β_2-adrenergic receptor stimulation and cAMP cause a G_1 arrest in human cultured ASM cells by opposing mitogen-induced elevation of cyclin D1 protein levels through a post-transcriptional action that seems to regulate the proteasome-dependent degradation of cyclin D1. Further elucidation of the targets for regulation of cell cycle progression by β_2-adrenergic receptor agonists and glucocorticoids that do reduce cyclin D1 mRNA levels (Fernandes et al., 1999) may indicate how these agents could be used optimally in combination to modulate airway-wall remodeling in asthma.

Acknowledgments

We thank Dr. Xun Li, John Bartolo, and the staff of the transplant unit, Alfred Hospital, Melbourne, Australia, for the provision of human lung specimens.

References

Brewster CEP, Howarth PH, Djukanovic R, Wilson J, Holgate ST and Roche WR (1990) Myofibroblasts and subepithelial fibrosis in bronchial asthma. *Am J Respir Cell Mol Biol* **3:**507–511.

Cocks BG, Vairo G, Bodrug SE and Hamilton JA (1992) Suppression of growth factor-induced CYL1 cyclin gene expression by antiproliferative agents. *J Biol Chem* **267:**12307–12310.

Cook SJ and McCormick F (1993) Inhibition by cAMP of *Ras*-dependent activation of *Raf*. *Science (Wash DC)* **262:**1069–1072.

Diehl JA, Zindy F and Sherr CJ (1997) Inhibition of cyclin D1 phosphorylation on threonine-286 prevents its rapid degradation via the ubiquitin-proteasome pathway. *Genes Dev* **11:**957–972.

Diehl JA, Cheng MG, Roussel MF and Sherr CJ (1998) Glycogen synthase kinase 3 beta regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev* **12:** 3499–3511.

Dudley DT, Pang L, Decker SJ, Bridges AJ and Saltiel AR (1995) A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc Nat Acad Sci USA* **92:**7686–7689.

Dunnill MS and Massarella GR (1969) A comparison of the quantitative anatomy of the bronchi in normal subjects, in status asthmaticus, in chronic bronchitis, and in emphysema. *Thorax* **24:**176–179, 1969.

Ebina M, Takahashi T, Chiba T and Motomiya M (1993) Cellular hypertrophy and hyperplasia of airway smooth muscles underlying bronchial asthma. A 3-D morphometric study. *Am Rev Respir Dis* **148:**720–726.

Fernandes D, Guida E, Koutsoubos V, Harris T, Vadiveloo P, Wislon JW and Stewart AG (1999) Glucocorticoids inhibit proliferation, cyclin D1 expression and retinoblastoma protein phosphorylation, but not activity of the extracellular regulated kinases (ERK) in human cultured airway smooth muscle. *Am J Resp Cell Mol Biol* **21:**77–88.

Gillzan KM and Stewart AG (1997) The role of potassium channels in the inhibitory effects of beta 2- adrenoceptor agonists on DNA synthesis in human cultured airway smooth muscle. *Pulm Pharmacol Ther* **10:**71–79.

Herwig S and Strauss M (1997) The retinoblastoma protein: a master regulator of cell cycle, differentiation and apoptosis. *Eur J Pharmacol* **246:**581–601.

Hinz M, Krappmann D, Eichten A, Heder A, Scheidereit C and Strauss M (1999) NF-κB function in growth control: Regulation of cyclin D1 expression and G_0/G_1-to-S-phase transition. *Mol Cell Biol* **19:**2690–2698.

Hershenson MB, Chao TS, Abe MK, Gomes I, Kelleher MD and Solway JR Mr (1995) Histamine antagonizes serotonin and growth factor-induced mitogen-activated protein kinase activation in bovine tracheal smooth muscle cells. *J Biol Chem* **270:**19908–19913.

James AL, Pare PD and Hogg JC (1989) The mechanics of airway narrowing in asthma. *Am Rev Resp Dis* **139:**242–246.

L'Allemain, G, Lavoie JN, Rivard N, Baldin V and Pouyssegur J (1991) Cyclin D1 expression is a major target of the cAMP-induced inhibition of cell cycle entry in fibroblasts. *Oncogene* **14:**1981–1990

Lavoie JN, L'Allemain G, Brunet A, Muller R and Pouyssegur J (1996) Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. *J Biol Chem* **271:**20608–20616.

Matsushime H, Roussel MF, Ashmun RA and Sherr CJ (1991) Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. *Cell* **71:**323–334.

Malarkey K, Chilvers ER, Lawson MF and Plevin R (1995) Stimulation by endothelin-1 of mitogen-activated protein kinases and DNA synthesis in bovine tracheal smooth muscle cells. *Br J Pharm* **116:**2267–2273.

Maruno K, Absood A and Said SI (1995) VIP inhibits basal and histamine-stimulated proliferation of human airway smooth muscle cells. *Am J Physiol* **268:**L1047–L1051.

Meloche S, Seuwen K, Pages G and Pouyssegur J (1992) Biphasic and synergistic activation of $p44^{mapk}$ (ERK1) by growth factors: Correlation between late phase activation and mitogenicity. *Mol Endocrinol* **6:**845–854.

Moule SK, Welsh GI, Edgell NJ, Foulstone EJ, Proud CG and Denton RM (1997) Regulation of protein kinase B and glycogen synthase kinase-3 by insulin and beta-adrenergic agonists in rat epididymal fat cells-activation of protein kinase B by wortmannin-sensitive and -insensitive mechanisms. *J Biol Chem* **272:**7713–7719.

Nakagawa R, Sato R, Futai M, Yokosawa H and Maeda M (1997) Gastric Gata-6 DNA-binding protein-proteolysis induced by cAMP. *FEBS Lett* **408:**301–305.

Noveral JP and Grunstein MM (1994) Adrenergic receptor-mediated regulation of cultured rabbit airway smooth muscle cell proliferation. *Am J Physiol* **267:**L291–9.

Pardee AB (1974) A restriction point for control of normal animal cell proliferation. *Proc Natl Acad Sci USA* **71:**1286–1290.

Ramakrishnan M, Musa NL, Li J, Liu PT, Pestell RG and Hershenson MB (1998) Catalytic Activation of Extracellular Signal-regulated Kinases Induces Cyclin D1 Expression in Primary Tracheal Myocytes. *Am J Respir Cell Mol Biol* **18:**736–740.

Resnitzky D and Reed SI (1995) Different roles for cyclins D1 and E in regulation of the G1-to-S transition. *Mol Cell Biol* **15:**3463–3469.

Rock KL, Gramm L, Rothstein L, Clark K, Stein R, Dick L, Hwang D and Goladberg AL (1994) Inhibitors of the proteasome block the degradation of most proteins and the generation of peptides presented on MHC class I molecules. *Cell* **78:**761–771.

Schramm CM, Omlor GJ, Quinn LM and Noveral JP (1996) Methylprednisolone and isoproterenol inhibit airway smooth muscle proliferation by separate and additive mechanisms. *Life Sci* **59:**9–14.

Scott PH, Belham CM, Al-Hafidh J, Chilvers ER, Peacock AJ, Gould GW and Plevin R (1996) 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. *Biochem J* **318:**965–971.

Sewen A, Burger C, Brusselbach S, Schalk C, Lucibello FC. and Muller R (1993) Human cyclin D1 encodes a labile nuclear protein whose synthesis is directly induced by growth factors and suppressed by cyclic AMP. *J Cell Sci* **104:**545–555.

Stewart AG, Grigoriadis G and Harris T (1994) Mitogenic actions of endothelin-1 and epidermal growth factor in cultured airway smooth muscle. *Clin Exp Pharmacol Physiol* **21:**277–285.

Stewart AG, Tomlinson PR and Wilson J (1993) Airway wall remodelling in asthma: a novel target for the development of anti-asthma drugs. *Trends Pharmacol Sci* **14:**275–279.

Stewart AG, Tomlinson PR and Wilson JW (1997) Beta 2-adrenoceptor agonist-mediated inhibition of human airway smooth muscle cell proliferation: Importance of the duration of beta 2- adrenoceptor stimulation. *Br J Pharmacol* **121:**361–368.

Tomlinson PR, Wilson JW and Stewart AG (1994) Inhibition by salbutamol of the proliferation of human airway smooth muscle cells grown in culture. *Br J Pharmacol* **111:**641–647.

Tomlinson PR, Wilson JW and Stewart AG (1995) Salbutamol inhibits the proliferation of human airway smooth muscle cells grown in culture: Relationship to elevated cAMP levels. *Biochem Pharmacol* **49:**1809–1819.

Walker TR, Moore SM, Lawson MF, Panettieri RA and Chilvers ER (1998) Platelet-derived growth factor B and thrombin activate phosphoinositide 3-kinase and protein kinase B: Role in mediating airway smooth muscle proliferation. *Mol Pharmacol* **54:**1007–1015.

Weber JD, Raben DM, Phillips, PJ and Baldassare, JJ (1997) Sustained activation of extracellular-signal-regulated kinase 1 (ERK1) is required for the continued expression of cyclin D1 in G1 phase. *Biochem J* **326:**, 61–68.

Vlahos R and Stewart AG (1999) Interleukin-1α and tumour necrosis factor-α modulate airway smooth muscle DNA synthesis by induction of cyclo-oxygenase-2: Inhibition by dexamethasone and fluticasone propionate. *Br J Pharmacol* **126:** 1315–1324.

Xiong Y, Conolly T, Futcher B and Beach D (1991) Human D-type cyclin. *Cell* **65:**691–699.

Young PG, Skinner SJ and Black PN (1995) Effects of glucocorticoids and beta-adrenoceptor agonists on the proliferation of airway smooth muscle. *Eur J Pharmacol* **273:**137–143.

Send reprint requests to: Alastair G. Stewart, Ph.D., Department of Pharmacology, University of Melbourne, Parkville, Victoria 3052, Australia. E-mail: a.stewart@pharmacology.unimelb.edu.au