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SALBUTAMOL INHIBITS THE PROLIFERATION OF HUMAN AIRWAY SMOOTH MUSCLE CELLS GROWN IN CULTURE: RELATIONSHIP TO ELEVATED cAMP LEVELS

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Abstract—The link between increased usage of β-adrenoceptor agonists and worsening of asthma symptoms has raised interest in the effects of agents such as salbutamol on airway wall remodelling, and particularly airway smooth muscle proliferation. In the present study we have investigated the role of increases in intracellular cAMP in the inhibitory effect of salbutamol on airway smooth muscle proliferation. The inhibitory effects of a combination of submaximally effective concentrations of salbutamol (10 nM) and the non-selective phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX, 100 μM) on thrombin (0.3 U/mL)-induced mitogenesis in human cultured airway smooth muscle cells was greater than that for either agent alone. In addition, agents known to increase cAMP-dependent protein kinase activity including forskolin (10 μM), 8-bromoadenosine-3′,5′-cyclic monophosphate (100 μM), and prostaglandin E_2 (1 μM) have an inhibitory effect on thrombin (0.3 U/mL)-induced proliferation. Furthermore, the cAMP antagonist, 8-bromoadenosine-3′,5′-cyclic monophosphorothioate, Rp-isomer (300 μM) significantly reduced the inhibitory effect of salbutamol (10 nM) on thrombin (0.3 U/mL)-induced DNA synthesis. In IBMX (100 μM)-pretreated cells, salbutamol (100 nM) increased intracellular cAMP levels via stimulation of a β_2-adrenoceptor. Salbutamol (10 μM), at concentrations supramaximally effective for inhibition of mitogenesis, had no effect on thrombin (0.3 U/mL)-induced increases in intracellular calcium levels. Therefore, our results suggest that the previously reported inhibition of mitogen-induced proliferation in human cultured airway smooth muscle cells by the β_2-adrenoceptor agonist, salbutamol (100 nM), is at least partly due to elevation of intracellular cAMP, while there is no effect of salbutamol on initial mitogen-induced increases in intracellular calcium.

Key words: asthma; airway smooth muscle; proliferation; thrombin; β_2-adrenoceptor agonist; cAMP

Bronchial asthma is characterized by chronic airway inflammation, reversible airways obstruction and non-specific bronchial hyper-responsiveness. Until recently, treatment of asthma has relied primarily upon the reduction of bronchospasm by the use of β_2-adrenoceptor agonists [1]. However, there is concern over an increase in asthma morbidity and mortality linked to regular β_2-adrenoceptor agonist usage [2–4]. In contrast, glucocorticoid usage has been reported to partially reverse airways hyper-responsiveness [5] and reduce the numbers of eosinophils, mast cells and T-lymphocytes in the airway submucosa [6]. These observations have led to the contention that asthma is largely a chronic inflammatory disorder [7] and that drug treatment should consist of the early introduction of anti-inflammatory agents, rather than bronchodilators that may exacerbate asthma [8].

Airway wall remodelling is considered to be one consequence of chronic airways inflammation [9]. In *post mortem* tissues of asthmatic patients who either had fatal asthma attacks or died of unrelated causes, hypertrophy and hyperplasia of bronchial smooth muscle have been described [10–12]. The thickened airway wall could be as important as the extent of smooth muscle shortening in determining airway responsiveness in asthmatics [13, 14].

The β_2-adrenoceptor agonists act to increase airways calibre via β_2-adrenoceptor mediated activation of adenylate cyclase in airway smooth muscle cells, resulting in the elevation of intracellular cAMP [15]. Relaxation of airway smooth muscle has been ascribed to a range of intracellular effects of cAMP, such as the inhibition of bronchoconstrictor-induced inositol phospholipid hydrolysis [16]; the reduction of bronchoconstrictor-induced increases in intracellular calcium by either increasing calcium extrusion and/or sequestration [17], or by decreasing calcium entry and inositol-(1,4,5)-trisphosphate-induced calcium release [18]; the inhibition of myosin-light chain kinase [19]; and the activation of either calcium-dependent potassium channels [20–22] or the Na^+/K^+ ATPase [23] and consequent membrane hyperpolarization.

Elevation of intracellular cAMP content has been linked to increases in proliferation of rat parotid cells *in vivo* and *in vitro* [24] and of dog thyroid cells [25]. However, in rat arterial smooth muscle cells, increases in intracellular concentrations of cAMP elicited by prostaglandin E_1 [26] or adenosine [27] have been shown to inhibit proliferation. In human tracheal smooth muscle cells grown in culture, β_2-adrenoceptor stimulation by isoprenaline elicits a

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rise in intracellular cAMP levels [28]. High concentrations of isoprenaline reduce proliferation of human airway smooth muscle grown in culture [29]. In addition, a recent study indicates that injection of the catalytic subunit of cAMP-dependent protein kinase inhibits porcine cultured airway smooth muscle cell proliferation induced by epidermal growth factor [30]. In rat 1 fibroblasts, it has been reported that elevated levels of cAMP inhibit the *Ras*-dependent activation of *Raf*-1, a component in the signalling pathway that leads to the activation of MAP* kinases $p42^{mapk}$ and $p44^{mapk}$ [31, 32].

In the present study, the role of cAMP in the anti-proliferative effects of salbutamol has been examined [33]. The present findings establish that salbutamol stimulates an increase in cAMP, and both this increase and the anti-proliferative effects are blocked by the β_2-adrenoceptor antagonist, ICI 118551. The anti-proliferative effects of salbutamol are mimicked by the membrane permeable cAMP analogue, 8-Br-cAMP, PGE_2 and the direct activator of adenylate cyclase, forskolin, and enhanced by phosphodiesterase inhibition. In addition, the cAMP antagonist, Rp-8-Br-cAMPS, reduced the anti-proliferative effects of salbutamol. These observations are consistent with the proposal that persistent increases in intracellular cAMP are inhibitory to airway smooth muscle proliferation.

MATERIALS AND METHODS

Materials. Plasticware was obtained from Falcon (Becton Dickinson Labware, Lincoln Park, NJ, U.S.A.). Glass eight-well tissue culture chamber slides were obtained from Nunc Inc. (Naperville, IL, U.S.A.). All chemicals used were of analytical grade or higher. The compounds used and their sources were as follows: L-glutamine, essentially fatty acid free BSA fraction V, Tyrode buffer (HEPES, 10 mM; NaCl 135 mM; KCl, 2.6 mM; $MgCl_2 \cdot 6H_2O$, 0.25 mM; $NaH_2PO_4 \cdot 2H_2O$, 0.45 mM; $NaHCO_3$, 12 mM; and D-glucose, 15 mM), thrombin (bovine plasma), salbutamol, 8-Br-cAMP, IBMX, PGE_2, adenosine, FURA-2 acetoxy methyl ester, Sigma (St. Louis, MO, U.S.A.); forskolin, ICI 118551 (Research Biochemicals International, Natick, MA, U.S.A.); Rp-8-Br-cAMPS (BioLog, Bremen, Germany); amphotericin B (Fungizone; Gibco Laboratories, NY, U.S.A.); elastase (Worthington Biochemical, Freehold, NJ, U.S.A.); Dulbecco 'A' phosphate buffer saline (Oxoid, U.K.); trypsin, versene, penicillin-G, streptomycin, collagenase type 1, Monomed A (CSL, Parkville, Victoria, Australia); foetal calf serum (Flow Laboratories, North Ryde, NSW, Australia); DMEM (Flow Laboratories, Irvine, U.K.); [6-^{3}H]thymidine (185 GBq/mmol, 5 Ci/mmol), cAMP [^{3}H] assay system (TRK 432, Amersham, U.K.); emulsifier-safe scintillant (Canberra-Packard, Australia). The antibodies used for immunocytochemistry were anti-smooth muscle α-actin (mouse monoclonal) (Dako M851), monoclonal mouse anti-human endothelial cell, CD31 (DAKO-CD31, JC/70A) (Dako M823), Dako Corporation (Santa Barbara, CA, U.S.A.); and anti-mouse Ig F(ab')2 fragment FITC-conjugate (host sheep) (Silenus DDAF; Silenus, Hawthorn, Victoria, Australia).

Cell culture. Human airway smooth muscle cells were harvested from bronchi obtained from lung resections. The tissue was initially immersed in DMEM (supplemented with 2 mM L-glutamine, 0.25% (w/v) BSA, 100 U/mL penicillin-G, 100 μg/mL streptomycin and 2 μg/mL amphotericin B) containing 3 mg/mL collagenase for 25 min at 37° in a humidified incubator containing 5% CO_2 in air. Following the partial digestion, the tissue was rinsed in phosphate buffered saline to remove the epithelium. For each cell culture, at least 0.1 g of smooth muscle tissue was stripped from the wall of the bronchus and was cut into small pieces, approximately 2 mm^3. The chopped tissue was digested further by incubation in DMEM (supplemented with 2 mM L-glutamine, 0.25% (w/v) BSA, 100 U/mL penicillin-G, 100 μg/mL streptomycin and 2 μg/mL amphotericin B) containing elastase (0.5 mg/mL) for 2 hr, followed by a 12 hr incubation in collagenase (1 mg/mL), at 37°. Examination of the tissue upon completion of the digestion revealed a high proportion of single cells. The cells were centrifuged (5 min, 100 *g*, room temperature) and washed three times in DMEM (supplemented with 2 mM L-glutamine, 0.25% (w/v) BSA, 100 U/mL penicillin-G, 100 μg/mL streptomycin and 2 μg/mL amphotericin B). The final cell resuspension was made in 25 mL of DMEM (supplemented with 10% (v/v) heat-inactivated foetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin-G, 100 μg/mL streptomycin and 2 μg/mL amphotericin B) and the cells were seeded in one 25 cm^2 culture flask. The primary isolates were incubated for 7 days to reach confluency. Thereafter, cells were harvested weekly by 10 min exposure to 0.5% trypsin, 1 mM EDTA and passaged at a 1:3 ratio in 75 cm^2 culture flasks. Cells at passage numbers 3–18 were used for experiments.

Immunocytochemistry. The expression of smooth muscle specific α-actin was used to determine the identity of the cultures [34]. Cells were subcultured into eight-well glass tissue culture chamber slides. They were allowed to grow on 100% monolayer confluency, in the presence of DMEM (supplemented with 10% (v/v) heat-inactivated foetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin-G, 100 μg/mL streptomycin and 2 μg/mL amphotericin B). The cells were then washed three times in phosphate buffered saline before being fixed in acetone for 7 min at 4°. Fixed cells were then stored at 4° for up to 4 weeks before staining. Prior to staining, the fixed cells were rehydrated in phosphate buffered saline containing 1% (w/v) BSA for 20 min and then

* Abbreviations: IBMX, 3-isobutyl-1-methylxanthine; 8-Br-cAMP, 8-bromoadenosine-3',5'-cyclic monophosphate; Rp-8-Br-cAMPS, 8-bromoadenosine-3',5'-cyclic monophosphorothioate, Rp-isomer; CRE, cAMP response element; CREB, cAMP response element binding protein; DMEM, Dulbecco's Modified Eagle's Medium; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; PDE, phosphodiesterases; PGE_2, prostaglandin E_2; salbutamol, α-((*t*-butylamino)methyl)-4-hydroxy-*m*-xylene-α,α'-diol; ICI 118551, erythro-DL-1-(1-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol.

exposed to the primary antibody, anti-smooth muscle α-actin (mouse monoclonal), for 1 hr at room temperature. This was followed by exposure to the secondary antibody, anti-mouse Ig F(ab')2 fragment FITC-conjugate, for 1 hr at room temperature. Background staining controls were provided by reversal of the order of antibody exposure. In addition, cells were stained with a monoclonal antibody for the endothelial cell marker, CD31. The staining of the fixed cells was then observed by fluorescence microscopy. Each of the cell lines used in these studies showed uniform staining for smooth muscle specific α-actin, but did not express CD31. Moreover, there did not appear to be any relationship between passage number and the intensity of staining for smooth muscle α-actin.

Proliferation assay. Cells were subcultured into 24-well plates at a 1:3 split ratio and allowed to grow to confluency over a 72 hr period. Twenty-four hours before stimulation the medium was replaced with DMEM (supplemented with 2 mM L-glutamine, 0.25% (w/v) BSA, 100 U/mL penicillin-G, 100 μg/mL streptomycin and 2 μg/mL amphotericin B). Where indicated IBMX (100 μM) was added 60 min before preincubation with salbutamol and remained in the medium until cell harvesting. Where indicated the cAMP antagonist, Rp-8-Br-cAMPS (300 μM) was added 30 min before preincubation with salbutamol and remained in the medium until cell harvesting. At 30 min prior to stimulation salbutamol (10 nM), forskolin (10 μM), 8-Br-cAMP (100 μM), PGE_2 (1 μM) or adenosine (10 μM) was added as indicated and each compound was left in the medium until cell harvesting. Upon stimulation, the putative mitogen and a supplement containing insulin, transferrin and selenium (Monomed A, 1%, v/v) were added to the allotted wells. The duration of stimulation with growth factors and growth-promoting substances was 28 hr for [^{3}H]thymidine incorporation assays. Cells were pulsed for the final 4 hr with [^{3}H]thymidine at a final concentration of 1 μCi/mL. Cell harvesting procedures followed the method outlined by Dicker and Rozengurt [35] as described previously [33].

cAMP assay. Cells were subcultured into six-well plates at a 1:3 split ratio and allowed to grow to confluency over a 72 hr period. Twenty-four hours before the experiment, the medium was replaced with serum free DMEM (supplemented with 2 mM L-glutamine, 0.25% (w/v) BSA, 100 U/mL penicillin-G, 100 μg/mL streptomycin and 2 μg/mL amphotericin B) in order to establish conditions identical to those for proliferation assays. All cells were pretreated with IBMX (100 μM) for 1 hr, which remained in the medium for the duration of the experiment. Cells were then incubated for 30 min in medium or in medium to which either ICI 118551 (50 nM), or thrombin (0.3 U/mL) was added and left in the medium for the duration of the experiment. These pretreatments were followed by a further 30 min incubation period, conducted as described below: for cells incubated in medium, the following 30 min treatment period consisted of incubation with either medium, salbutamol (100 nM), thrombin (0.3 U/mL), PGE_2 (1 μM), forskolin (10 μM) or adenosine (100 μM); for cells preincubated with ICI 118551 (50 nM), a 30 min stimulation with either salbutamol (100 nM) or forskolin (10 μM) followed; while in cells preincubated with thrombin (0.3 U/mL), the subsequent 30 min incubation was with salbutamol (100 nM). Cell extracts were made by aspiration of supernatants and addition of assay buffer (50 mM Tris/HCl, 4 mM EDTA, pH 7.5) to the cell monolayer. The cells were then scraped from the culture plates and the samples were heated for 5 min in a boiling water bath to aggregate and remove protein. Samples were centrifuged (10 min, 10,000 *g*, 4°) and the cAMP contents of these supernatants were assayed in duplicate following the suppliers instructions (Amersham Cyclic AMP [^{3}H] assay system, TRK 432).

Intracellular calcium measurements using FURA-2AM. Glass coverslips were sterilized in 70% (v/v) ethanol and placed on the bottom of the tissue culture chambers in the six-well culture plates. The cells were seeded at a 1:3 split ratio and allowed to grow to confluency. To load the cells with the calcium-sensitive dye, the medium was replaced with DMEM containing 1 μM of the FURA-2 acetoxy methyl ester. The cells were incubated at room temperature in a shaker apparatus (60 strokes/min) for 40 min. The medium was replaced with Tyrode buffer (pH 7.4) containing 0.25% (w/v) BSA and incubated for 15 min at 37°. The measurement of intracellular calcium was carried out using an F-2000 fluorescence spectrophotometer (Hitachi, Japan) equipped with filters, magnetic stirring, and heating (37°). The emission wavelength was set at 510 nm and the excitation wavelengths alternating at 0.5 sec intervals between 340 and 380 nm. Basal and agonist-stimulated elevations in intracellular calcium were calculated from the (340/380 nm) fluorescence ratio [36].

Data analysis. Incubations in the proliferation experiments were carried out in quadruplicate and in at least two cell lines derived from lung resection specimens obtained from two individuals. A total of six cell lines from six individuals were used in this study. Results are expressed as mean values ± SEM. Student's unpaired *t*-test was used for statistical comparisons between the means of quadruplicate incubations within a cell line. Differences were considered to be statistically significant when $P < 0.05$. Fold increases were calculated by dividing the mean dpm from quadruplicate incubations of mitogen-stimulated cells by the mean value from unstimulated cells. Percentage decreases in mitogenic responses were calculated from the mean of the [^{3}H]thymidine incorporation in agonist-stimulated cells (100%) in individual experiments compared with the response in treated cells and the results are presented as the means and SEM of N experiments. Intracellular cAMP determinations were carried out in triplicate and in three cell lines derived from lung resection specimens obtained from three individuals. Results are expressed as mean values ± SEM for N cell lines. Intracellular calcium experiments were carried out in at least two cells lines derived from lung resection specimens obtained from separate individuals. Bracket control experiments, carried out at equal times before and after a single test experiment, were conducted where indicated.

RESULTS

Inhibition of thrombin-stimulated [^{3}H]thymidine incorporation by salbutamol is increased by phosphodiesterase inhibition

Thrombin (0.3 U/mL) elicited a mitogenic response in each of the cell lines examined, causing an average 8.3 ± 1.2-fold increase in [^{3}H]thymidine incorporation (N = 6). Thrombin was used to elicit proliferation in all the current experiments, but we have previously shown that salbutamol also inhibits [^{3}H]thymidine incorporation induced by epidermal growth factor and the thromboxane A_2 mimetic, U46619 [33].

Preincubation of smooth muscle cells with the β_2-adrenoceptor agonist, salbutamol (10 nM), for 30 min prior to thrombin (0.3 U/mL) addition, significantly inhibited the incorporation of [^{3}H]-thymidine by 55.3 ± 3.0% ($P < 0.05$, N = 3 cell lines). A 1 hr preincubation with the non-selective phosphodiesterase inhibitor, IBMX (100 μM), had no significant effect on unstimulated cells ($P > 0.05$, N = 3), though it inhibited thrombin (0.3 U/mL)-stimulated incorporation of [^{3}H]thymidine by 72.2 ± 4.6% ($P < 0.05$, N = 3). IBMX preincubation prior to salbutamol (10 nM) preincubation resulted in an 87.9 ± 2.3% ($P < 0.05$, N = 3) inhibition of thrombin-induced [^{3}H]thymidine incorporation (Fig. 1). Therefore, while IBMX (100 μM) or salbutamol (10 nM) pretreatment partially inhibited thrombin (0.3 U/mL)-induced mitogenesis, the combination of these pretreatments reduced [^{3}H]thymidine incorporation to a level not different to the resting levels.

Stimulants of cAMP inhibit thrombin-stimulated [^{3}H]thymidine incorporation

A 30 min preincubation with the direct activator of adenylate cyclase, forskolin (10 μM), significantly reduced the basal incorporation of [^{3}H]thymidine by 45.0 ± 12.4% ($P < 0.05$, N = 3) and inhibited [^{3}H]thymidine incorporation in thrombin (0.3 U/mL)-stimulated cells by 61.9 ± 7.7% ($P < 0.05$, N = 3) (Fig. 2A). Preincubation with the stable, membrane-permeable analogue of cAMP, 8-Br-cAMP (100 μM) had no significant effect on basal [^{3}H]thymidine incorporation ($P > 0.05$, N = 3), whereas it inhibited [^{3}H]thymidine incorporation in thrombin (0.3 U/mL)-stimulated cells by 29.0 ± 4.9% ($P < 0.05$, N = 3) (Fig. 2B). PGE_2

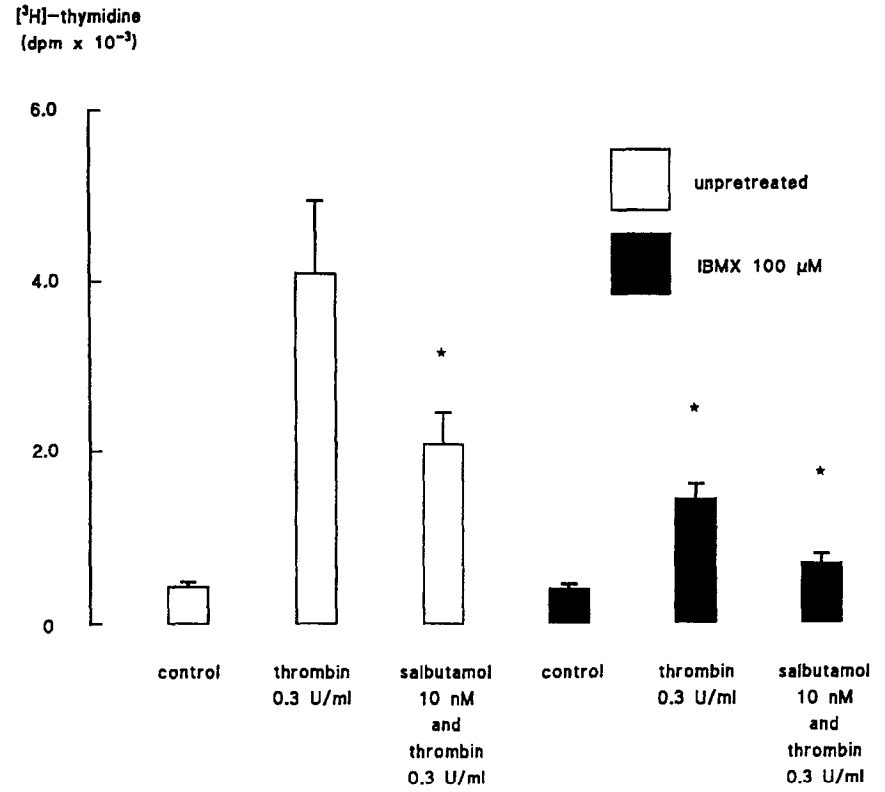

Fig. 1. Effect of preincubation of human cultured airway smooth muscle cells with IBMX (100 μM) on [^{3}H]thymidine incorporation. Histograms represent [^{3}H]thymidine incorporation in cells that were either unstimulated; thrombin (0.3 U/mL)-stimulated; or preincubated with salbutamol (10 nM) then stimulated with thrombin (0.3 U/mL); in the absence (open) or presence (filled) of IBMX (100 μM, added 1 hr before salbutamol). Data are presented as the mean ± SEM of dpm from quadruplicate incubations of one experiment which was representative of those conducted in three separate cell lines.
* $P < 0.05$, Student's unpaired *t*-test, compared to thrombin-stimulated cells.

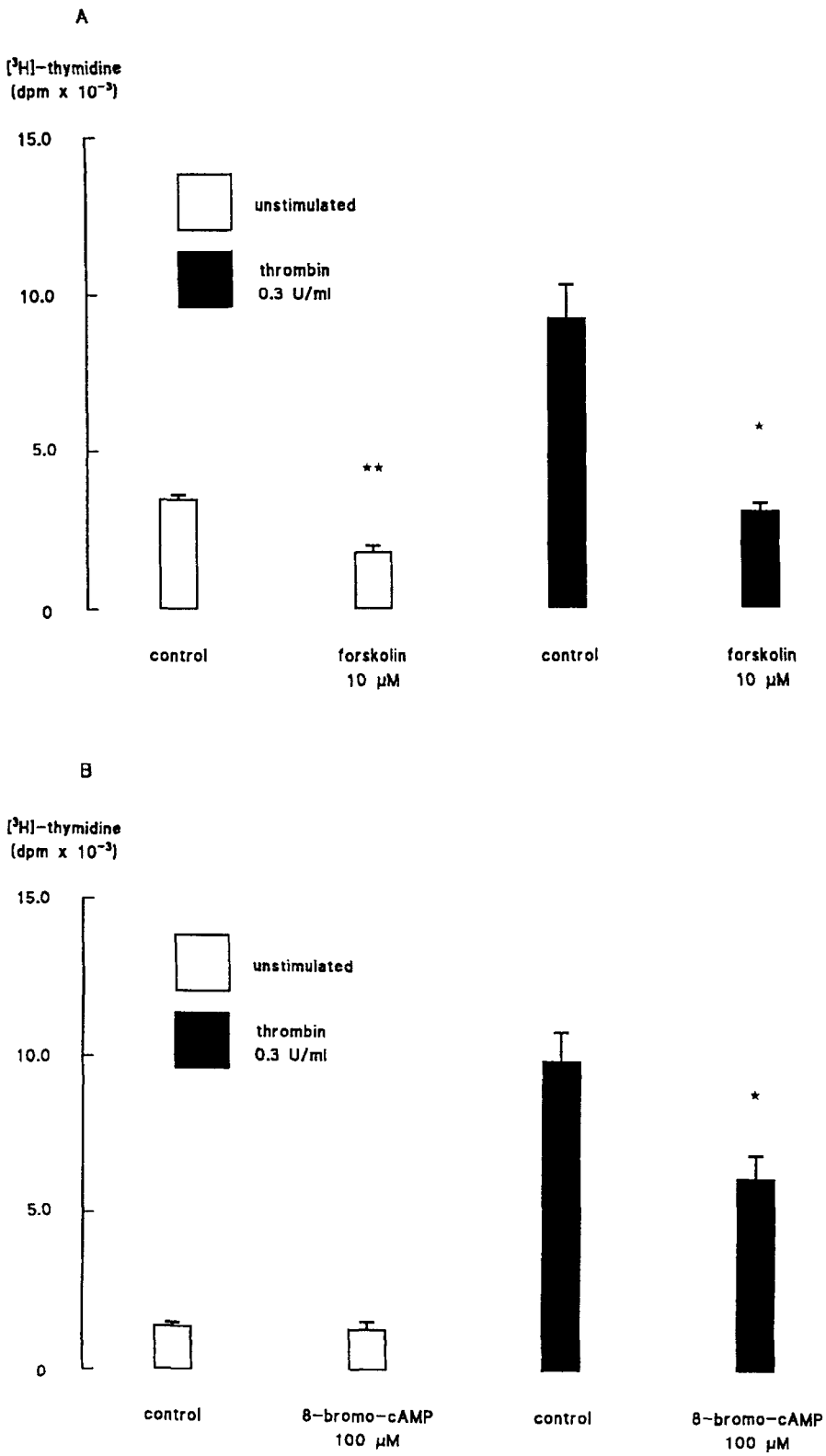

Fig. 2. The effect of preincubation with (A) forskolin (10 μM), (B) 8-Br-cAMP (100 μM), on [^{3}H]-thymidine incorporation in unstimulated (open histograms) and thrombin (0.3 U/mL)-stimulated (filled histograms) cells. Data are presented as the mean ± SEM of dpm from quadruplicate incubations of one experiment which was representative of those conducted in three separate cell lines. * $P < 0.05$, Student's unpaired t-test, compared to thrombin-stimulated cells. ** $P < 0.05$, Student's unpaired t-test, compared to unstimulated cells.

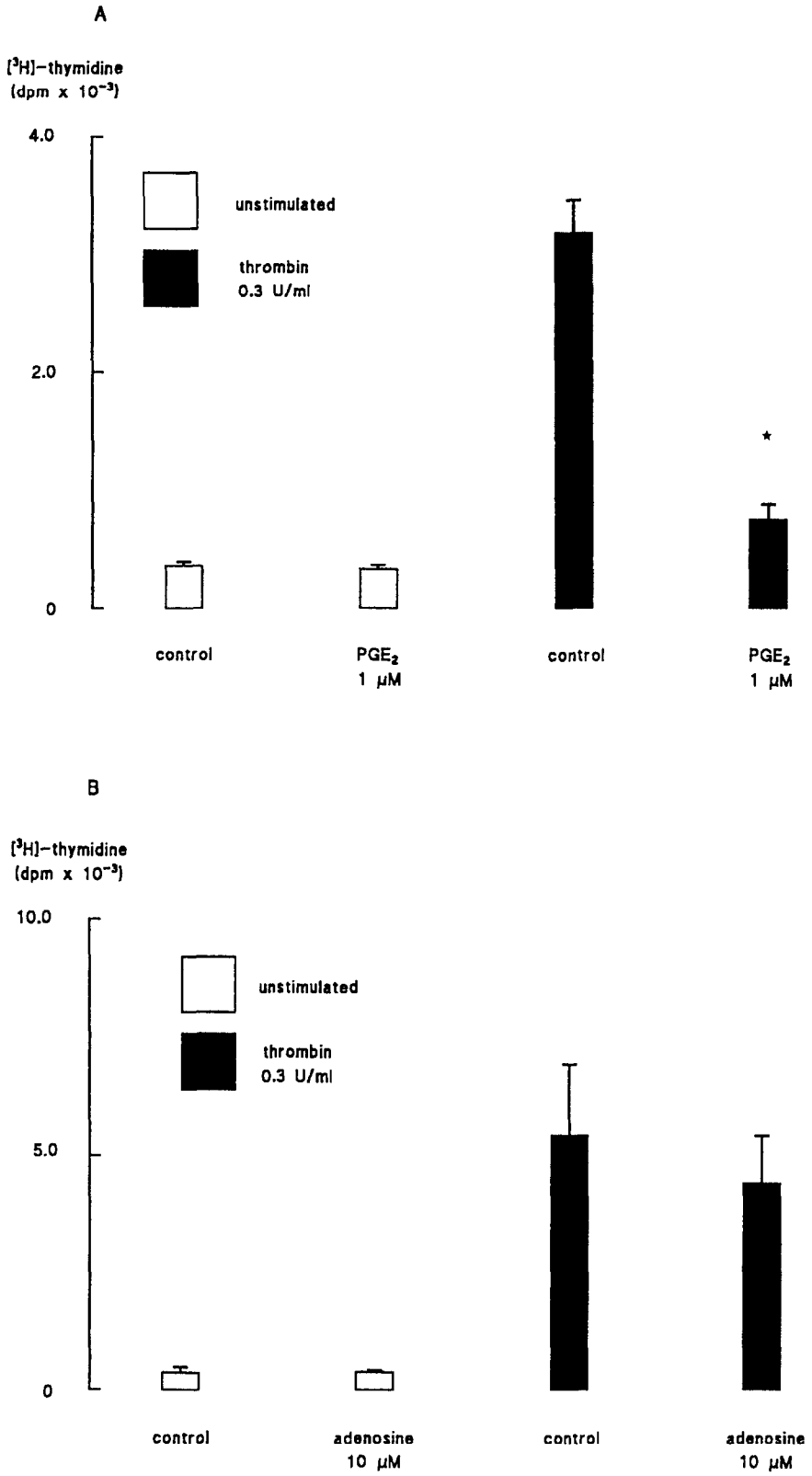

Fig. 3. The effect of preincubation with (A) PGE_2 (1 μM), and (B) adenosine (10 μM) on [^{3}H]thymidine incorporation in unstimulated (open histograms) and thrombin (0.3 U/mL)-stimulated (filled histograms) cells. Data are presented as the mean ± SEM of dpm from quadruplicate incubations of one experiment which was representative of those conducted in three separate cell lines. * $P < 0.05$, Student's unpaired *t*-test, compared to thrombin-stimulated cells.

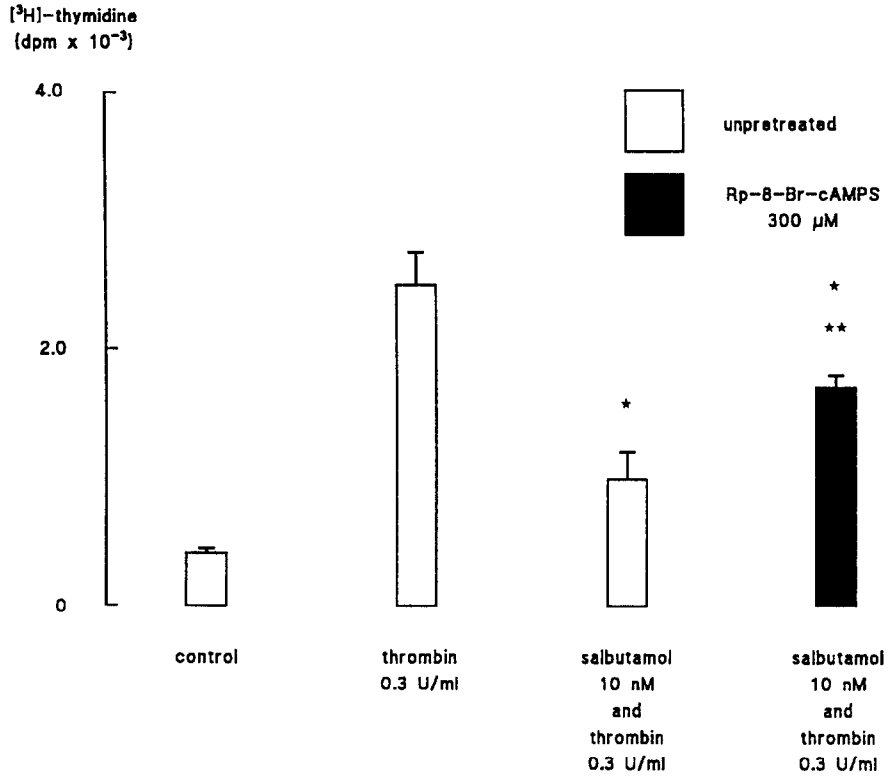

Fig. 4. The effect of preincubation with Rp-8-Br-cAMPS (300 μM) (filled histogram) on salbutamol (10 nM) inhibition of [^{3}H]thymidine incorporation in thrombin (0.3 U/mL)-stimulated (open histogram) cells. Data are presented as the mean ± SEM of dpm from quadruplicate incubations of one experiment which was representative of those conducted in two separate cell lines. * $P < 0.05$, Student's unpaired *t*-test, compared to thrombin-stimulated cells. ** $P < 0.05$, Student's unpaired *t*-test, compared to thrombin-stimulated cells, pretreated with salbutamol.

(1 μM) preincubation had no significant effect on the basal [^{3}H]thymidine incorporation ($P > 0.05$, $N = 3$), but inhibited that induced by thrombin (0.3 U/mL), by 76.5 ± 1.0% ($P < 0.05$, $N = 3$) (Fig. 3A). In contrast, adenosine (10 μM) had no significant effect on either basal or thrombin (0.3 U/mL)-stimulated [^{3}H]thymidine incorporation ($P > 0.05$, $N = 3$) (Fig. 3B).

Inhibition of cAMP-dependent protein kinase

Addition of the cAMP antagonist, Rp-8-Br-cAMPS (300 μM) 30 min before salbutamol significantly reduced the inhibitory effect of salbutamol (10 nM) from a 55.3 ± 3.0% inhibition with salbutamol alone, to a 40% inhibition of thrombin (0.3 U/mL)-induced [^{3}H]thymidine incorporation in the presence of Rp-8-Br-cAMPS ($P < 0.05$, $N = 2$) (Fig. 4).

Agonist stimulation of increases in intracellular cAMP

All cells were pretreated for one hour with IBMX (100 μM). A 30 min incubation with salbutamol

Table 1. Agonist-induced increases in intracellular cAMP (fmol/μg protein)

	Control (30 min)	Salbutamol (100 nM) (30 min)	ICI 118511 (50 nM, 30 min) and salbutamol (100 nM, 30 min)
Control (30 min)	17 ± 6.0	83 ± 16*	10 ± 4.0 NS
Thrombin (0.3 U/mL) (30 min)	4.0 ± 2.0*	95 ± 18*	ND

Data represent the means ± SEM of three experiments using three cell lines, each experiment being carried out in triplicate. * $P < 0.05$, paired Student's *t*-test compared to control; NS not significant; ND not done.

Table 2. Stimulant-induced increases in intracellular cAMP (fmol/μg protein)

Control (60 min)	11 ± 3.9 (N = 6)
Control (30 min) and forskolin (10 μM) (30 min)	104 ± 29 (N = 3)*
ICI 118551 (50 nM) (30 min) and forskolin (10 μM) (30 min)	102 ± 24 (N = 3)*
Control (30 min) and PGE_2 (1 μM) (30 min)	454 (N = 2)
Control (30 min) and adenosine (100 μM) (30 min)	18 ± 6.0 (N = 3) NS

Data represent the means ± SEM of three experiments using N cell lines, each experiment being carried out in triplicate. * $P < 0.05$, paired Student's t-test compared to control. NS, not significant.

(100 nM) elicited a significant increase in intracellular cAMP levels compared to control. Pretreatment for 30 min with the selective β_2-adrenoceptor antagonist, ICI 118551 (50 nM) prevented salbutamol (100 nM)-induced increases in intracellular cAMP. Incubation for 30 min with thrombin (0.3 U/mL) induced a significant decrease in intracellular cAMP levels (Table 1). However, incubation with thrombin (0.3 U/mL) for 30 min had no effect on increases in intracellular cAMP elicited by a subsequent 30 min incubation with salbutamol (100 nM) (Table 1). A 30 min incubation with either forskolin (10 μM) or PGE_2 (1 μM) elicited increases in intracellular cAMP. Pretreatment with ICI 118551 (50 nM) did not inhibit forskolin (10 μM)-induced increases in intracellular cAMP. Adenosine (100 μM) was reported to elicit increases in intracellular cAMP in rat vascular smooth muscle cells [27]. However, after a 30 min incubation in airway smooth muscle cells, intracellular cAMP levels were not different from control (Table 2).

Measurement of intracellular calcium concentrations

Stimulation of human airway smooth muscle cells with thrombin (0.3 U/mL) (Fig. 5A) led to increases in intracellular calcium concentrations which were resistant to inhibition by concentrations of salbutamol (10 μM) that were supramaximal for anti-proliferative effects. Lower concentrations of salbutamol (100 nM) that have been previously reported to have a maximal effect on thrombin-induced mitogenesis [33] also

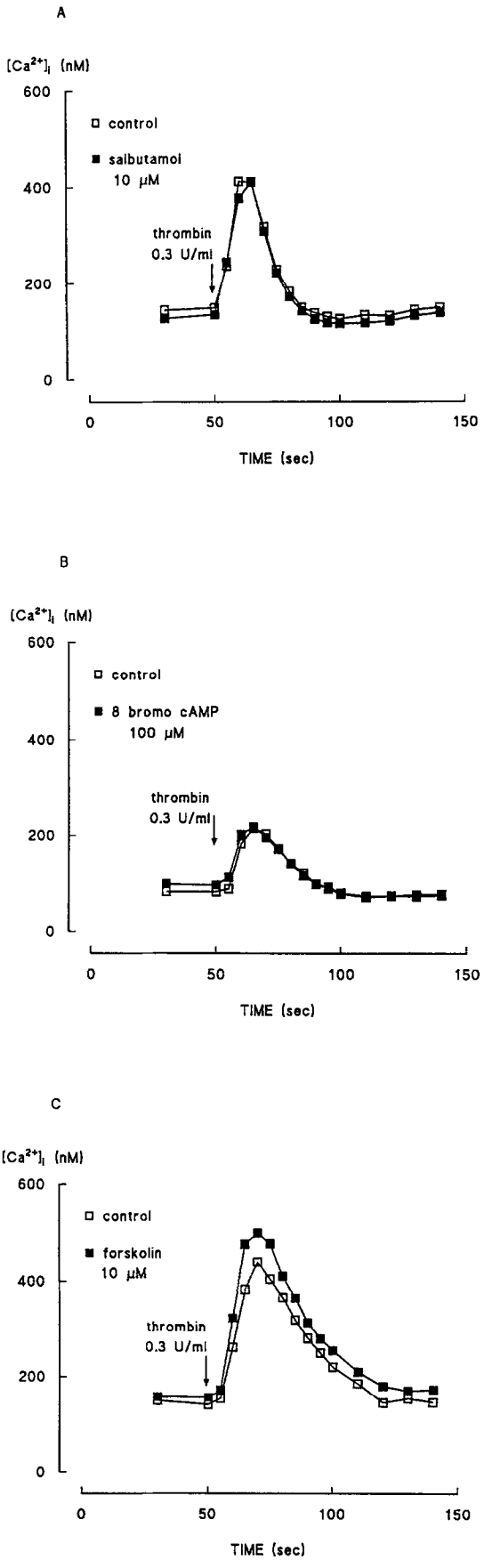

Fig 5. Time-course relationship for (A) thrombin (0.3 U/mL) stimulation of intracellular calcium in control and salbutamol (10 μM, 2 min at 37°) pretreated human airway smooth muscle cells. Time-course relationship for (B) thrombin (0.3 U/mL) stimulation of intracellular calcium in control and 8-Br-cAMP (100 μM, 2 min at 37°) pretreated human airway smooth muscle cells. Time-course relationship for (C) thrombin (0.3 U/mL) stimulation of intracellular calcium in control and forskolin (10 μM, 2 min at 37°) pretreated human airway smooth muscle cells. Data for the control values represent the mean of two time-course experiments carried out at equal times before and after the single salbutamol (10 μM) pretreatment experiment. Data are representative of experiments conducted in two cell lines.

failed to inhibit thrombin (0.3 U/mL)-induced increases in intracellular calcium (data not shown). Pretreatment with either 8-Br-cAMP at a concentration of 100 μM (Fig. 5B), or forskolin (10 μM) (Fig. 5C) had no effect on thrombin (0.3 U/mL)-induced increases in intracellular calcium. Adenosine (100 μM) stimulated increases in intracellular calcium of 151 ± 37 nM (N = 3) above the basal concentration (56 ± 19 nM, N = 3) indicating the existence of a functional response to adenosine.

DISCUSSION

We have previously demonstrated that the widely used bronchodilator, salbutamol, has a direct inhibitory effect on mitogen-induced proliferation of human airway smooth muscle cells [33]. Here we provide evidence that this inhibitory effect may be at least partly mediated by increases in intracellular cAMP content.

Earlier reports from work conducted on rat and porcine vascular smooth muscle demonstrate that agonists that increase intracellular cAMP have an inhibitory effect on mitogen-induced proliferation [26, 27]. Very high concentrations of the non-selective β-adrenoceptor agonist, isoprenaline (100 μM), reduced proliferation of human airway smooth muscle cells [29]. Hall *et al.* [16] have shown that isoprenaline increased intracellular cAMP levels in human tracheal smooth muscle cells grown in culture. We have extended these observations to show that the clinically used bronchodilator salbutamol elicits increases in intracellular cAMP above basal levels via activation of β_2-adrenoceptors. Other agents known to activate cAMP-dependent protein kinase, such as forskolin, 8-Br-cAMP, and PGE_2 also inhibit the mitogen-induced proliferation of human airway smooth muscle cells. Pretreatment with the non-selective phosphodiesterase inhibitor, IBMX, which increases both basal and salbutamol-stimulated cAMP levels, partially inhibited thrombin-induced mitogenesis. The combination of IBMX with salbutamol produced a greater inhibition of proliferation than that obtained with either agent alone. This observation is similar to the finding that inhibitors of PDE III and IV alone, or in combination with an adenylate cyclase stimulant, reduce proliferation of pig cultured vascular smooth muscle cells [37]. Therefore combined use of β_2-adrenoceptor agonists and specific phosphodiesterase inhibitors, particularly inhibitors of the PDEs III and IV, which have been demonstrated to be present in human airway smooth muscle [38] may be a useful therapeutic regimen in asthma.

The relaxant effects of elevated intracellular cAMP on airway smooth muscle, which are rapid in onset, may be due to a number of factors such as inhibition of inositol phospholipid hydrolysis [16]; increasing calcium extrusion and/or sequestration [17]; decreasing calcium entry and inositol-(1,4,5)-triphosphate-induced calcium release [18]; the inhibition of myosin-light chain kinase [19]; the activation of calcium-dependent potassium channels [20–22]; the stimulation of Na^+/K^+ ATPase [23] and consequent membrane hyperpolarization. Proliferation was measured as [^{3}H]thymidine incorporation between 24 and 28 hr after exposure to thrombin in cells that had been pretreated for 30 min and subsequently continuously exposed to agonists reported to elevate intracellular cAMP levels, whereas measurement of intracellular cAMP levels were carried out only after a brief exposure (30 min) to agonists known to elevate intracellular cAMP levels. cAMP measurements were made after 30 min exposure to stimuli because the relatively rapid effects of elevated intracellular cAMP were considered likely to influence mitogen-stimulated DNA synthesis. However, it is clear that at least one of the early mitogenic signals, the increase in intracellular calcium, is not influenced by agents that increase intracellular cAMP at the time of mitogen addition.

Injection of the catalytic subunit of cAMP-dependent protein kinase is reported to inhibit mitogen-induced proliferation in porcine airway smooth muscle cells [30]. Therefore, we used the cAMP antagonist Rp-8-Br-cAMPS in an attempt to prevent the anti-proliferative effect of salbutamol [37]. Inhibition by salbutamol of thrombin-induced DNA synthesis was reduced by Rp-8-Br-cAMPS (300 μM), which suggests that this effect is mediated at least in part by an increase in intracellular cAMP levels and activation of protein kinase A.

Adenosine (100 μM) had no effect on the mitogen-induced proliferation of airway smooth muscle cells, in contrast to its inhibitory effect on rat vascular smooth muscle cells [27]. Adenosine (100 μM) did not elicit increases in intracellular cAMP levels, despite evidence from studies using other smooth muscle cell types, indicating that adenosine elevates levels of this second messenger. Nevertheless, the human airway smooth muscle appears to express adenosine receptors, since adenosine (100 μM) elicited a rise in intracellular calcium. In other cell types the adenosine A_1-receptor is linked to release of calcium from intracellular stores and influx of extracellular calcium [39]. Further investigations with specific adenosine receptor agonists and antagonists are underway to establish the adenosine receptor subtype(s) present in human cultured airway smooth muscle.

Interestingly, thrombin (0.3 U/mL) reduced the basal intracellular cAMP levels, which parallels findings in vascular smooth muscle cells [40] and suggests the involvement of a Gi-like protein which negatively regulates adenylate cyclase, as reported for platelets [41]. Although lowering of basal cAMP levels may be a component of the mitogenic activity of thrombin, it is clear that thrombin does not influence the ability of salbutamol to increase cAMP.

Salbutamol had no effect on thrombin (0.3 U/mL)-induced increases in intracellular calcium at concentrations which were maximal (100 nM) [32] or supramaximal (10 μM) for inhibition of mitogen-induced proliferation. Pretreatment with the phosphodiesterase inhibitor IBMX for 1 hr did not unmask any inhibitory effect of salbutamol (10 μM) on thrombin-induced rises in intracellular calcium (data not shown). In addition, neither the membrane permeable analogue of cAMP, 8-Br-cAMP (100 μM), nor the direct activator of adenylate cyclase, forskolin (10 μM), had any effect on increases in intracellular calcium stimulated by thrombin (0.3 U/mL), but

each compound inhibited thrombin (0.3 U/mL)-induced increases in [^{3}H]thymidine incorporation. This inhibitory effect of salbutamol on mitogen-induced cell proliferation appears not to rely upon the inhibition of mitogen-induced calcium mobilization. The dissociation of an antiproliferative effect from inhibition of calcium mobilizaton is not unexpected, since it has been reported recently that, in rabbit vascular smooth muscle cells grown in culture, calcium mobilization is unaffected by concentrations of cAMP analogues that inhibit proliferation [42].

Elevated levels of cAMP have been shown to lead to the protein kinase A phosphorylation of the 43 kDa CREB which then results in CREB protein binding to the CRE on the promoter region of target genes and subsequent gene transcription [43, 44]. Salbutamol has been shown to increase CREB protein binding to CRE in human lung preparations [45], and therefore it is possible that the inhibitory effect of salbutamol on mitogen-induced proliferation may be due to this effect. Alternatively, elevated levels of cAMP in rat 1 fibroblasts have been demonstrated to inhibit the Ras-dependent activation of *Raf*-1, a component in the signalling pathway that leads to the activation of mitogen-activated protein kinases p42^{mapk} and p44^{mapk} [31, 32]. These potential mechanisms of action of salbutamol are currently being examined.

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