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Effects of glucocorticoids and β-adrenoceptor agonists on the proliferation of airway smooth muscle

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Abstract

An increase in airway smooth muscle is a characteristic feature of asthma. Because β-adrenoceptor agonists and corticosteroids are commonly used in the treatment of asthma we have studied the effects of these medicines on the growth of airway smooth muscle. These agents were incubated with bovine airway smooth muscle cells for 40 h for measurement of thymidine incorporation and 64 h for measurement of cell counts. Salbutamol inhibited thymidine incorporation (IC_{50} = 60 nM) and led to a reduction in cell number (IC_{50} = 10 nM). At 10 μM there was a 14.6 ± 2.6% reduction in cell number. Salmeterol also inhibited the growth of the airway smooth muscle cells but the effect did not plateau at 10 μM. At this concentration there was an 89.5 ± 3.6% reduction in thymidine incorporation and a 44.1 ± 5.2% reduction in cell number. Cortisol and beclomethasone dipropionate were more potent than salbutamol in inhibiting thymidine incorporation with IC_{50} values of 5 nM and 0.2 nM respectively. Cortisol 100 nM led to a 16.6 ± 6.5% reduction and beclomethasone dipropionate 3 nM led to a 17.8 ± 5.8% reduction in cell number. If similar effects occur in man and in vivo, these medicines could act directly on airway smooth muscle to inhibit the development of hyperplasia.

Keywords: Smooth muscle, airway; Asthma; Glucocorticoid; β-Adrenoceptor agonist, cell culture; Cell proliferation

1. Introduction

Morphometric studies which have compared the airways of patients dying from asthma with those of other individuals show that the airway wall is thickened in asthma (James et al., 1989). Computer modelling shows that this increase in airway wall thickness could account for bronchial hyperresponsiveness (Hogg et al., 1987; Lambert et al., 1993), which is an extreme sensitivity of the airways to a wide range of physical and chemical stimuli, and is a characteristic feature of asthma (Boushey et al., 1980). Patients with asthma have an increase in maximal airway narrowing and this observation can also be explained by increases in airway wall thickness (Lambert et al., 1992). There is an increase in airway smooth muscle in asthma (Dunnill, 1960; Heard and Hussain, 1971) which is believed to be due primarily to smooth muscle hyperplasia although in some individuals hypertrophy of smooth muscle cells may be important (Ebina et al., 1993). This increase in airway smooth muscle is one of the most important components of the airway wall thickening in asthma (James et al., 1989).

In view of these observations it would clearly be advantageous if drugs used in the treatment of asthma inhibited the growth of airway smooth muscle. Inhaled corticosteroids now have an important role in the treatment of asthma. The actions of corticosteroids in asthma are usually explained in terms of their actions on eosinophils and other inflammatory cells (Schleimer, 1990). They may however have an additional direct effect on airway smooth muscle to prevent its hyperplasia. Corticosteroids certainly inhibit the growth of vascular smooth muscle cells (Jarvelinen et al., 1982; Longnecker et al., 1982; Longnecker et al., 1984; Hirosumi et al., 1987; Berk et al., 1987) and of many other cell types but their effects on airway smooth muscle

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cells have not previously been reported. We have studied the effect of cortisol and beclomethasone dipropionate on the proliferation of bovine airway smooth muscle cells.

There has been considerable controversy about the effects of treatment with β-adrenoceptor agonists in asthma. Recently it has been suggested that the use of fenoterol, a β-adrenoceptor agonist, was associated with an increase in asthma deaths in New Zealand (Crane et al., 1989). Sears et al. (1990) conducted a double-blind crossover study comparing 6 months of treatment with regular fenoterol with the as required use of inhaled β-adrenoceptor agonists. They found that the regular use of fenoterol was associated with worse control of the asthma and an increase in bronchial responsiveness. If regular β-adrenoceptor agonists do have a deleterious effect in asthma the mechanism is not at all clear. One possibility would be that β-adrenoceptor agonists could promote the growth of airway smooth muscle leading to an increase in bronchial responsiveness.

Catecholamines stimulate the growth of vascular smooth muscle cells in vitro (Blaes and Boissel, 1983; Nakaki et al., 1990). Whether this is only an α-adrenoceptor effect or whether β-adrenoceptor agonists can also stimulate the proliferation of vascular smooth muscle is less clear. Blaes and Boissel (1983) found that both phentolamine, an α-adrenoceptor antagonist, and propranolol, a β-adrenoceptor antagonist, inhibited the effects of adrenaline on the growth of rat arterial smooth muscle. In contrast Nakaki et al. (1990) found that α_1-adrenoceptor agonists promoted the growth of rat aortic smooth muscle whereas β_2-adrenoceptor agonists had an inhibitory effect. We have studied the effects of β-adrenoceptor agonists and antagonists on the proliferation of bovine airway smooth muscle.

2. Materials and methods

2.1. Materials

Unless otherwise stated chemicals were obtained from Sigma, St. Louis, MO, USA. Fetal bovine serum was obtained from Life Technologies (Auckland, New Zealand). Salbutamol, salmeterol and beclomethasone dipropionate were a gift from Glaxo Group Research (Greenford, UK). Biodegradable Counting Scintillant (Amersham, UK) was used for scintillation counting.

2.2. Cell culture

Primary cultures were prepared using a modification of the method described by Panettieri et al. (1990). Bovine trachea was obtained from the local abattoir and transported back to the laboratory on ice. The trachealis muscle was dissected out in a laminar flow hood, with removal of the mucosa and adventitia. The airway smooth muscle was diced then serially digested in Krebs-Ringer-Henseleit buffer (NaCl 115 mM, KCl 5 mM, KH_2PO_4 1 mM, $MgSO_4$ 1 mM, $CaCl_2$ 1 mM, glucose 15 mM) containing collagenase type IV (350 U/ml) and protease (*Bacillus polymyxa* 0.3 mg/ml) for 1 h at 37°C followed by trypsin (0.025%) and EDTA (0.01%), in Hanks' balanced salt solution without Mg^{2+} or Ca^{2+}, for 35 min. All the digestion and culture media contained penicillin-streptomycin (2.5 μg/ml). After each digestion the dissociated cells were filtered through a Nitex 100 mesh filter, centrifuged then resuspended in Ham's F12 medium containing 10% fetal bovine serum. The cells were then plated at 37°C for 15 min to allow fibroblasts to adhere to plastic. The cell suspension was removed and plated for a further 30–40 min to allow smooth muscle cells but not epithelial cells to adhere. The cells were then grown to confluence in Ham's F12 containing 10% fetal bovine serum with the media being changed every 72 h.

2.3. Cell characterisation

Immunocytochemistry was performed using monoclonal anti-sera (all at 1:250 dilution) to α smooth muscle actin and pan cytokeratin (Amersham, UK). Fluorescein isothiocyanate-labelled goat anti-mouse serum (1:200) was used to produce a fluorescent image by UV epi-fluorescence microscopy (Olympus BH2).

2.4. [^{3}H]Thymidine incorporation / cell proliferation studies

Once confluent, the cells were trypsinized then seeded into 24-well plates (Flow Labs, VA, USA) at a density of 35 000 cells per well. After 60 h incubation in Ham's F12 with 10% fetal bovine serum, the cells were washed with Krebs-Ringer-Henseleit buffer and the media changed to Ham's F12 containing 5 μg/ml each of bovine insulin and bovine transferrin and 3% fetal bovine serum for all experiments. β-adrenoceptor agonists or corticosteroids were added 8 h later and the cells were then incubated with these agents for 40 h. 1 μCi/ml of [^{3}H]thymidine (Amersham) was added for the last 16 h of each experiment. [^{3}H]Thymidine incorporation was halted by the addition of unlabelled thymidine (100 μg), the cells were washed twice with Hanks' balanced salt solution (2 ml) as previously described (Shevach, 1992) and then trypsinized. The volume of cell suspension was adjusted to 1.5 ml with Hanks' balanced salt solution and 2 aliquots were taken, mixed with Biodegradable Counting Scintillant (3 ml) and counted in an LKB 1219 liquid scintillation

counter. Results obtained with this method are the same as those where extraction with trichloroacetic acid is performed prior to scintillation counting. Trypsinized cells were pipetted up and down several times to prevent clumping and cell counts were then performed using a Coulter counter. Cell numbers were determined after the smooth muscle cells had been incubated with β-adrenoceptor agonists or corticosteroids for 64 h. Changes in thymidine incorporation are detectable earlier than changes in cell number and this is why a longer interval was used when assessing changes in cell number.

In time-course experiments 100 nM salbutamol was incubated with cells for 40 h as described above or for 16 h when the salbutamol was added at the same time as the [^{3}H]thymidine. We also determined the effect of adding this concentration of salbutamol together with [^{3}H]thymidine for the last 6 h of the experiment.

Each experiment was carried out using a primary culture of airway smooth muscle cells from a fresh bovine trachea. There were 4–8 replicates (wells) for each concentration. Cell numbers and thymidine incorporation are expressed as a percentage of the mean value for the control wells ± standard deviation. (n) indicates the number of times each experiment was performed. IC_{50} values were calculated by interpolation from plots of the mean data. Statistical significance was assessed by one-way analysis of variance and the Bonferroni modification of the t-test.

3. Results

3.1. Immunocytochemistry

Immunocytochemical characterization of the cultured cells showed that > 98% stained positively with a monoclonal antibody specific for smooth muscle α-actin indicating that these were smooth muscle cells rather than fibrobasts or epithelial cells. No staining was seen with an antibody for pan cytokeratin providing further evidence that there was no contamination with epithelial cells.

3.2. Effects on growth of fetal bovine serum

In preliminary experiments incubation with 10% fetal bovine serum stimulated the growth of the airway smooth muscle cells with a doubling time of 22–26 h. After 60 h cells were changed to media supplemented with insulin/transferrin and 3% fetal bovine serum. The growth rate was determined after a further 48 h. In cells incubated with 3% fetal bovine serum the growth rate was reduced by 66% compared with those incubated in 10% fetal bovine serum.

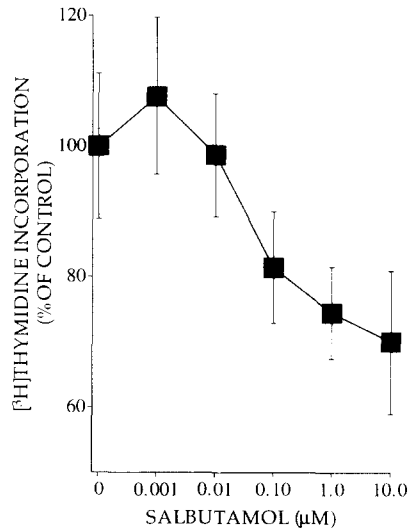

Fig. 1. Changes in thymidine incorporation following incubation of bovine airway smooth muscle cells with salbutamol for 40 h. Changes are expressed as a percentage of control. Values are shown as mean ± S.D. ($n = 5$). There was a significant reduction in thymidine incorporation ($P < 0.001$) for concentrations of salbutamol ≥ 100 nM.

3.3. Inhibition of growth by β-adrenoceptor agonists

Salbutamol, a β_2-adrenoceptor agonist, inhibited thymidine incorporation with an IC_{50} of 60 nM (Fig. 1) and led to a reduction in cell number with an IC_{50} of 10 nM (Fig. 2B). The highest concentration of salbutamol (10 μM) led to a 30.0 ± 10.9% reduction in thymidine incorporation and a 14.6 ± 2.6% reduction in cell number. At 1 nM salbutamol there was a small increase in thymidine incorporation but there were no corresponding changes in cell count at this concentration. The long acting β_2-adrenoceptor agonist salmeterol also inhibited thymidine incorporation and reduced cell number but in contrast to salbutamol there was no evidence of this response plateauing at 10 μM (Fig. 3). At this concentration salmeterol reduced thymidine incorporation by 89.5 ± 3.6% and cell number by 44.1 ± 5.2% of control values. Both propranolol 1 μM (Fig. 2A) and the selective β_2-adrenoceptor antagonist ICI 118551 10 nM (data not shown) caused a shift to the right in the dose-response curve for salbutamol. The same concentration of propranolol abolished the inhibitory effect of salbutamol on the change in cell number (Fig. 2B). These findings are consistent with the inhibitory effect of salbutamol being mediated by β-adrenoceptors. The time-course experiments showed no evidence of downregulation of the response to salbutamol 100 nM during a 40 h incubation compared with a 16 h incubation. After 40 h thymidine incorporation was reduced to 80.3 ± 8.8% ($n = 4$) of control values compared with 86.2 ± 6.9%

($n = 4$) for the 16 h incubation. Addition of salbutamol 100 nM for 6 h led to a reduction in thymidine incorporation to 94.9 ± 7.6% ($n = 4$) of control values.

3.4. Inhibition of growth by glucocorticoids

The inhibitory effects of cortisol and beclomethasone dipropionate on the growth of bovine tracheal smooth muscle cells were more potent than those of salbutamol. The IC_{50} for thymidine incorporation was 5 nM for cortisol (Fig. 4A) and 0.2 nM for beclomethasone dipropionate (Fig. 4B). At a concentration of 100 nM cortisol, there was a 47.0 ± 11.9% reduction in thymidine incorporation (Fig. 4) and a 16 ± 6.5% ($n = 3$, $P < 0.001$) reduction in cell number. The maximum reduction in thymidine incorporation with beclomethasone dipropionate was 42.3 ± 5.9% at a concentration of 3 nM (Fig. 4B). This concentration of beclometha-

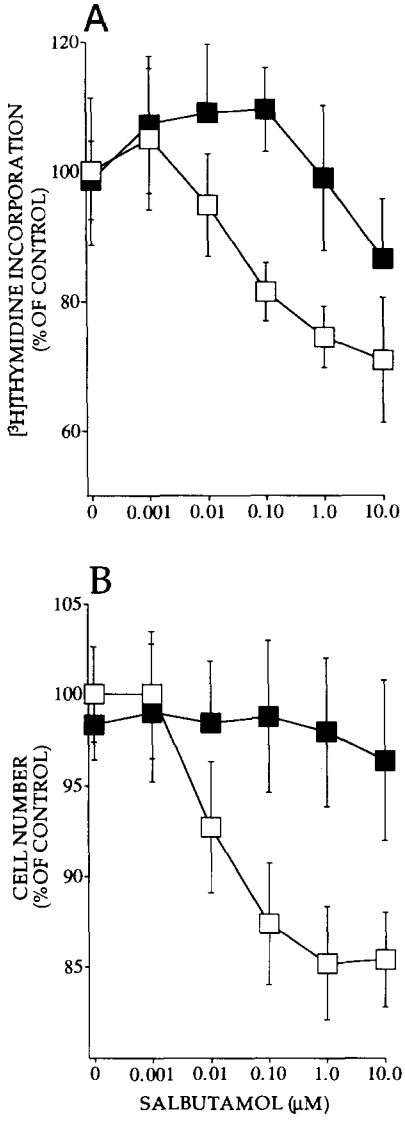

Fig. 2. Effect of salbutamol on the proliferation of bovine airway smooth muscle cells in the presence (□) and absence (■) of propranolol 1 μM. Changes are expressed as a percentage of control. Values are shown as mean ± S.D. ($n = 3$). A: Changes in thymidine incorporation after incubation with salbutamol for 40 h. B: Changes in cell number after incubation with salbutamol for 64 h ($n = 5$). In the presence of propranolol there was no significant change in cell number at any concentration of salbutamol. In the absence of propranolol there was a significant reduction in cell number ($P < 0.001$) at concentrations of salbutamol ≥ 10 nM.

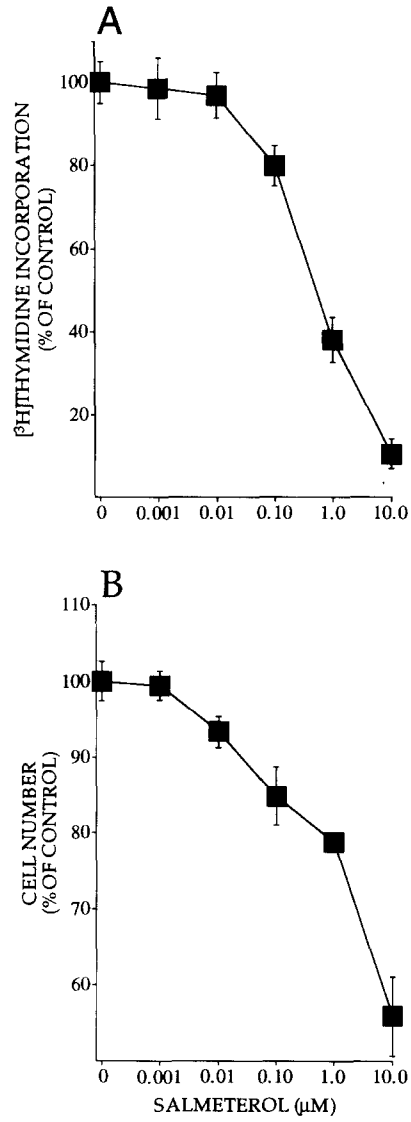

Fig. 3. Effect of salmeterol on the proliferation of bovine airway smooth muscle cells. Changes are expressed as a percentage of control. Values are shown as mean ± S.D. ($n = 4$). A: Changes in thymidine incorporation following incubation with salmeterol for 40 h. There was a significant reduction in thymidine incorporation ($P < 0.001$) for concentrations of salmeterol ≥ 100 nM. B: Changes in cell number following incubation with salmeterol for 64 h ($n = 3$). There was a significant reduction in cell number ($P < 0.001$) for concentrations of salmeterol ≥ 100 nM.

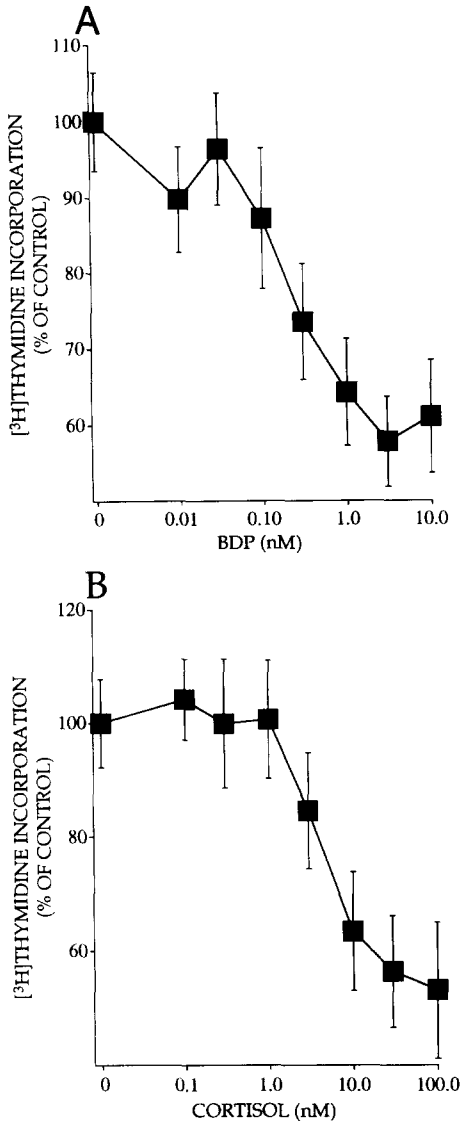

Fig. 4. Effects of cortisol and beclomethasone dipropionate on proliferation of bovine airway smooth muscle cells. Changes are expressed as percentage of control. Values are shown as mean ± S.D. A: Changes in thymidine incorporation following incubation with cortisol for 40 h ($n = 3$). There was a significant reduction in thymidine incorporation ($P < 0.001$) for concentrations of cortisol ≥ 3 nM. B: Changes in thymidine incorporation following incubation with beclomethasone dipropionate for 40 h ($n = 3$). There was a significant reduction in thymidine incorporation ($P < 0.001$) for concentrations of beclomethasone dipropionate ≥ 0.1 nM.

sone dipropionate led to a $17.8 \pm 5.7\%$ ($n = 3$, $P < 0.001$) reduction in cell number.

4. Discussion

We have shown that glucocorticoids cause a significant reduction in cell number and thymidine incorporation by bovine airway smooth muscle cells. There have been no previous reports of the effects of glucocorticoids on airway smooth muscle but a number of workers have studied the effects of corticosteroids on vascular smooth muscle. The extent to which corticosteroids inhibit the growth of vascular smooth muscle cells in culture varies with the species studied, the surface on which the cells are grown, the use of primary or secondary cultures and whether the experiments are conducted in the presence of foetal bovine serum or in serum-free medium (Jarvelinen et al., 1982; Longnecker et al., 1982, 1984; Hirosumi et al., 1987; Berk et al., 1988). Nonetheless corticosteroids inhibit thymidine incorporation by bovine airway smooth muscle cells at concentrations which are at least comparable if not lower than those which inhibit the growth of vascular smooth muscle. We studied beclomethasone dipropionate since it is the inhaled steroid most commonly used in the treatment of asthma. Beclomethasone dipropionate was 20 times more potent than cortisol in inhibiting thymidine incorporation by the airway smooth muscle cells. It is important to consider whether the concentrations of beclomethasone dipropionate which inhibit thymidine incorporation by cultured airway smooth muscle cells are therapeutically relevant. Sarnstrand et al. (1985) noted that following inhalation of 500 μg budesonide, the highest plasma concentration of budesonide was 1 nM. On this basis they estimated that concentrations of budesonide in the airways were likely to be less than 100 nM. It is probable that the concentrations of budesonide and beclomethasone dipropionate will be similar following inhalation. If human airway smooth muscle cells respond in the same way as bovine airway smooth muscle cells then the concentrations of beclomethasone dipropionate inhibiting thymidine incorporation (0.1–3 nM) are therapeutically relevant.

In the lung, esterases convert beclomethasone dipropionate to its more active metabolite beclomethasone monopropionate (Martin et al., 1974). We do not know if this occurs to the same extent in our cell cultures. Beclomethasone dipropionate may be more potent in vivo because it is converted to beclomethasone monopropionate to a greater extent. Although we have demonstrated that corticosteroids are effective in inhibiting the growth of airway smooth muscle, inhaled steroids in conventional doses only lead to modest improvements in bronchial hyperresponsiveness in patients with asthma. Compared with normal individuals, subjects with asthma have a 10- to 100-fold increase in bronchial responsiveness. Juniper et al. (1990) however found that treatment with inhaled budesonide 400 μg/day only led to a 4-fold reduction in bronchial responsiveness. If inhaled steroids are to lead to greater reductions in bronchial hyperresponsiveness they may have to cause regression of smooth muscle hyperplasia or a decrease in hypertrophy but our studies do not allow us to comment on the efficacy of corticosteroids

in reversing remodelling once it has occurred. Our findings do raise the possibility that inhaled steroids could help prevent further remodelling from occurring. A recently published study by Haahtela et al. (1994) is of interest in this regard. In this controlled, randomised, study one group of subjects with mild asthma was started on treatment with inhaled budesonide soon after the onset of symptoms. The other group was not treated with budesonide for another 2 years. The improvement in bronchial hyperresponsiveness and symptoms over the first year of treatment with budesonide was greater in the group who was treated early, compared with the group started on budesonide 2 years later.

Although salbutamol 1 nM led to a small increase in thymidine incorporation this was not associated with an increase in cell numbers. At 10 nM and higher concentrations of salbutamol there was a reduction in thymidine incorporation and cell number. These observations are consistent with a report by Tomlinson et al. (1994) that salbutamol inhibits the growth of human airway smooth muscle cells. Whereas we found that salbutamol inhibited the response to fetal bovine serum at concentrations of 10 nM and greater they showed that the responses to thrombin, epidermal growth factor and to a thromboxane mimetic were inhibited by salbutamol 100 nM. We also demonstrated that both propranolol and ICI 118551 (a β_2-adrenoceptor antagonist) led to a rightward shift in the dose response to salbutamol which is consistent with the inhibitory effect of salbutamol being mediated by β-adrenoceptors. Although the maximum effect of salbutamol on cell number was similar to that seen with cortisol and beclomethasone dipropionate, salbutamol was considerably less potent with an IC_{50} of 60 nM compared with 0.2 nM for beclomethasone dipropionate. It is also likely that in vivo smooth muscle is exposed to concentrations of salbutamol of 100 nM or more for shorter periods than those used in our experiments. This raises the question of whether the inhibitory effects of salbutamol would be seen in vivo. It should be noted however that the IC_{50} for the relaxation of isolated airway smooth muscle by salbutamol is approximately 100 nM (Decker et al., 1982; Goldie et al., 1986) and this is not dissimilar to the IC_{50} we observed for inhibition of thymidine incorporation. The possibility that salbutamol inhibits the proliferation of airway smooth muscle in vivo cannot be dismissed completely.

The recently introduced long-acting β-adrenoceptor agonists are of interest because airway smooth muscle is exposed to their effects for longer periods of time. Salmeterol is a long-acting β-adrenoceptor agonist whose duration of action is attributable to its large lipophilic side chain (Brittain, 1991; Pearlman et al., 1992). Like salbutamol, salmeterol inhibited thymidine incorporation and decreased cell number at concentrations greater than 10 nM. In contrast to salbutamol the response to salmeterol did not plateau at 10 μM. At this concentration salmeterol reduced thymidine incorporation to less than 10% of control and cell number was reduced by 44%. At high concentrations the effects of salmeterol are much greater than those of either salbutamol or corticosteroids. These observations would only be clinically relevant if concentrations of salmeterol as high as 10 μM were achieved in the airways. Whether this is the case is uncertain.

Clenbuterol is a β-adrenoceptor agonist which has an anabolic effect on skeletal muscle but it acts by promoting protein synthesis and causing cell hypertrophy without increasing cell number (Symonds et al., 1990). While treatment with β-adrenoceptor agonists inhibits the proliferation of bovine airway smooth muscle cells, further studies are necessary to determine whether β-adrenoceptor agonists could cause hypertrophy of airway smooth muscle cells. Corticosteroids inhibit the growth of airway smooth muscle cells in low concentrations and this may be clinically important. The most effective way to prevent airway remodelling may be to treat asthma with inhaled steroids early in the course of the disease.

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