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β_2-Adrenoceptor agonist-mediated inhibition of human airway smooth muscle cell proliferation: importance of the duration of β_2-adrenoceptor stimulation

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1 Airway hyperresponsiveness in asthma has been ascribed to airway wall thickening as a result of smooth muscle proliferation and hypertrophy. We have previously shown that continuous exposure to the β_2-adrenoceptor agonist, salbutamol inhibits mitogen-induced proliferation of airway smooth muscle cells. In the present study, the effects of variable durations and repeated periods of exposure to β_2-adrenoceptor agonists on DNA synthesis in human cultured airway smooth muscle have been investigated to model some of the possible pharmacokinetic profiles of these agents following inhalation. DNA synthesis was measured by [^{3}H]-thymidine incorporation.

2 Shorter periods of exposure (up to 2.5 h) of airway smooth muscle cells to salbutamol (100 nM) commencing 30 min before thrombin (0.3 u ml^{-1}) stimulation had no effect on the subsequent increase in [^{3}H]-thymidine incorporation. However, inhibition by salbutamol was evident with a 4.5 h exposure and was maximal after an 8.5 h exposure. Similar patterns of results were observed when fenoterol (100 nM) was used in place of salbutamol as the β_2-adrenoceptor agonist or when epidermal growth factor (300 pM) was used in place of thrombin as the mitogen. Salbutamol had no effect on thrombin-stimulated [^{3}H]-leucine incorporation after 8.5 h of exposure, but a statistically significant effect was observed after 48 h of exposure.

3 Experiments in which DNA synthesis was measured up to 52 h after the addition of thrombin indicated that exposure to salbutamol during the first 8 h of mitogen stimulation delayed rather than inhibited the DNA synthesis.

4 Addition of salbutamol (100 nM) at different times either before or up to 24 h after the addition of thrombin indicated that [^{3}H]-thymidine incorporation (measured between 24 and 28 h after thrombin) could be significantly attenuated when salbutamol was added as late as 18 h after the addition of thrombin.

5 The effects of more prolonged exposure to salbutamol were investigated by the addition of salbutamol for either 15 or 24 h per day for a total of 3 days. There were no significant differences in the level of inhibition of thrombin-stimulated [^{3}H]-thymidine incorporation between continuous and intermittent salbutamol over the 3 day period and the inhibition was also not different to that achieved with a single continuous exposure to salbutamol over 28 h.

6 These results indicate that although exposure to β_2-adrenoceptor agonists during the first 8 h of mitogen stimulation does not have a sustained inhibitory effect on DNA synthesis, repeated intermittent or prolonged continuous exposures to salbutamol do inhibit DNA synthesis, without evidence of marked desensitization.

Keywords: Salbutamol; airway smooth muscle; fenoterol; cell proliferation; asthma; cell growth; β_2-adrenoceptors

Introduction

Asthma is a chronic inflammatory disease characterized by the presence of increased airway responsiveness to a range of constrictor substances (Barnes *et al*., 1995). Bronchodilator drugs, primarily β_2-selective adrenoceptor agonists, produce rapid symptomatic relief. However, these bronchodilators act directly to relax airway smooth muscle and do not appear to have marked effects on the underlying inflammation (Barnes & Chung, 1992; Morley, 1993; Barnes, 1995). There have been a series of studies which have identified the possibility of an association between the use of β_2-adrenoceptor agonists and both exacerbations of asthma (Anderson, 1989; Sears *et al*., 1990; Spitzer *et al*., 1992; Ernst *et al*., 1993) and increased airway reactivity (Van Shayck *et al*., 1990), leading to concern over the pattern of β_2-adrenoceptor agonist usage and its potential relationship to epidemiological findings of increasing morbidity and mortality from asthma (Barret & Strom, 1995), although other commentaries suggest that the confounding influence of asthma severity may explain these epidemiological findings (Devoy *et al*., 1995). Nevertheless, this concern has prompted a shift in emphasis in the treatment of asthma from the regular use of short-acting bronchodilator agents towards the prophylactic use of anti-inflammatory agents such as inhaled corticosteroids, since there is evidence that the early use of anti-inflammatory drugs may have a better outcome than the regular use of short-acting bronchodilators (Haahtela *et al*., 1991; Van Esen-Zandvliet *et al*., 1992).

Airway wall remodelling, likely to be a consequence of chronic airways inflammation, involves but is not limited to an increase in the volumetric proportion of airway smooth muscle (Hirst & Twort, 1992; Stewart *et al*., 1993; 1995a). Hypertrophy and hyperplasia of bronchial smooth muscle have been described in *post mortem* tissues obtained from individuals with fatal asthma, from asthmatics who died of unrelated causes and from lung resection specimens of known asthmatic patients (Dunnill *et al*., 1969; Hossain, 1973; Ebina *et al*., 1990; 1993; Kuwano *et al*., 1993). Mathematical studies modelling morphometric data suggest that the amount of airway wall thickening could be as important as the extent of smooth

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muscle shortening in determining airway responsiveness in asthmatics (James *et al*., 1989; Wiggs *et al*., 1992).

Continuous exposure to salbutamol has a substantial direct inhibitory effect on airway smooth muscle proliferation initiated by a range of mitogens (Tomlinson *et al*., 1994). In the present study, the influence of β_2-adrenoceptor agonists has been re-evaluated to take into account the pharmacokinetic profile of short-acting inhaled β_2-adrenoceptor agonists such as salbutamol (Ullman & Svedmyr, 1988) and the repeated and intermittent nature of β-agonist exposure.

Methods

Cell culture

Human airway smooth muscle cells were harvested from bronchi of 0.5–1.5 cm diameter obtained either at lung resection or from lungs of transplant recipients. For each cell culture, approximately 0.1 g of smooth muscle tissue was stripped from the wall of the bronchus. The tissue was initially immersed in Dulbecco's Modified Eagle's Medium (DMEM) (supplemented with 2 mM L-glutamine, 0.25% w/v BSA, 100 u ml^{-1} penicillin-G, 100 $\mu g\ ml^{-1}$ streptomycin and 2 $\mu g\ ml^{-1}$ amphotericin B) containing 3 mg ml^{-1} collagenase for 25 min at 37°C in a humidified incubator containing 5% CO_2 in air. Following partial digestion, the tissue was rinsed in phosphate buffered saline (PBS) to remove the epithelium and was cut into small pieces, approximately 2 mm cubed. The chopped tissue was digested further by incubation in DMEM containing elastase (0.5 mg ml^{-1}) for 2 h, followed by a 12 h incubation in collagenase (1 mg ml^{-1}), at 37°C. The cells were centrifuged (5 min, 100 *g*, room temperature) and washed three times in DMEM. The final cell resuspension was made in 25 ml of DMEM (supplemented with 10% v/v heat-inactivated foetal calf serum (FCS) in place of 0.25% BSA, the medium was otherwise identical to that described above) 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 at a density of approximately 1×10^4 cells/cm^2. Cells at passage numbers 3 to 12 were used for experiments. There was no relationship between passage number and responsiveness to either thrombin or salbutamol (correlation coefficients were −0.016 and −0.132, respectively) (Figure 1a and b). Furthermore, there was no relationship between responsiveness to thrombin and the inhibitory effect of salbutamol ($r^2 = 0.159$, $n = 24$, Figure 1c).

Immunocytochemistry

The expression of smooth muscle α-actin and smooth muscle myosin were used to determine the identity of the cultures (Skalli *et al*., 1986; Mohammad & Sparrow, 1989). Cells were subcultured into 8-well glass tissue culture chamber slides, allowed to grow to confluency in the presence of DMEM (supplemented with 10% FCS), washed three times in PBS before being fixed in ice-cold acetone for 20 s. Fixed cells were then stored at 4°C for up to four weeks before staining. Endogenous peroxidase activity was blocked by incubation in 2% H_2O_2 for 30 min at room temperature, cells were permeabilized in 0.5% triton X-100 in PBS, incubated in PBS containing 0.25% w/v BSA for 20 min then exposed to anti-smooth muscle α-actin, anti-smooth muscle myosin or anti-platelet endothelial cell adhesion molecule-1 (PECAM-1, Mouse monoclonal CD31, a marker of endothelial cells) for 1–2 h at room temperature. This was followed by exposure to anti-mouse or anti-rabbit IgG horseradish peroxidase conjugated immunoglobulin for 1 h at room temperature. Background staining controls were provided by reversal of the order of antibody exposure. The staining of the fixed cells was then observed by light microscopy. Each of the cell cultures used in these studies showed staining for smooth muscle α-actin and myosin in

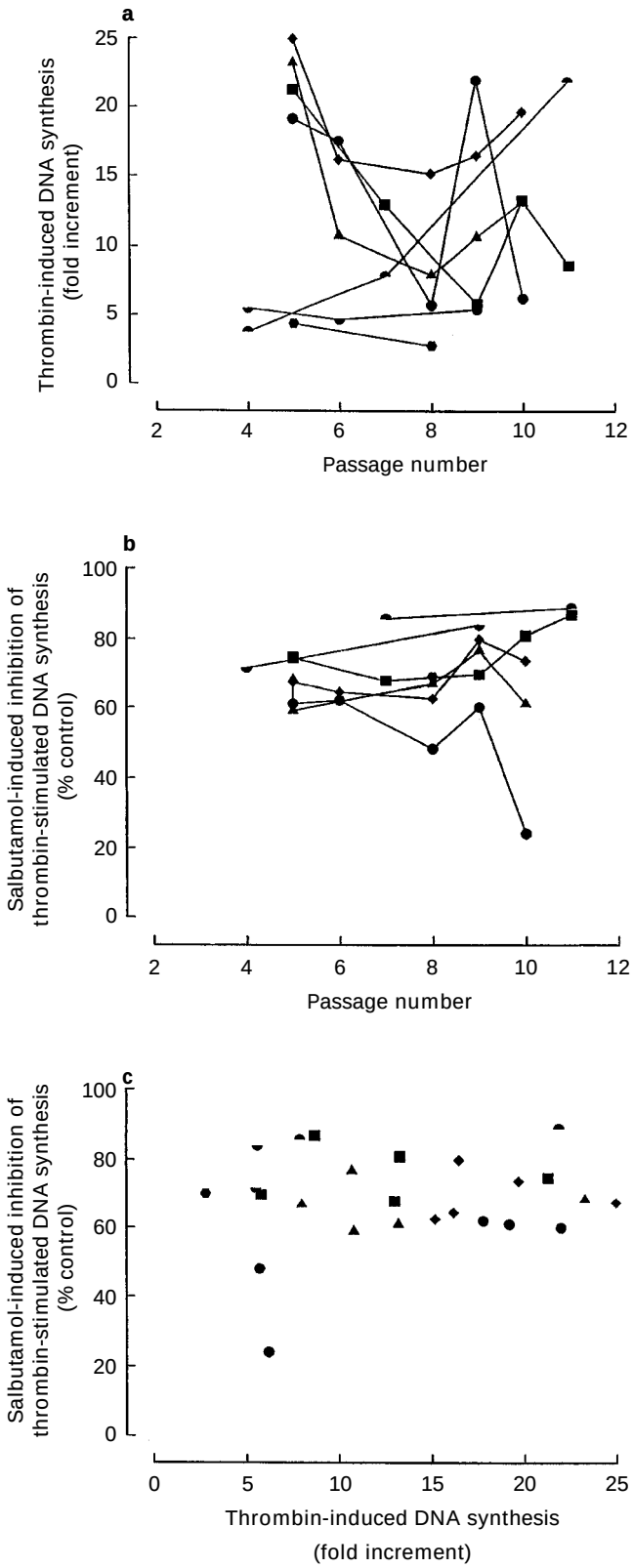

Figure 1 Relationships between passage number and (a) response to thrombin; (b) response to salbutamol and (c) relationship between response to thrombin and response to salbutamol. Responses to thrombin are expressed as fold increases in the incorporation of [^{3}H]-thymidine over the level detected in unstimulated cells. Responses to salbutamol are expressed as percentage inhibition of the response to thrombin. Each separate symbol represents data obtained on a cell culture derived from a different donor. In part (c) each symbol represents data obtained at a specific passage number.

more than 95% of the cells and between the 3rd and 12th passage of cells used in these studies, there did not appear to be any relationship between passage number and the intensity of staining for smooth muscle α-actin. No PECAM-1 staining was detected.

Preparation of reagents

3-Iso-butyl 1-methyl xanthine (IBMX), initially dissolved in 100% dimethyl suphoxide (DMSO) at 100 mM, was diluted 1 in 10 with DMEM (0.25% BSA) to produce a stock solution of 10 mM. The use of a final concentration of 100 μM IBMX resulted in a concentration of 0.1% DMSO which has no effect on DNA synthesis in response to thrombin or other mitogens, or on the inhibitory effects of salbutamol (data not shown). All other agents were added from stock solutions of 100 times the final concentration which were prepared in DMEM (0.25% BSA).

Proliferation assay

Cells were subcultured into 24 well plates at approximately 1.5×10^4 cells/cm^2 and allowed to grow to monolayer confluency over a 72 h period. Twenty-four hours before stimulation the medium was replaced with serum-free DMEM (containing 0.25% BSA) to produce growth arrest and to synchronize cell-cycle progression upon subsequent stimulation with thrombin (0.3 u ml^{-1}) and a supplement containing insulin, transferrin and selenium (Monomed A, 1% v/v). In experiments in which the exposure time to a β_2-adrenoceptor agonist was varied, cells were pretreated (30 min) with the β_2-adrenoceptor agonist, before mitogen stimulation. The β_2-adrenoceptor antagonist, ICI 118551 was added at varying time points to terminate action of the β_2-adrenoceptor agonist (Figure 2). The duration of stimulation with growth factors and growth-promoting substances was 24 h for the [^{3}H]-thymidine incorporation assays (with the exception of the time-course study presented in Figure 6 and Table 2) and 48 h for [^{3}H]-leucine incorporation assays. Cells were incubated for the last 4 h of these incubation periods in the presence of either [^{3}H]-thymidine or [^{3}H]-leucine at a final concentration of 1 μCi ml^{-1} to allow incorporation into DNA and protein, respectively. At the end of the pulse labelling period, the medium containing the radioactivity was aspirated and the cells were lysed by addition of 100 μl 0.1 M NaOH. The DNA and protein were isolated by filtration onto glass fibre filters (Packard, standard) mounted in a binding harvester (Packard Filtermate 196). The filters were then washed extensively with a total volume of 5 ml distilled water and drying was facilitated by a final wash with 2 ml ethanol. The dried filters were counted in a Packard Topcount liquid scintillation counter.

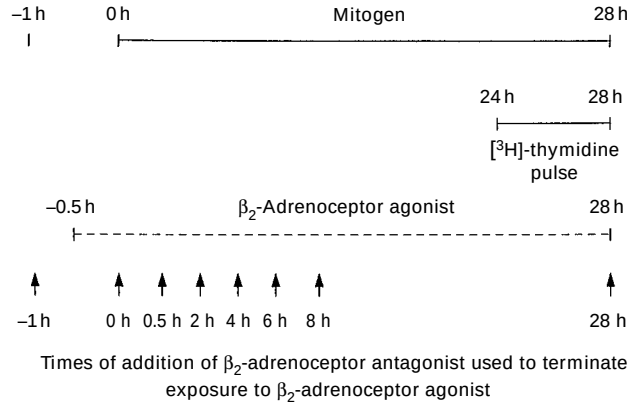

Figure 2 Represents a time line of one of the experimental protocols involved in varying time exposure to the β_2-adrenoceptor agonist, salbutamol. Total mitogen exposure time in proliferation assays with [^{3}H]-thymidine incorporation was 28 h in the data presented in Figures 2–5 (solid line). Cells were incubated with [^{3}H]-thymidine (1 μCi ml^{-1}) for the last four hours of the experiment. The β_2-adrenoceptor agonist, salbutamol (100 nM), was added 30 min before mitogen stimulation. Exposure time to the β_2-adrenoceptor agonist was varied (broken line) by the addition of the β_2-adrenoceptor antagonist, ICI 118551 (50 nM or 1 μM) (solid arrows).

Cyclic AMP assay

Cells were subcultured into 6 well plates at a 1 : 3 split ratio and allowed to grow to confluency over a 72 h period and serum-deprived as described earlier in order to establish conditions identical to those used in DNA and protein synthesis assays. Cells were pretreated either with medium or IBMX (100 μM) for 30 min. The cells were then incubated for a further 60 min period with agents as described in detail in the results section. Cell extracts were made by aspiration of supernatants and addition of assay buffer (50 mM Tris/HCl, 4 mM EDTA, pH 7.5), the cells were then scraped from the culture plates and the samples were heated for 5 min in a boiling water bath to aggregate protein which was removed by centrifugation (10 min, 10,000 *g*, 4°C). The adenosine 3′ : 5′-cyclic monophosphate (cyclic AMP) levels in these supernatants were assayed in duplicate following the suppliers instructions (Amersham Cyclic AMP ^{3}H assay system, TRK 432). The limit of detection of the assay was 5 fmol cyclic AMP μg^{-1} protein.

Materials

All chemicals used were of analytical grade or higher. The compounds used and their sources were as follows: L-glutamine, essentially fatty acid free bovine serum albumin fraction V (BSA), Tyrode buffer, thrombin (bovine plasma), human recombinant [Leu21]-epidermal growth factor (EGF), salbutamol (α-[(t-butylamino)methyl]-4-hydroxy-*m*-xylene-α, α′-diol), fenoterol (2-[3,5-dihydroxyphenyl]-2-hydroxy-2′-[4-hydroxyphenyl]-1′-methyldiethylamine), 3-isobutyl-1-methylxanthine (IBMX), from Sigma, U.S.A.; ICI 118551 (erythrodl-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol), from Research Biochemicals International, U.S.A.; amphotericin B (Fungizone), Gibco Laboratories, U.S.A.; collagenase type CLS 1, elastase, from Worthington Biochemical, U.S.A.; Dulbecco 'A' phosphate buffer saline (PBS), from Oxoid, U.K.; trypsin, versene, penicillin-G, streptomycin, Monomed A, from CSL, Australia; foetal calf serum (FCS), from Flow Laboratories, Australia; Dulbecco's Modified Eagle's Medium (DMEM), from Flow Laboratories, Scotland; [6-^{3}H]-thymidine (185 GBq mmol^{-1}, 5 Ci mmol^{-1}), cyclic AMP ^{3}H assay system (TRK 432), from Amersham, U.K.; L-[4,5-^{3}H(N)]-leucine (2.0 TBq mmol^{-1}, 54 Ci mmol^{-1}) ICN Radiochemicals, U.S.A.; emulsifier-safe scintillant, Canberra-Packard, Australia. Anti-smooth muscle α-actin (mouse monoclonal) (M851), monoclonal mouse anti-human endothelial cell, CD31 (JC/70A) (M823), from Dako Corporation, U.S.A.; rabbit anti-mouse IgG horseradish peroxidase-conjugated; mouse anti-rabbit IgG horseradish peroxidase-conjugated from Silenus Laboratories, Hawthorn, Australia.

Statistics

Incorporation assays with [^{3}H]-thymidine and [^{3}H]-leucine were carried out in quadruplicate and in at least three cell cultures derived from lung resection specimens obtained from separate individuals (*n* denotes the number of different cultures, i.e., the number of cultures, each one derived from a different individual that was used in each protocol). Results are expressed as mean d.p.m. $\pm$ s.e.mean. Student's *t* test was used for comparisons between incubations, and Bonferroni's correction for multiple comparisons was used to correct the *P* value (Wallenstein *et al*., 1980). Data pooled from individual experiments were subjected to statistical analysis, with a *P*

value less than 0.05 being considered significant. Agonist-stimulated [^{3}H]-thymidine or [^{3}H]-leucine incorporation was normalized to the baseline incorporation of ^{3}H to adjust for variations in the level of [^{3}H]-thymidine or [^{3}H]-leucine incorporation between cell cultures. Intracellular cyclic AMP determinations were carried out in triplicate and in 2 to 3 cell cultures derived from lung resection specimens obtained from 2–3 individuals. Results are expressed as mean values ± s.e.-means for n cell cultures.

Results

Mitogen stimulation of [^{3}H]-thymidine incorporation

Thrombin (0.3 u ml^{-1}) elicited a mitogenic response in each of the cell cultures examined, causing an average 11.4 ± 1.7 fold increase in [^{3}H]-thymidine incorporation (n = 8). EGF (300 pM) elicited a 8.4 ± 2.5 fold increase in [^{3}H]-thymidine incorporation (n = 3).

Effect of varying the duration of exposure to salbutamol on inhibition of thrombin-stimulated [^{3}H]-thymidine and [^{3}H]-leucine incorporation

Incubation of smooth muscle cells with the β_2-adrenoceptor agonist salbutamol (100 nM, from 30 min before thrombin until the end of the incubation), at a concentration previously shown to be optimal for inhibition of DNA synthesis (Tomlinson *et al*., 1994), significantly reduced thrombin-induced incorporation of [^{3}H]-thymidine to 26 ± 4% of the control response (P < 0.05, Student's t test, n = 8). The inhibitory effect of salbutamol (100 nM) on [^{3}H]-thymidine incorporation in cells stimulated with thrombin was reduced (82 ± 4% of thrombin, n = 6) by the addition 30 min beforehand of the selective β_2-adrenoceptor antagonist ICI 118551 at 50 nM. ICI 118551 had no direct effect on thrombin-stimulated [^{3}H]-thymidine incorporation (Tomlinson *et al*., 1995). Addition of ICI 118511 (50 nM) at 0.5, 1, 2.5, and 4.5 h after the commencement of the salbutamol exposure prevented the consequent inhibition of thrombin-stimulated [^{3}H]-thymidine incorporation, but when it was added at 8.5 h or later, the action of salbutamol was unaffected (P > 0.05, Student's t test, n = 3) (Figure 3).

Incubation with thrombin for 48 h elicited a 2.20 ± 0.02 fold increase in the rate of [^{3}H]-leucine incorporation (n = 3). In cells incubated for 48.5 h with salbutamol (100 nM), the thrombin-stimulated [^{3}H]-leucine incorporation was reduced to 62 ± 6% of the control response (P < 0.05, paired Student's t test, n = 3). Addition of ICI 118551 (50 nM) 30 min before, or up to 8.5 h after salbutamol prevented the inhibition of thrombin-stimulated [^{3}H]-leucine incorporation (Figure 3).

Effect of varying the duration of exposure to β_2-adrenoceptor agonists on inhibition of mitogen-stimulated [^{3}H]-thymidine incorporation

The initial time-course experiments were carried out with 50 nM ICI 118551 (Figure 3), but the incomplete inhibition of the effect of salbutamol necessitated the repetition of these experiments at the higher concentration of ICI 118551 (1 μM) which prevented the inhibition by salbutamol of thrombin-stimulated [^{3}H]-thymidine incorporation (134 ± 17% of thrombin response, n = 3). Repeating the experiment with ICI 1185551 (1 μM) showed that β_2-adrenoceptor blockade prevented the inhibition by salbutamol of thrombin-stimulated [^{3}H]-thymidine incorporation when the receptor antagonist was added at 0.5, 1 and 2.5 h after salbutamol, but not when it was added at or later than 4.5 h after salbutamol (P > 0.05, Student's t test, n = 3) (Figure 4).

Incubation with salbutamol (100 nM, 30 min before the addition of EGF until the end of the experiment) significantly reduced the EGF (300 pM)-stimulated incorporation of [^{3}H]-thymidine to 38 ± 13% of the control response (P < 0.05, Student's t test, n = 3) and this reduction was prevented by the addition of ICI 118551 (1 μM, 30 min before salbutamol). Addition of ICI 118551 at 0.5 and 2.5 h after salbutamol also prevented the inhibition of EGF (300 pM)-stimulated [^{3}H]-thymidine incorporation, whereas no effect was observed after the addition at 4.5, 8.5 or 28.5 h (P > 0.05, Student's t test, n = 3) (Figure 4).

Incubation of smooth muscle cells with another β_2-adrenoceptor agonist, fenoterol (100 nM, 30 min before thrombin until the end of the experiment) significantly inhibited the

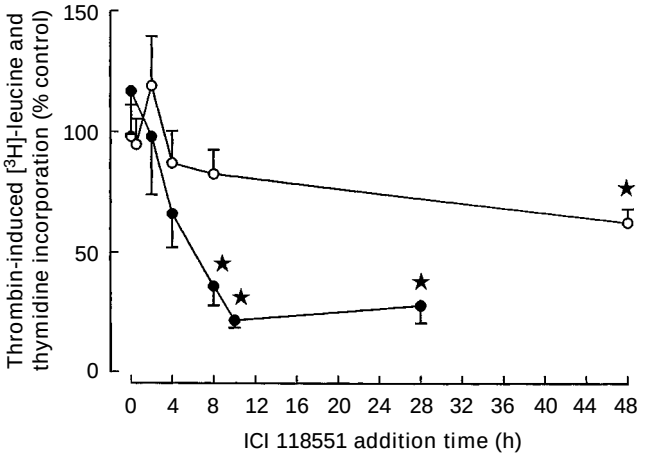

Figure 3 Effect of salbutamol (100 nM, added 30 min before thrombin) on [^{3}H]-thymidine (●) or [^{3}H]-leucine (○) incorporation in cells stimulated with thrombin (0.3 u ml^{-1}). ICI 118551 (50 nM) was added at a concentration of 50 nM at various times from 1 h before thrombin (defined as zero time) up to 48 h after thrombin to terminate the action of salbutamol. The data for [^{3}H]-thymidine or [^{3}H]-leucine incorporation in cells incubated with salbutamol represent percentages of the response to thrombin in the absence of salbutamol and are presented as the mean of three experiments with three cell cultures, each experiment being carried out in quadruplicate; vertical lines show s.e.mean. *P < 0.05, Student's unpaired t test, compared to thrombin-stimulated cells.

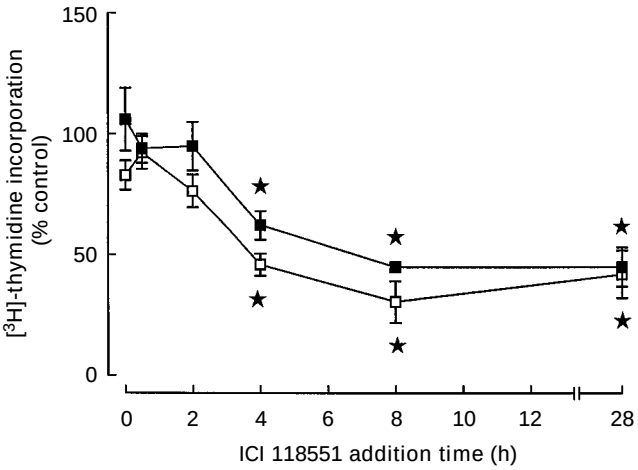

Figure 4 Effect of salbutamol on [^{3}H]-thymidine incorporation in cells stimulated with thrombin (0.3 u ml^{-1}, ■) or EGF (300 pM, □). The design of the experiment was identical to that shown in Figure 3 with the exception that ICI 188551 (1 μM) was used at a concentration of 1 μM. The data are expressed as percentages of the [^{3}H]-thymidine incorporation in response to either EGF or thrombin in the absence of salbutamol treatment and represent the means and s.e.mean (vertical lines). *P < 0.05, Student's unpaired t test, compared to cells stimulated with thrombin in the absence of salbutamol treatment.

incorporation of [^{3}H]-thymidine to 21±4% of the control response ($P<0.05$, Student's t test, $n=3$). Addition of ICI 118551 (1 μM) 30 min beforehand (106±6% control) or at 0.5, 1 or 2.5 h after fenoterol (101±3%; 107±9%; 74±7%, respectively) prevented the inhibitory effect on [^{3}H]-thymidine incorporation ($P<0.05$, Student's t test, $n=3$), whereas addition of ICI 118551 at 4.5 h (62±7% control) or 8.5 h (34±4% control) failed to prevent the inhibitory effect of fenoterol as observed with salbutamol ($P>0.05$, Student's t test, $n=3$).

Effects of ICI 118551 on salbutamol-induced increases in intracellular cyclic AMP

Experiments were conducted to establish whether the treatment with ICI 118551 reduced increases in cyclic AMP following stimulation by salbutamol. To enhance the effects of salbutamol in increasing levels of cyclic AMP IBMX (100 μM) was added 1 h before salbutamol and left in the medium until the end of the experiment. No differences in basal levels of intracellular cyclic AMP were observed after IBMX treatment (data not shown). Incubation with salbutamol (100 nM) alone for 30 min, with a further 30 min incubation in the presence of the selective β_2-adrenoceptor antagonist, ICI 118551 (50 nM), reduced intracellular cyclic AMP to levels which were not different from those of unstimulated cells. The basal level of cyclic AMP in cells incubated for 24 h (matching the duration of most experiments in which DNA synthesis was measured) was not different from that during the 1 h incubation, whereas the level in salbutamol-stimulated cells was reduced compared with that following stimulation by salbutamol (Table 1).

Effects of an 8.5 h exposure to salbutamol on DNA synthesis determined at different times after mitogen stimulation

Data from experiments examining the inhibitory effects of salbutamol, during the first 8 h of thrombin-stimulation indicated that there was maximal inhibition of DNA synthesis (Figures 3 and 4). However, measurement of [^{3}H]-leucine incorporation, as an index of changes in cell mass, was not affected by the 8.5 h exposure to salbutamol (Figure 3). To investigate whether the 8.5 h exposure to salbutamol caused a sustained inhibition of DNA synthesis, the cells were exposed to [^{3}H]-thymidine for 4 h commencing at 24, 28, 32 or 48 h after exposure to thrombin and the results obtained were compared with those obtained with a continuous exposure to salbutamol. The level of inhibition of the thrombin response by continuous exposure to salbutamol was significantly greater than that observed with exposure during the first 8.5 h (Figure 5). When the [^{3}H]-thymidine incorporation was examined after longer periods of incubation in thrombin up to 52 h, the magnitude of the inhibitory effect of continuous exposure to salbutamol was maintained, whereas the inhibitory effect of the 8.5 h exposure to salbutamol was significantly reduced ($P<0.05$, unpaired Student's t test) from 55% at 28 h to 24% at 52 h (Figure 5).

Effects of prolonged exposure to salbutamol on DNA synthesis

The preceding experiments suggested that a single exposure exceeding the normal duration of action of salbutamol had no sustained inhibitory effect on DNA synthesis in the continued presence of thrombin. However, this protocol is not analogous to the pattern of exposure observed with regular use of salbutamol. Therefore, the effects of two different incubation patterns of salbutamol were examined: in one series, salbutamol was present from 9 h after the beginning of incubation with thrombin until the end of the 81 h incubation period; in the second series, salbutamol was added 9 h after the beginning of the incubation and was removed after a further 15 h by aspirating the DMEM, washing with 1 ml of PBS (0.25% BSA) then adding 1 ml of DMEM without salbutamol for a 9 h period at the end of which salbutamol was reintroduced for 15 h and the cycle was repeated once more. Thrombin was present from 9 h before the addition of salbutamol to the end of the 81 h incubation period. In the second series, the thrombin was replaced after each washing step.

The thrombin (0.3 u ml^{-1})-stimulated incorporation of [^{3}H]-thymidine was 17.4±7.6 (fold increase over the basal level of incorporation). The level of inhibition of thrombin- stimulated incorporation of [^{3}H]-thymidine produced by salbutamol present continuously was not significantly different ($P>0.05$, paired Student's t test) from that of exposure to salbutamol for 15 h per day (Table 2).

The effects of post-*mitogen addition of salbutamol on [^{3}H]-thymidine incorporation*

All previous experiments with salbutamol had involved its addition to the culture medium before the cells were exposed to mitogen. A time-course experiment was therefore carried out to determine whether *post*-mitogen addition of salbutamol inhibited [^{3}H]-thymidine incorporation and to identify the latest point in the progression of the cells through G1 phase of

Table 1 Salbutamol-induced increases in intracellular cyclic AMP in IBMX (100 μM) pretreated cells

	Cyclic AMP (fmol μg^{-1} protein) *Incubation time*	
Treatment	*1 h*	*24 h*
Vehicle	5±2 ($n=3$)	6 ($n=2$)
Salbutamol (100 nM)	103±13 ($n=3$)*	22 ($n=2$)
Salbutamol (100 nM) +ICI 118551 (50 nM)	15±7 ($n=3$)	–

Data represent the means and s.e.mean of experiments with n cell lines, each experiment being carried out in triplicate. *$P<0.05$, paired Student's t test compared to vehicle.

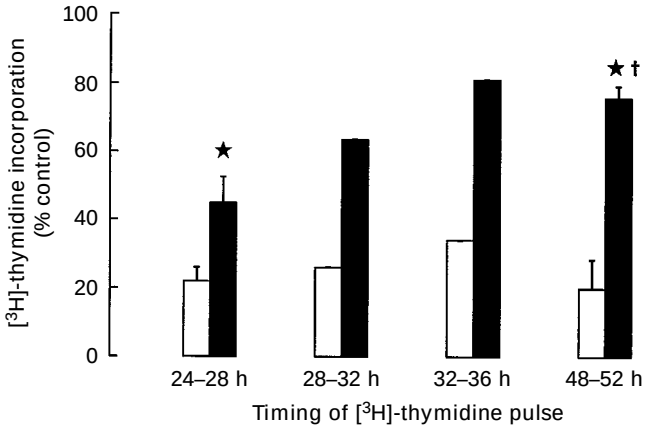

Figure 5 Comparison of the effects of continuous and brief (8.5 h) exposure to salbutamol (100 nM, added 30 min before thrombin) on [^{3}H]-thymidine incorporation in cells stimulated with thrombin (0.3 u ml^{-1}). Either vehicle (open columns) or ICI 118551 (1 μM) was added 8 h after the addition of thrombin (solid columns). The [^{3}H]-thymidine pulse was carried out over a four hour period commencing (in separate groups of cells) at 24, 28, 32 or 48 h after the addition of thrombin. Data are presented as the mean±s.e.mean of 4 experiments conducted in 4 cell cultures (24 and 48 h time points; $n=2$ cell cultures at 28 and 32 h time points). *$P<0.05$, Student's paired t test, between continuous and 8 h exposures at the same time point (i.e. 24 or 48 h). †$P<0.05$, Student's unpaired t test, between incorporation at 48 h and that at 24 h under either continuous or 8.5 h exposure to salbutamol.

the cell cycle at which inhibition still occurred. The maximum level of inhibition by salbutamol was achieved when it was added between 2 and 12 h after thrombin addition and left in the medium until the end of the [^{3}H]-thymidine pulse (24–28 h). Addition of salbutamol 12 h or more after thrombin resulted in a decline in the inhibition and no inhibition was observed when the salbutamol was added at 22 h or later (Figure 6).

Discussion

Continuous exposure to the bronchodilator, salbutamol, inhibits mitogen-induced proliferation of human airway smooth muscle cells (Tomlinson *et al*., 1994) through elevation of intracellular cyclic AMP (Tomlinson *et al*., 1995). We now show that short durations of exposure to β_2-adrenoceptor agonists of up to 8.5 h have either no effect or only a short-lasting inhibitory effect on DNA synthesis in the continued presence of mitogen. However, importantly, more prolonged exposure to salbutamol over 72 h, either continuously or for 15 h per day inhibits thrombin-stimulated DNA synthesis without evidence of marked desensitization.

Table 2 Effects of repeated or continuous exposures to salbutamol (100 nM) on inhibition of thrombin (0.3 u ml^{-1})-stimulated [^{3}H]-thymidine incorporation

Treatment	*[^{3}H]-thymidine incorporation (% response to thrombin in untreated cells)*
Vehicle	100
Salbutamol continuous	29.2 ± 2.8*
Salbutamol 15 h /day	38.3 ± 10.3*

Data represent the means and s.e.mean of 4 experiments with 4 different cell cultures, each experiment being carried out in quadruplicate. *$P<0.05$, paired Student's t test compared to vehicle.

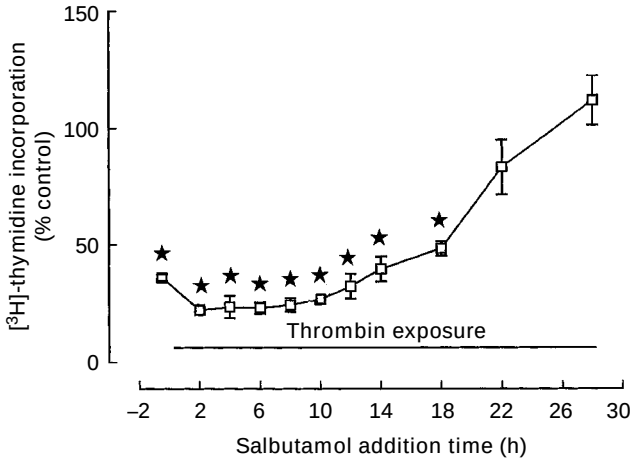

Figure 6 The effect of salbutamol (100 nM), added at different times from 0.5 h before thrombin up to 28 h afterwards, on thrombin-stimulated DNA synthesis, measured between 24 and 28 h after the addition of thrombin (defined as time zero). The results are presented as means and s.e.mean (vertical lines) of the grouped data from 3 individual experiments in 3 separate cell lines (each carried out in quadruplicate) and represent [^{3}H]-thymidine incorporation expressed as a percentage of the incorporation in non-pretreated, thrombin-stimulated cells. *$P<0.05$, paired Student's t test, compared with DNA synthesis observed in non-pretreated, thrombin-stimulated cells.

In conventional cell culture studies, cells are bathed continuously in medium containing fixed levels of test substances. In studies of airway smooth muscle cell proliferation, the minimum period of incubation required to measure DNA synthesis is between 24 and 28 h, since these cells enter S-phase approximately 22 h after exposure to thrombin (Stewart *et al*., 1995a). Other parameters indicative of cell proliferation, such as changes in cell number or increases in protein synthesis (Stewart *et al*., 1995b), require longer incubation periods (36–48 h). Many of the agents which are now being evaluated for effects on cell proliferation in culture are unlikely to be at active concentrations *in vivo* continuously over these periods of time.

Salbutamol is a relatively short acting bronchodilator with an effective duration of action of 3–5 h (Ullman & Svedmyr, 1988), but is often used repeatedly for symptomatic relief or regularly for prophylaxis and therefore the possible range of exposure periods is large and variable. The current study specifically examined the effects of salbutamol exposure periods of between 1 and 72 h to cover both longer and shorter periods of exposure than the 28 h that was examined previously (Tomlinson *et al*., 1994), since the previous protocol represented only one of the many patterns of exposure to this agent.

The effectiveness of the selective β_2-adrenoceptor antagonist, ICI 118551 in terminating the action of salbutamol was confirmed by the reduction in the level of cyclic AMP to control levels within 30 min of its addition, consistent with previous findings (Hall *et al*., 1992; Tomlinson *et al*., 1994). There was no decline in the degree of stimulation of cyclic AMP levels by salbutamol 1 h after its addition. However, after 24 h exposure to salbutamol the levels of cyclic AMP were considerably reduced, but remained above those in unstimulated cells. This desensitization is similar to that described by Hall *et al*. (1993), but does not appear to be sufficient to cause any marked desensitization in the level of inhibition of the thrombin-stimulated DNA synthesis. It remains possible that the desensitization of elevation cyclic AMP in response to salbutamol occurs more rapidly than the inhibitory effect on DNA synthesis can be observed, which is with a minimum of 2.5 to 4.5 h of exposure to salbutamol.

Salbutamol treatment of airway smooth muscle for the first 2 h of exposure to thrombin had no effect on subsequent DNA synthesis, but when the treatment was extended to the first 4 h, an inhibitory effect was observed which became maximal by 8 h and similar observations were made with another short-acting β_2-adrenoceptor agonist, fenoterol (Nials *et al*., 1993). The pattern of inhibition by salbutamol was not specific to thrombin as a mitogen since similar results were obtained when EGF was used.

The discrepancy between the ability of 8.5 h exposure to salbutamol to inhibit DNA synthesis, measured at 24 h, and its failure to inhibit protein synthesis measured at 48 h suggested that the increase in cell mass had been delayed rather than inhibited. In contrast, continuous exposure to salbutamol reduced the thrombin-stimulated increase in cell mass. In cells exposed to salbutamol for the first 8 h of thrombin exposure, the inhibition of the thrombin-stimulated DNA synthesis declined from approximately 70% at 24 h to 20% at 48 h indicating that this duration of exposure delays rather than inhibits DNA synthesis.

There is a large range of possible sequences and durations of exposure to the agonists and mitogens which would depend on the frequency and pattern of β_2-adrenoceptor agonist usage and the severity of the asthma. The number of experiments required to examine all the possible permutations and combinations in the timing and durations of exposure to β_2-adrenoceptor agonist and mitogens is beyond practical limits. Therefore, caution is required in extrapolating from the results of our cell culture studies to events in asthmatics with β_2-adrenoceptor agonists. Notwithstanding this need for caution, in our experiments several scenarios have been examined. The more extensive studies involved the addition of the β_2-adre-

noceptor agonist before mitogen stimulation in an attempt to mimic a pattern of regular prophylactic β_2-adrenoceptor agonist usage. An additional protocol was followed to provide a pattern of exposure more analogous to that of regular usage. The decline in levels of β_2-adrenoceptor agonist during sleep may release from inhibition cells that had been previously exposed to mitogen in the presence of β_2-adrenoceptor agonist. Thus, the effects of exposure to salbutamol continuously or for 15 h per day were contrasted. These distinct patterns of exposure showed the same level of inhibition by salbutamol, indicating that over the 3 days of exposure, the 9 h per day during which salbutamol was absent from the medium did not result in any loss of inhibitory effect. The addition of salbutamol up to 18 h after the mitogen and its continuous presence throughout the remainder of the experiment inhibited DNA synthesis, indicating the potential for brief periods of exposure to have inhibitory effects depending on the timing of this exposure relative to the entry of the cells into S-phase. This pattern of exposure, in which mitogen is present before the β_2-adrenoceptor agonist, may occur *in vivo* when salbutamol is used symptomatically. Inhibition was completely lost if salbutamol was added at 20 h or more after the addition of thrombin, whereas there was approximately 50% inhibition of DNA synthesis when salbutamol was added at 18 h. Thus, stimulation of β_2-adrenoceptors for a period of 2 h is sufficient to inhibit DNA synthesis, suggesting that both the timing of the β_2-adrenoceptor agonist exposure and its duration are determinants of the level of inhibition.

One intriguing, and as yet unexplained effect of regular use of β_2-adrenoceptor agonists, is an apparent link to increases in airway hyperresponsiveness (Anderson, 1989; Sears *et al*., 1990; VanShayck *et al*., 1990; Page, 1991; Spitzer *et al*., 1992; Ernst *et al*., 1993). Much of the increased airway responsiveness in asthma may result from an airway wall remodelling process (Hirst & Twort, 1992; Stewart *et al*., 1993; 1995b) comprising loss of epithelial cells, subepithelial fibrosis, mucous cell hyperplasia (Djukanovic *et al*., 1990) and an increase in the volume of airway smooth muscle (James *et al*., 1989; Wiggs *et al*., 1992) which correlates with disease severity (Kuwano *et al*., 1993). These changes may be a response to chronic airway inflammation produced by resident and infiltrating inflammatory cells such as eosinophils and mast cells (Jeffery *et al*., 1989; Djukanovic *et al*., 1990). Our findings suggest that both continuous and intermittent chronic exposure to β_2-agonists inhibits airway smooth muscle hyperplasia *in vitro*. In view of the duration of action of short-acting β_2-agonists, it seems less likely that their sporadic, symptomatic use would inhibit an underlying chronic airway smooth muscle proliferation. The closest clinical corollary of continuous exposure of cells in culture, with the exception of the newly introduced long-acting β_2-adrenoceptor agonists, is that of regular usage of β_2-adrenoceptor agonists. This latter pattern of usage is the one most clearly (Sears *et al*., 1990), but not universally (Chapman *et al*., 1994) linked to increased airway hyperresponsiveness. Our findings predict that regular usage of β_2-agonists would attenuate rather than enhance airway smooth muscle proliferation and consequently diminish rather than exacerbate airway hyperresponsiveness, but do not exclude the possibility that β_2-adrenoceptor agonists could enhance airway hyperresponsiveness by mechanisms unrelated to airway smooth muscle cell proliferation.

In summary, periods of a single exposure to short-acting β_2-adrenoceptor agonists, that match or exceed the clinical duration of action of a single inhalation, have no inhibitory effect on airway smooth muscle mitogenic responses. However, more importantly, repeated exposure to salbutamol in airway smooth muscle stimulated with thrombin does not reduce the capacity of salbutamol to inhibit DNA synthesis.

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References

ANDERSON, H.R. (1989). Increase in hospital admissions for childhood asthma: trends in referral, severity, and readmissions from 1970 to 1985 in a health region of the United Kingdom. *Thorax*, **44,** 614–619.

BARNES, P.J. (1995). Beta-adrenergic receptors and their regulation. *Am. J. Respir. Crit. Care Med*., **152,** 838–860.

BARNES, P.J. & CHUNG, K.F. (1992). Questions about inhaled β_2-adrenoceptor agonists in asthma. *Trends Pharmacol. Sci*., **13,** 20–23.

BARNES, P.J., HOLGATE, S.T., LAITINEN, L.A. & PAUWELS, R. (1995). Asthma mechanisms, determinants of severity and treatment: the role of nedocromil sodium. *Clin. Exp. Allergy*, **25,** 771–787.

BARRET, T.E. & STROM, B.L. (1995). Inhaled beta-adrenoceptor agonists: more harm than good? *Am. J. Resp. Crit. Care Med*., **151,** 574–577.

CHAPMAN, K.R., KESTEN, S. & SZALAI, J.P. (1994). Regular vs as-needed inhaled salbutamol in asthma control. *Lancet*, **343,** 1379–1382.

DEVOY, M.A.B., FULLER, R.W. & PALMER, J.B.D. (1995). Are there any detrimental effects of the use of long-acting β_2-agonists in the treatment of asthma and chronic obstructive pulmonary disease? *Chest*, **107,** 1116–1124.

DJUKANOVIC, R., ROCHE, W.R., WILSON, J.W., BEASLEY, C.R., TWENTYMAN, O. & HOLGATE, S.T. (1990). The role of mucosal inflammation in asthma. State of the art review. *Am. Rev. Respir. Dis*., **142,** 434–457.

DUNNILL, M.S., MASSARELLA, G.R. & ANDERSON, J.A. (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.

EBINA, M., TAKAHASHI, T., CHIBA, T. & MOTOMIYA, M. (1993). Cellular hypertrophy and hyperplasia of airway smooth muscle underlying bronchial asthma. A 3-D morphometric study. *Am. Rev. Resp. Dis*., **148,** 720–726.

EBINA, M., YAEGASHI, H., CHIBA, R., TAKAHASHI, T., MOTOMIYA, M. & TANEMURA, M. (1990). Hyperreactive site in the airway tree of asthmatic patients revealed by thickening of bronchial muscles. *Am. Rev. Resp. Dis*., **141,** 1327–1332.

ERNST, P., HABBICK, B., SUISSA, S., HEMMELGARN, B., COCKROFT, D., BUIST, A.S., HORWITZ, R.I., MCNUTT, M. & SPITZER, W.O. (1993). Is the association between inhaled beta-agonist use and life-threatening asthma because of confounding by severity? *Am. Rev. Respir. Dis*., **148,** 75–79.

HAAHTELA, T., JARVINEN, M., KAVA, T., KIVIRANTA, K., KOSKINEN, S., LEHTONEN, K., NIKANDER, K., PERSSON, T., REINIKAINEN, K., SELROOS, O., SOVIJARVI, A., STENIUS-AARNIALA, B., SVAHN, T., TAMMIVAARA, R. & LAITINEN, L.A. (1991). Comparison of β_2-agonist, terbutaline, with inhaled corticosteroid, budesonide, in newly detected asthma. *N. Engl. J. Med*., **325,** 388–392.

HALL, I.P., DAYKIN, K. & WIDDOP, S. (1993). β_2-adrenoceptor desensitization in cultured human airway smooth muscle. *Clin. Sci*., **84,** 151–157.

HALL, I.P., WIDDOP, S., TOWNSEND, P. & DAYKIN, K. (1992). Control of cyclic AMP levels in primary cultures of tracheal smooth muscle cells. *Br. J. Pharmacol*., **107,** 422–428.

HIRST, S.J. & TWORT, C.H. (1992). The proliferative response of airway smooth muscle. *Clin. Exp. Allergy*, **22,** 907–915.

HOSSAIN, S. (1973). Quantitative measurement of bronchial muscle in men with asthma. *Am. Rev. Respir. Dis*., **107,** 99–109.

JAMES, A.L., PARE, P.D. & HOGG, J.C. (1989). The mechanisms of airway narrowing in asthma. *Am. Rev. Respir. Dis.*, **139,** 242–246.

JEFFERY, P.K., WARDLAW, A.L., NELSON, F.C., COLLINS, J.V. & KAY, A.B. (1989). Bronchial biopsies in asthma: an ultrastructural, quantitative study and correlation with hyperreactivity. *Am. Rev. Respir. Dis.*, **140,** 1745–1753.

KUWANO, K., BOSKEN, C.H., PARE, P.D., BAI, T.R., WIGGS, B.R. & HOGG, J.C. (1993). Small airways dimensions in asthma and chronic obstructive pulmonary disease. *Am. Rev. Respir. Dis.*, **148,** 1220–1225.

MOHAMMAD, M.A. & SPARROW, M.P. (1989). The distribution of heavy chain isoforms of myosin in airways smooth muscle from adult and neonatal humans. *Biochem. J.*, **260,** 421–426.

MORLEY, J. (1993). Immunopharmacology of asthma. *Trends Pharmacol. Sci.*, **14,** 208–213.

NIALS, A.T., COLEMAN, R.A., JOHNSON, M., MAGNUSSEN, H., RABE, K.F. & VARDEY, C.J. (1993). Effects of β-adrenoceptor agonists in human bronchial smooth muscle. *Br. J. Pharmacol.*, **110,** 1112–1116.

PAGE, C.P. (1991). One explanation for the asthma paradox: inhibition of natural anti-inflammatory mechanism by β_2-agonists. *Lancet*, **337,** 717–720.

SEARS, M.R., TAYLOR, D.R., PRINT, C.G., LAKE, D.C., LI, Q., FLANNERY, E.M., YATES, D.M., LUCAS, M.K. & HERBISON, G.P. (1990). Regular inhaled beta-agonist treatment in bronchial asthma. *Lancet*, **336,** 1391–1396.

SKALLI, O., ROPRAZ, P., TRZECIAK, A., BENZONANA, G., GILLESSEN, G. & GABBIANI, G. (1986). A monoclonal antibody against α-smooth muscle actin: a new probe for smooth muscle differentiation. *J. Cell Biol.*, **103,** 2787–2796.

SPITZER, W.O., SUISSA, S., ERNST, P., HORWITZ, R.I., HABBICK, B., COCKCROFT, D., BOIVIN, J-F., MCNUTT, M., BUIST, A.S. & REBUCK, A.S. (1992). The use of β_2-agonists and the risk of death and near death from asthma. *N. Engl. J. Med.*, **326,** 501–506.

STEWART, A.G., FERNANDES, D. & TOMLINSON, P.R. (1995a). The effect of glucocorticoids on proliferation of human cultured airway smooth muscle. *Br. J. Pharmacol.*, **116,** 3219–3226.

STEWART, A.G., TOMLINSON, P.R. & J. WILSON. (1993). Airway wall remodelling in asthma: a novel target for the development of anti-asthma drugs. *Trends Pharmacol. Sci.*, **14,** 275–279.

STEWART, A.G., TOMLINSON, P.R. & J. WILSON. (1995b). Regulation of airway wall remodelling: prospects for the development of novel anti-asthma drugs. *Adv. Pharmacol.*, **33,** 209–253.

TOMLINSON, P.R., WILSON, J.W. & STEWART, A.G. (1994). Inhibition by salbutamol of the proliferation of human airway smooth muscle cells grown in culture. *Br. J. Pharmacol.*, **111,** 641–647.

TOMLINSON, P.R., WILSON, J.W. & STEWART, A.G. (1995). Salbutamol inhibits the proliferation of human airway smooth muscle cells grown in culture: relationship to elevated cAMP levels. *Biochem. Pharmacol.*, **49,** 1809–1819.

ULLMAN, A. & SVEDMYR, N. (1988). Salmeterol, a new long acting inhaled β_2-adrenoceptor agonist: comparison with salbutamol in adult asthmatic patients. *Thorax*, **43,** 674–678.

VAN ESSEN-ZANDVLIET, E.E., HUGHES, M.D., WAALKENS, H.J., DUIVERMAN, E.J., POCOCK, S.J., KERREBIJN, K.F. AND THE DUTCH CHRONIC NON-SPECIFIC LUNG DISEASE STUDY GROUP. (1992). Effects of 22 months of treatment with inhaled corticosteroids and/or beta-2-agonists on lung function, airway responsiveness, and symptoms in children with asthma. *Am. Rev. Respir. Dis.*, **146,** 547–554.

VAN SHAYCK, C.P., GRAAFSMA, S.J., VISCH, M.B., DOMPELING, E., VAN WEEL, C. & VAN HERWAARDEN, C.L.A. (1990). Increase bronchial hyperresponsiveness after inhaling salbutamol during 1 year is not caused by subsensitisation to salbutamol. *J. Allergy Clin. Immunol.*, **86,** 792–800.

WALLENSTEIN, S., ZUCKER, C.L. & FLEISS, J.L. (1980). Some statistical methods useful in circulation research. *Circ. Res.*, **47,** 1–9.

WIGGS, B.R., BOSKEN, C., PARE, P.D. & HOGG, J.C. (1992). A model of airway narrowing in asthma and in chronic obstructive pulmonary disease. *Am. Rev. Respir. Dis.*, **145,** 1251–1258.

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