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Inhibition by salmeterol of increased vascular permeability and granulocyte accumulation in guinea-pig lung and skin

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1. The long-acting β_2-adrenoceptor agonist, salmeterol has been evaluated for its anti-inflammatory effects in the guinea-pig lung and skin.

2. Salmeterol, administered in bronchodilator doses to conscious guinea-pigs by both oral (0.01–1.0 mg kg^{-1}) and inhaled (nebulizer concentration, 0.001–1.0 mg ml^{-1}) routes, inhibited histamine-induced plasma protein extravasation (PPE) into the airway lumen.

3. Inhibition of PPE by salmeterol was long-lasting (>6 h) and was inhibited by prior administration of propranolol (1 mg kg^{-1}, s.c.), indicating an effect mediated by β-adrenoceptors.

4. Inhaled salbutamol (nebulizer concentration, 0.001– 1.0 mg ml^{-1}) also inhibited PPE in guinea-pig lung but, in contrast to salmeterol, this effect was short-lived with substantial loss of activity 2 h after administration.

5. Inhaled salmeterol (0.1 mg ml^{-1}) and salbutamol (1.0 mg ml^{-1}) inhibited the accumulation of neutrophils in guinea-pig lung in response to lipopolysaccharide (100 μg ml^{-1}). Salmeterol, but not salbutamol, inhibited the infiltration of eosinophils into the airway lumen in response to platelet activating factor (100 μg ml^{-1}). These effects of salmeterol were blocked by prior administration of propranolol (5 mg kg^{-1}, s.c.), indicating that they were also β-adrenoceptor-mediated.

6. Oral salmeterol (10 mg kg^{-1}, p.o.), but not salbutamol (10 and 100 mg kg^{-1}, p.o.), inhibited zymosan-induced granulocyte accumulation and PPE in guinea-pig skin. Lower doses of salmeterol (0.1 and 1 mg kg^{-1}) inhibited PPE, but not granulocyte accumulation. The effects of salmeterol were blocked by prior administration of propranolol (1 mg kg^{-1}, s.c.). Both salmeterol and salbutamol inhibited histamine-induced PPE in guinea-pig skin.

7. Intradermal salmeterol (10^{-8} mol per site), but not salbutamol, was also effective in inhibiting zymosan-induced granulocyte accumulation and PPE in guinea-pig skin.

8. It is concluded that salmeterol, at bronchodilator doses in the guinea-pig, inhibits granulocyte accumulation and PPE, possibly by an action on the vasculature. As this profile of activity is not shared by the shorter-acting compound, salbutamol, it would seem that anti-inflammatory activity is associated with β-adrenoceptor agonism of long duration. The implications of these findings for the use of salmeterol in the treatment of bronchial asthma are discussed.

Keywords: Salmeterol; salbutamol; anti-inflammatory; β-adrenoceptor agonist; vascular permeability; neutrophil; eosinophil; lung; skin; granulocyte

Introduction

The anti-inflammatory properties of β-adrenoceptor agonists in experimental animals are well documented (Spector & Willoughby, 1960; Green, 1972; Persson *et al.*, 1978). Adrenaline inhibits oedema formation following thermal injury to rats (Spector & Willoughby, 1960), while salbutamol has been shown to inhibit carrageenin-induced oedema in the mouse paw following local and intraperitoneal injection (Green, 1972), and PAF-induced oedema in the rat following oral administration (Bonnet, 1981). These anti-inflammatory actions were due to stimulation of β-adrenoceptors, as they were inhibited by β-adrenoceptor blocking agents (Green, 1972; Arntzen & Briseid, 1973; Persson *et al.*, 1979). Moreover, the anti-inflammatory effects of β-agonists are not restricted to skin and subcutaneous tissue, since terbutaline has been shown to inhibit plasma protein extravasation (PPE) and the increase in lung weight induced by histamine in the guinea-pig (Persson *et al.*, 1979).

One explanation for the inhibitory actions of β-adrenoceptor agonists in some of the studies described above is via inhibition of the release of inflammatory mediators (Butchers *et al.*, 1980). However, this mechanism is unlikely to account for all the effects seen, since in many experiments the direct actions of inflammatory mediators are inhibited (Svensjo *et al.*, 1976).

β-Adrenoceptor agonists inhibit capillary permeability by a mechanism which does not appear to be secondary to vasodilatation. Rippe & Grega (1978) demonstrated that isoprenaline reduced capillary filtration in a maximally vasodilated, rat perfused hindquarter preparation. Further studies showed that isoprenaline and terbutaline inhibited PPE, whereas equi-vasodilator doses of acetylcholine or papaverine were ineffective (Svensjo *et al.*, 1976; Grega *et al.*, 1980; Raymond *et al.*, 1980; Persson *et al.*, 1982; Prasad *et al.*, 1982). Joyner *et al.* (1979) and Svensjo & Grega (1986) suggested that β-agonists modulate PPE by an action on β-adrenoceptors on post-capillary venules.

This hypothesis was supported by Gudgeon & Martin (1989) who demonstrated that isoprenaline inhibits phorbol myristate acetate-induced transit of albumin across porcine endothelial monolayers in culture, by a mechanism which was blocked by propranolol.

To our knowledge, few studies have examined the effect of β-adrenoceptor agonists on other aspects of acute-inflammation, although Spicer *et al.* (1990) demonstrated that a number of β-adrenoceptor agonists prevent sephadex-induced blood eosinophilia in rats while subcutaneous isoprenaline also inhibited sephadex-induced eosinophil accumulation in rat lung. Furthermore, in spite of the experimental evidence,

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currently available β_2-adrenoceptor agonists with a short duration of action, such as salbutamol, do not appear to have any significant anti-inflammatory activity in therapeutic situations such as atopic dermatitis or the late phase response in asthmatics following antigen challenge (Cockroft & Murdock, 1987; Archer & Macdonald, 1987; Green *et al.*, 1988). The duration of action of the β-agonist may be an important factor, since many inflammatory processes both develop and resolve slowly.

The present paper compares the anti-inflammatory properties of the new long-acting β_2-adrenoceptor agonist, salmeterol (Bradshaw *et al.*, 1987), with those of salbutamol in guinea-pig lung and skin. Preliminary accounts of part of this work have been published in abstract form (Whelan & Johnson, 1990; 1991a; 1991b).

Methods

Lung inflammation

Histamine-induced plasma protein extravasation (PPE) Male guinea-pigs (300–400 g) were given an intra-cardiac injection of iodinated (^{125}I) human serum albumin (0.5 μCi, 0.3 ml) in heparinised saline (10 u ml^{-1}) under light isoflurane anaesthesia. Following recovery, animals were placed in a plexiglas chamber and exposed to an aerosol of histamine (0.5 mg ml^{-1}) generated by a Devilbiss nebuliser for 30 s, followed by a further 30 s exposure to the atmosphere in the chamber.

Thirty minutes after histamine challenge, guinea-pigs were killed with an overdose of pentobarbitone sodium (Expiral) administrated intraperitoneally and a blood sample was taken by cardiac puncture. The trachea of each animal was cannulated, and the lungs lavaged twice with 10 ml heparinised (10 u ml^{-1}), phosphate-buffered saline at 37°C. The radioactivity in both an aliquot of plasma and a 5 ml sample of the pooled bronchoalveolar lavage fluid (BALF) was measured in a scintillation counter (LKB Compugamma). From these data, plasma protein extravasation (expressed as μl plasma ml^{-1} BALF) was calculated.

Where potency was estimated, drugs were administered to the animals by the oral or aerosol route 30 min before histamine challenge. In duration of action studies, the time interval between β-agonist administration and histamine exposure was extended for periods of up to 8 h. In experiments where salmeterol, salbutamol or prostaglandin E_2 (PGE_2) was administered by inhalation, guinea-pigs were placed in a plexiglas chamber and exposed to aerosols of these agents as described by Ball *et al.* (1991), in that solutions of these agents were nebulised into the chamber for 1 min and the animals breathed the atmosphere within the chamber for a further 2 min.

Lipopolysaccharide (LPS) and platelet activating factor (PAF)-induced granulocyte accumulation Male guinea-pigs (300–400 g) were placed in plexiglas chambers and exposed to aerosols of LPS (100 μg ml^{-1}) or PAF (100 μg ml^{-1}) for 10 min as described by Folkerts *et al.* (1988) and Aoki *et al.* (1987) respectively. Four hours after LPS, or 24 h after PAF, guinea-pigs were killed and the lungs lavaged with heparinised, phosphate-buffered saline as described above.

A total leucocyte count was performed, and a cytospin preparation was made from the BALF recovered. Cytospin preparations were fixed with methanol, stained with Wright's stain and differential leucocyte counts carried out by an operator who was unaware of the treatments given to the animals during the experiment.

Dermal inflammation

Granulocyte accumulation and granulocyte-dependent vascular permeability:- preparation and labelling of guinea-pig granulocytes Guinea-pig peritoneal granulocytes, elicited by intraperitoneal injection of glycogen (20 ml, 0.1% w/v), were harvested from exudates (18 h after injection of glycogen) by centrifugation on histopaque 1077 (20 min, 200 *g*). Granulocytes recovered in the pellet were washed twice with heparinised Tyrode solution. Cells were then resuspended in 2 ml of heparinised Tyrode solution, mixed with ^{111}indium oxine (150 μCi: 5.55 MBq), and incubated at room temperature for 15 min. Labelled granulocytes (>85% pure) were washed twice with heparinised Tyrode solution and suspended in an appropriate volume of heparinised Tyrode solution for reinjection (usually 2–5 ml). In experiments where vascular permeability was measured, (^{125}I) iodinated human serum albumin (10 μCi: 0.37 MBq) was added to the labelled-granulocyte suspension.

Experimental protocol Male guinea-pigs (300–400 g) were anaesthetized with ketamine (40 mg kg^{-1}, i.m.)/xylazine (8 mg kg^{-1}, i.m.) and the ventral surface of the abdomen shaved. Each guinea-pig received an intra-cardiac injection of indium-labelled granulocytes (usually 0.3 ml) and six intradermal injections of either sterile saline (100 μl) or a suspension of zymosan (0.06–2 mg) in saline. The animals were then allowed to recover from the anaesthetic. After 4 h, guinea-pigs were killed by the intraperitoneal administration of pentobarbitone sodium. A blood sample (2 ml) was taken into heparin (50 u ml^{-1} final conc.) and a full thickness biopsy of the injection site taken with an 18 mm hollow punch.

An aliquot (500 μl) of the blood sample was removed for counting, whilst the remainder was centrifuged. An aliquot of the resulting plasma (200 μl) was then also taken for counting.

The radioactivity of the blood, plasma and skin biopsies was determined. From these data, the granulocyte content of each skin site was calculated according to equation (1), given a granulocyte count of 2.25×10^3 cells μl^{-1} of blood (determined from differential counts of the blood from 6 control guinea-pigs).

$$\text{Tissue granulocytes} = 2.25 \times 10^3 \times \text{tissue } ^{111}\text{In count} \div \frac{\text{blood } ^{111}\text{In count}}{\text{vol blood counted } (\mu\text{l})} \quad (1)$$

Similarly, plasma protein extravasation, expressed as μl plasma equivalents, was calculated according to equation (2).

$$\text{Plasma protein } (\mu\text{l}) = \text{Tissue } ^{125}\text{I count} \div \frac{\text{plasma } ^{125}\text{I count}}{\text{vol plasma counted } (\mu\text{l})} \quad (2)$$

In the experiments described, 83.4 ± 1.5% of ^{111}In was incorporated into the granulocytes. Approximately 10^7 cells were injected into the circulation of each guinea-pig and 4 h after injection <20% of ^{111}In in the blood was recovered from the plasma, indicating that >80% of the ^{111}In administered remained associated with the blood cells. However less than 20% of the injected neutrophils were circulating in the blood 4 h after injection. All values quoted for granulocyte accumulation and plasma protein extravasation (vascular permeability) are corrected for the increases induced by an intradermal injection of sterile saline.

Granulocyte-independent vascular permeability Male guinea-pigs (250–350 g) were anaesthetised with ketamine/xylazine as described above. Each guinea-pig was given an intracardiac injection of iodinated human serum albumin (≃0.5 μCi in 0.3 ml heparinised saline) followed by a series of intradermal injections of sterile saline or histamine in sterile saline. After 30 min, the animals were killed by an injection of pentobarbitone sodium. A blood sample and full thickness biopsies of the injection sites were taken. The radioactivity of a sample of plasma (200 μl) and each biopsy was determined and the

plasma protein extravasation, expressed as μl plasma, calculated as described in equation (2).

Statistical analysis

In lung, histamine-induced increases in plasma protein extravasation were log normally distributed. These data are expressed as geometric means and 95% confidence limits. EC_{50} values were calculated from a regression analysis. Plasma protein extravasation in skin, and granulocyte accumulation in skin and lung were normally distributed and are expressed as arithmetic means ± s.e.mean. Where appropriate, levels of statistical significance were calculated, for normally distributed data, by Student's *t* test.

Drugs and reagents

Iodinated human serum albumin (specific activity 2.5 μCi mg^{-1} albumin) and indium oxine were purchased from Amersham International. Zymosan, histamine, propranolol, platelet activating factor (PAF) and LPS (*E. coli* 026:B6) were obtained from Sigma Ltd and PGE_2 (Prostin E_2) from Upjohn Ltd. Salmeterol and salbutamol (Glaxo Group Research Ltd) were dissolved in distilled water to an initial concentration of 10 mg ml^{-1} with the addition of a few drops of glacial acetic acid. This solution was diluted further in distilled water prior to oral or inhaled administration. In experiments where compounds were administered intradermally, they were dissolved in dimethyl sulphoxide (DMSO, final concentration 1%) and co-injected with histamine or zymosan.

Results

Lung inflammation

Histamine-induced plasma protein extravasation in the guinea-pig lung Thirty minutes after inhalation of histamine (0.5 mg ml^{-1}) the plasma protein content of BALF increased (Figure 1) from 0.80 μl ml^{-1} (geometric mean, 95% confidence limits 0.6–1.08 μl ml^{-1}, $n = 10$) to 5.26 μl ml^{-1} (4.82–5.72 μl ml^{-1}, $n = 13$). Although bronchoconstriction was not measured in these experiments, this level of histamine challenge did not provoke respiratory distress, and was well tolerated by all animals.

Effect of inhaled salmeterol and salbutamol on histamine-induced PPE When salmeterol (nebulizer concentration, 0.001–1.0 mg ml^{-1}) was administered to guinea-pigs by aerosol, 30 min prior to challenge with histamine, a concentration-related inhibition of PPE was observed. At a dose of 0.1 mg ml^{-1} salmeterol, total inhibition of histamine-induced PPE was achieved (Figure 1). The aerosol concentration of salmeterol required to reduce histamine-induced PPE by 50% (ED_{50}) was found by regression analysis to be 0.015 mg ml^{-1}. The vehicle for salmeterol (acetic acid/water) had no effect on histamine-induced PPE.

Like salmeterol, inhaled salbutamol (0.001–1.0 mg ml^{-1}) also inhibited histamine-induced PPE (Figure 1). An ED_{50} of 0.038 mg ml^{-1} was obtained for salbutamol which was not significantly different from that of salmeterol. Pretreatment of guinea-pigs with a β-adrenoceptor blocking dose of propranolol (1 mg kg^{-1} s.c.; Daly *et al.*, 1975), prevented the inhibition of histamine-induced PPE by salmeterol (Figure 1).

Effect of inhaled prostaglandin E_2 on histamine-induced PPE In contrast to the β_2-adrenoceptor agonists, administration of an equi-effective bronchodilator dose of PGE_2 (0.1 mg ml^{-1}; Ball *et al.*, 1987), 30 min prior to histamine, did not inhibit PPE in guinea-pig lung. When administered at shorter time intervals before histamine challenge, PGE_2 also had no effect on PPE.

Effect of oral salmeterol on histamine-induced PPE Salmeterol (0.01–1.0 mg kg^{-1}, p.o.), 30 min prior to histamine, also inhibited PPE in a dose-related manner, with the highest dose producing total inhibition (Table 1). Analysis of the

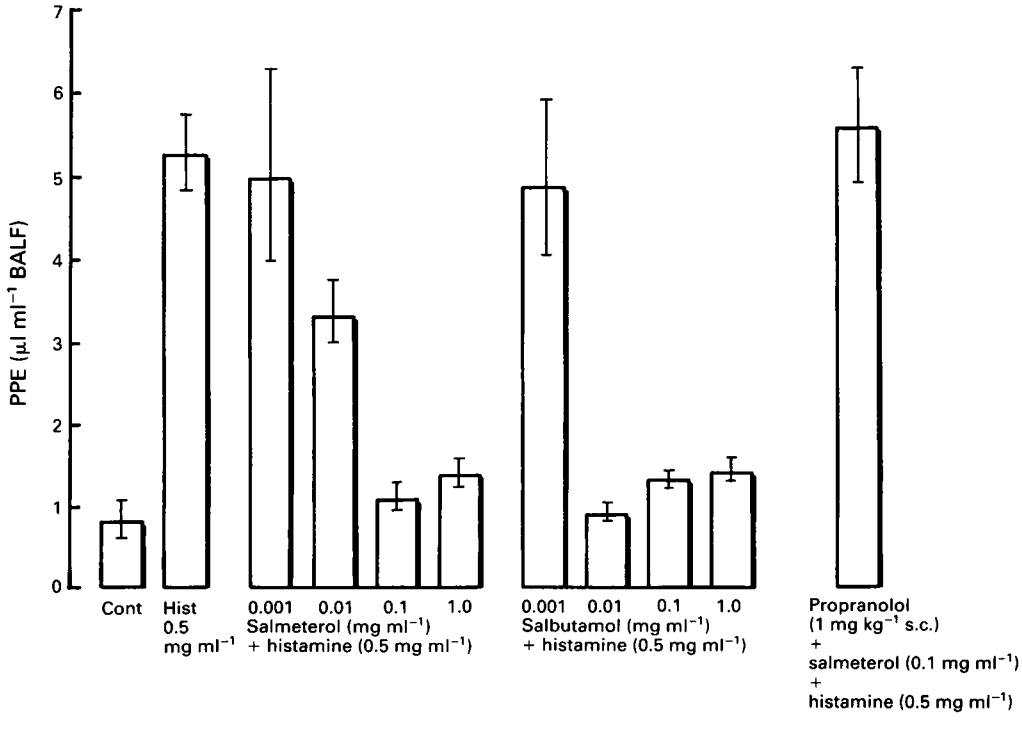

Figure 1 Inhibition of histamine-induced plasma protein extravasation (PPE) in guinea-pig lung by inhaled salmeterol (0.001–1 mg ml^{-1}) and salbutamol (0.001–1 mg ml^{-1}) and blockade of the effects of salmeterol by propranolol (1 mg kg^{-1}, s.c.). Each column is the geometric mean and 95% confidence limits of at least 6 determinations. Concentrations refer to the concentration of the β-agonists in the nebuliser solution.

Table 1 Inhibition of histamine (0.5 mg ml^{-1})-induced plasma protein extravasation (PPE) in guinea-pig lung by orally administered salmeterol (0.01–1 mg kg^{-1})

Treatment	Dose (mg kg^{-1})	PPE* (µl plasma ml^{-1} BALF)	n
Control	–	1.18 (1.10–1.28)	21
Vehicle	–	4.38 (4.02–4.78)	25
Salmeterol	0.01	5.40 (4.36–6.70)	6
Salmeterol	0.1	1.52 (1.32–1.74)	6
Salmeterol	1.0	0.62 (0.58–0.66)	6

*Values shown are geometric means and 95% confidence limits, in parentheses, of *n* determinations.
BALF = bronchoalveolar lavage fluid.

data obtained revealed that the ED_{50} oral dose of salmeterol was 0.02 mg kg^{-1}. The effect of orally administered salbutamol on histamine-induced PPE was not determined.

The duration of inhibition of PPE by salmeterol and salbutamol Inhibition of histamine-induced PPE by inhaled salmeterol (0.1 mg ml^{-1}) was long-lasting, being still evident for at least 6 h after administration (Figure 2). In contrast, salbutamol (0.1 mg ml^{-1}) had a shorter duration of action, with substantial loss of activity within 2 h (Figure 2).

Inhibition of lipopolysaccharide and PAF-induced granulocyte accumulation by salmeterol and salbutamol Exposure of guinea-pigs to a sub-maximal, aerosol dose of LPS (100 µg ml^{-1}) for 10 min resulted in an increase in the BALF neutrophil count from 3.8 ± 1.2 × 10^3 cells ml^{-1} (mean ± s.e.mean) to 141.3 ± 33.3 × 10^3 cells ml^{-1}, 4 h after challenge. No significant ($P > 0.05$) change in any other cell type was observed. Pretreatment of guinea-pigs with an aerosol of salmeterol (0.1 mg ml^{-1}), 30 min before challenge with LPS, significantly ($P < 0.05$) reduced the BALF neutrophil count to 46.8 ± 9.1 × 10^3 cells ml^{-1} (Figure 3), a reduction of 69%. Lower aerosol concentrations of salmeterol (0.01 mg ml^{-1}) were without effect.

In a parallel series of experiments, a ten fold higher dose of salbutamol (aerosol concentration 1.0 mg ml^{-1}), also significantly reduced LPS-induced BALF neutrophilia by 67% ($n = 6$), ($P < 0.05$). Lower aerosol concentrations of salbutamol (0.1 mg ml^{-1}) were without effect. When guinea-pigs were pretreated with propranolol (5.0 mg kg^{-1}, s.c., 30 min prior to salmeterol; Kellet, 1966), salmeterol had no significant inhibitory effect on LPS-induced neutrophil accumulation ($P > 0.05$).

Similarly, exposure of guinea-pigs to an aerosol of PAF (100 µg ml^{-1}; Aoki *et al.*, 1987) for 10 min resulted in a significant increase in the number of eosinophils in BALF from 53.3 ± 12.5 × 10^3 cells ml^{-1} to 97.7 ± 18.8 × 10^3 cells ml^{-1}, 24 h after challenge ($P < 0.05$). Pretreatment of guinea-pigs with salmeterol (aerosol concentration, 0.1 mg ml^{-1}), 30 min prior to challenge with PAF, completely abolished PAF-induced eosinophil accumulation (eosinophil count 59.2 ± 19.8 × 10^3 cells ml^{-1}; Figure 4). A lower dose of salmeterol (0.01 mg ml^{-1}) also had an inhibitory effect.

In contrast to salmeterol, salbutamol (aerosol concentration 1.0 mg ml^{-1}) did not inhibit PAF-induced infiltration of eosinophils in guinea-pig lung. Inhibition of PAF-induced eosinophilia by salmeterol was abolished when guinea-pigs were pretreated with propranolol (Figure 5).

Dermal inflammation

Zymosan-induced granulocyte accumulation and plasma protein extravasation Skin samples which had been injected with sterile saline contained 34.5 ± 6.0 × 10^3 ($n = 20$) granulocytes, although this value varied from experiment to experiment. Histological examination of these samples revealed no granulocytes in the extravascular compartment, therefore this value presumably reflects the granulocyte content of the vasculature within the biopsy. Similarly skin samples injected with saline had a plasma volume of 9.65 ± 0.86 µl ($n = 20$).

Four hours after the intradermal administration of zymosan (0.06–2 mg per site), a dose-related increase in the number of granulocytes (measured as an accumulation of ^{111}In) and plasma protein extravasation (measured as the accumulation of $^{125}I_2$-HSA) was observed in the injection

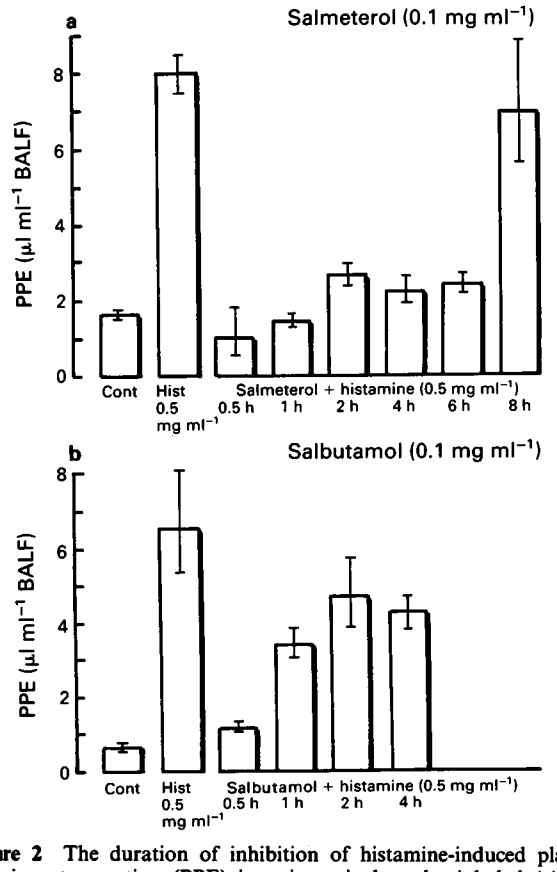

Figure 2 The duration of inhibition of histamine-induced plasma protein extravasation (PPE) in guinea-pig lung by inhaled (a) salmeterol (0.1 mg ml^{-1}) and (b) salbutamol (0.1 mg ml^{-1}). Each column represents the geometric mean and 95% confidence limits of at least 6 determinations. Values illustrated are the plasma exudation measured following challenge with histamine (0.5 mg ml^{-1}) at the times indicated after administration of β_2-adrenoceptor agonists.

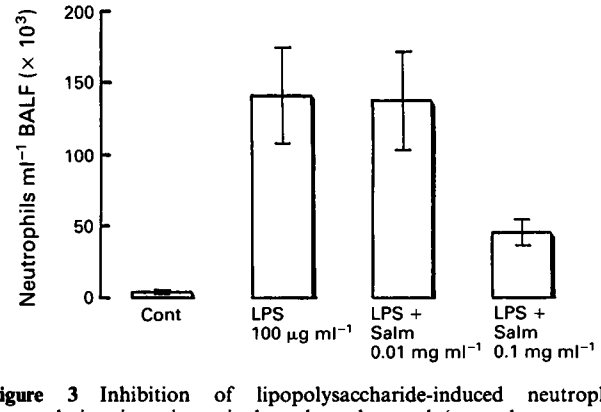

Figure 3 Inhibition of lipopolysaccharide-induced neutrophil accumulation in guinea-pig lung by salmeterol (aerosol concentrations 0.01–0.1 mg ml^{-1}). Cont = control, LPS = lipopolysaccharide (100 µg ml^{-1}), Salm = salmeterol. Each column is the mean of 6 determinations; s.e.mean shown by vertical bars.

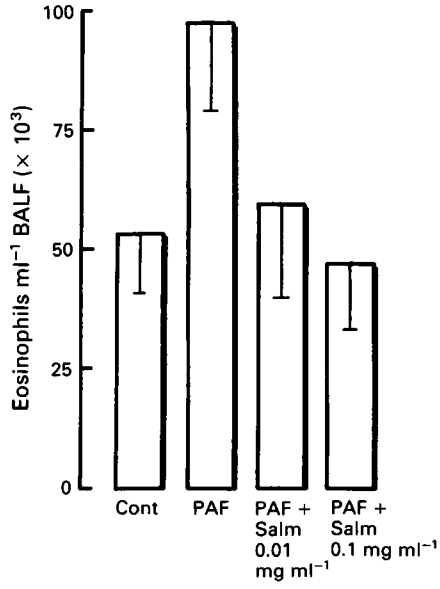

Figure 4 Inhibition of PAF-induced eosinophil accumulation in guinea-pig lung by salmeterol (aerosol concentrations, 0.01–0.1 mg ml^{-1}). Cont = control, PAF = PAF (0.1 mg ml^{-1}), Salm = salmeterol. Each column is the mean of 6 determinations; s.e.mean shown by vertical bars.

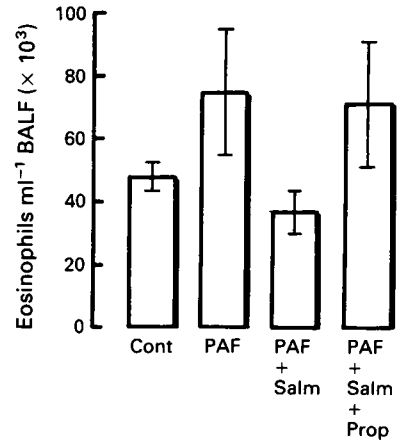

Figure 5 Blockade by propranolol (5 mg kg^{-1}, s.c.) of the inhibitory actions of salmeterol (0.1 mg ml^{-1}) on PAF-induced eosinophil accumulation in guinea-pig lung. Cont = control, PAF = PAF (0.1 mg ml^{-1}), Salm = salmeterol 0.1 mg ml^{-1}, Prop = propranolol (5 mg kg^{-1}, s.c.). Each column is the mean of at least 6 determinations; s.e.mean shown by vertical bars.

sites. Granulocyte accumulation was confirmed by histology, which showed extensive cell infiltration of the dermis and subcutaneous tissue. Identification of individual cell types was not possible although the predominant cells appeared to be neutrophils. A sub-maximal dose of 0.2 mg per site zymosan was selected for further experiments. Granulocyte accumulation, but not PPE, induced by zymosan (0.2 mg per site) varied from experiment to experiment (Table 2), however, within any given experiment this response was consistent between animals.

Oral administration of salmeterol (10 mg kg^{-1}) significantly ($P<0.05$) decreased zymosan-induced (0.2 mg per site) granulocyte accumulation and PPE (Table 2). Lower doses of salmeterol (1.0 and 0.1 mg kg^{-1}, p.o) significantly ($P<0.05$) reduced PPE, but had no significant effect on granulocyte accumulation (Table 2). In contrast salbutamol (10 and 100 mg kg^{-1}, p.o.) had no significant inhibitory effect on zymosan-induced granulocyte accumulation or PPE. The highest dose of salmeterol used in this study (10 mg kg^{-1}, p.o.) had no significant ($P>0.05$) effect on circulating neutrophil numbers.

Pretreatment of guinea-pigs with propranolol (1 mg kg^{-1}, s.c.), 30 min prior to salmeterol (10 mg kg^{-1}, p.o.), prevented the inhibitory action of salmeterol on zymosan-induced granulocyte accumulation and PPE.

Salmeterol was also effective by the intradermal route. Intradermal administration of 10^{-8} mol per site significantly ($P<0.05$) reduced granulocyte accumulation from 79.6 ± 13.3 (mean ± s.e.mean) to 39.0 ± 10.9 × 10^3 cells per site ($n=6$) and PPE from 15.2 ± 4.1 to 7.3 ± 1.8 ($n=6$) µl plasma per site ($n=6$). Lower doses of salmeterol (10^{-9} mol per site) had no significant effect. Intradermal injection of salbutamol (10^{-9}–10^{-7} mol per site) had no effect on zymosan-induced granulocyte accumulation or PPE.

Granulocyte-independent vascular permeability

Histamine (10 ng–10 µg per site), unlike zymosan, caused plasma protein extravasation in guinea-pig skin without granulocyte accumulation. A dose of 100 ng per site histamine was selected for studies with salmeterol since this submaximal dose caused consistent increases in PPE, whereas a greater variation in response was seen with higher doses. When guinea-pigs were pretreated with salmeterol (10 mg kg^{-1}, p.o.) 1 h prior to injection of histamine (100 ng per site), PPE was significantly ($P<0.05$) reduced from 6.5 ± 1.0 µl plasma per site to 2.7 ± 0.7 µl plasma per site ($n=5$), a reduction of 58.5%. Lower doses of salmeterol (1 mg kg^{-1}, p.o.) had no inhibitory effect on histamine-induced PPE (5.9 ± 1.6 µl plasma per site) ($P>0.05$). Like salmeterol, salbutamol also inhibited histamine-induced PPE, but at a ten fold higher dose. Oral administration of 100 mg kg^{-1} salbutamol significantly ($P<0.05$) reduced PPE from a control value of 11.9 ± 2.2 to 5.7 ± 1.7 ($n=4$) µl plasma per site. A lower dose of salbutamol (10 mg kg^{-1}) did not inhibit histamine-induced PPE (10.5 ± 2.5 to 9.8 ± 3.0 µl plasma per site ($n=8$)).

Table 2 Effect of salmeterol and salbutamol on zymosan (0.2 mg per site)-induced inflammation in guinea-pig skin

		*PMN cell accumulation** (*cells per site × 10^3*)			*Plasma protein extravasation** (*µl*)		
Agonist	*Dose* (*mg kg^{-1}, p.o.*)	*Control*	*Test*	n	*Control*	*Test*	n
Salmeterol	0.1	141.1 ± 19.0	129.1 ± 30.4	6	14.6 ± 2.2	7.7 ± 2.5	6
	1.0	53.3 ± 13.3	39.8 ± 12.7	6	17.9 ± 2.1	8.2 ± 3.5	6
	10.0	460.0 ± 82.0	190.0 ± 35.0	8	12.7 ± 2.8	5.8 ± 1.3	6
Salbutamol	10	153.3 ± 24.1	135.6 ± 43.0	4	11.0 ± 2.6	6.4 ± 2.8	4
	100	153.3 ± 24.1	118.8 ± 37.7	6	11.0 ± 2.6	10.0 ± 3.9	6

*Values shown are arithmetic mean ± s.e.mean of n determinations.
PMN = polymorphonuclear.

Pretreatment of guinea-pigs with propranolol (1 mg kg^{-1}, s.c.), 30 min prior to salmeterol (10 mg kg^{-1}, p.o.), abolished the inhibitory action of salmeterol on histamine-induced PPE in guinea-pig skin.

Discussion

In acute inflammatory reactions, pharmacologically-active substances (kinins, histamine, C_{5a} etc.) are released, and lead to increased venular permeability to plasma proteins, thus promoting oedema formation (Haddy *et al.*, 1976), and granulocyte accumulation (Hurley, 1972). Bronchial asthma is a disease increasingly regarded as being due to airway inflammation. In the asthmatic lung, the release of mast cell-derived inflammatory mediators has been detected (Wenzel *et al.*, 1990; 1991) and there is histological evidence of inflammatory cell activation and infiltration (Beasley *et al.*, 1989; Djukanovic *et al.*, 1991). Plasma proteins are also found in the bronchial lumen (Dunnill, 1960; Ryley & Brogan, 1968; Wanner, 1977; Persson, 1986) and the aqueous (non-mucus) phase of asthmatic sputum has been compared with inflammatory exudate (Ryley & Brogan, 1968). The presence of plasma protein in the bronchial secretions thickens mucus, thus impairing mucociliary clearance (Wanner, 1977), and can impair surfactant function, reducing ventilation (Seeger *et al.*, 1985). Additionally, bronchial mucosal oedema would produce a significant narrowing of the airway, thereby augmenting any bronchoconstriction (Hogg, 1985).

The inhibitory actions of β-adrenoceptor agonists on inflammatory mediator release and plasma protein extravasation are well documented, data coming from studies both in animals (Persson *et al.*, 1982) and in man (Basran *et al.*, 1982; Howarth *et al.*, 1985). However, clinical experience has not shown currently available β-adrenoceptor agonists to have significant anti-inflammatory activity (Archer & Macdonald, 1987; Cockroft & Murdock, 1987; Green *et al.*, 1988). This may be a consequence of the relatively short duration of action of such drugs. The experiments described above were therefore designed to investigate whether salmeterol, a novel long-acting β_2-agonist, would exhibit anti-inflammatory activity in addition to its long-lasting bronchodilator effects. Data published previously had shown that salmeterol inhibits inflammatory mediator release from human lung (Butchers *et al.*, 1991) and that this inhibition was sustained for up to 20 h.

The findings of this study indicate that salmeterol inhibits histamine-induced PPE in guinea-pig lung following aerosol or oral administration at bronchodilator doses (Ball *et al.*, 1991) and that these effects were mediated by β-adrenoceptors, since they were prevented by propranolol pretreatment. Furthermore, the inhibitory action of salmeterol on PPE is of long duration (>6 h) and is similar to that reported for the bronchodilator effects of the drug (Ball *et al.*, 1991).

Histamine increases PPE in lung by an action on the bronchial vasculature (Barnes *et al.*, 1988). Although the concentration of histamine used in these experiments did not cause overt bronchoconstriction, it is possible that histamine promotes PPE indirectly through a bronchoconstrictor action and that by inhibiting bronchoconstriction, salmeterol also inhibited PPE. This is unlikely since PGE_2 at bronchodilator doses did not inhibit histamine-induced PPE. In addition, salmeterol also inhibited histamine and zymosan-induced PPE in the skin of these animals. Instead, it is likely that salmeterol acts on the vasculature, probably on the endothelium of post-capillary venules (Persson *et al.*, 1982; Gudgeon & Martin, 1989), to prevent PPE.

The long duration of action of salmeterol against PPE may be of the therapeutic relevance. In animal studies, following the administration of bradykinin or histamine, the rate of PPE increases rapidly and returns to basal levels even when the stimulus persists (Adamski *et al.*, 1987). However, the resolution of this process is much slower, particularly in lung (Hurley, 1972; Sanchis *et al.*, 1972). Short-acting β_2-adrenoceptor agonists, such as salbutamol, may decrease the rate of extravasation, but only transiently. Since the rate-limiting step in clearing extravascular protein is the rate of removal (Hurley, 1972; Grega & Adamski, 1988), any transient reduction in the rate of extravasation would not result in a reduction of interstitial protein levels. In contrast, a long-acting compound, such as salmeterol, may be capable of reducing the rate of PPE for a sufficient period to allow the clearance mechanism to have an effect. This would be expected to reduce tissue oedema formation.

Salmeterol also inhibits granulocyte accumulation in the lung at bronchodilator doses and granulocyte accumulation and granulocyte-dependent PPE in the skin. These effects were again antagonized by propranolol and thus also appear to be mediated by an action on β-adrenoceptors. Eosinophil accumulation in the bronchial lumen is one of the hallmarks of asthma (Horn *et al.*, 1975; Venge, 1985), while neutrophil accumulation is characteristic of other forms of inflammation (Rylander & Haglind, 1986; Thompson *et al.*, 1989). Furthermore, eosinophil and neutrophil accumulation in the airways have been linked to nocturnal asthma (Martin *et al.*, 1991). Inhibition of granulocyte accumulation by β_2-adrenoceptor agonists is unlikely to be due to an action on the granulocyte itself, since high concentrations of β_2-adrenoceptor agonists are generally required to inhibit inflammatory cells *in vitro* (Busse & Sosman, 1984; Baker & Fuller, 1990) and these effects are not reversed by propranolol (Baker & Fuller, 1990). It is possible that, as for PPE, salmeterol inhibited granulocyte accumulation by an action on the venular endothelium. The finding that salmeterol, but not salbutamol, inhibited PAF-induced eosinophil accumulation over 24 h, and zymosan-induced granulocyte infiltration in the skin following systemic (oral) and local administration, suggests that inhibition of granulocyte accumulation is a property of long-acting β_2-adrenoceptor agonists. Thus, the finding that salbutamol albeit at ten fold higher concentrations than required to inhibit PPE, inhibited neutrophil infiltration in lung, but not in the skin, is perhaps surprising. However, the results obtained in the lung are consistent with data in the literature demonstrating that salbutamol blocked antigen-induced neutrophil, but not eosinophil, accumulation (Hutson *et al.*, 1988). This discrepancy between guinea-pig lung and skin requires further investigation, but may reflect a difference in the kinetics of neutrophil infiltration into these tissues or in the duration of action of salbutamol at higher doses.

In summary, the studies described above demonstrate that salmeterol has anti-inflammatory properties. It inhibits granulocyte accumulation, granulocyte-dependent PPE and granulocyte-independent PPE in guinea-pig lung and skin, following topical, local and oral administration. These effects occur at doses which have been shown to produce bronchodilatation in this species (Ball *et al.*, 1991). Like the effects on inflammatory mediator release (Butchers *et al.*, 1991), salmeterol has a long duration of action against both PPE and granulocyte accumulation. The duration of the anti-inflammatory effects of salmeterol is similar to that for bronchodilatation which, in man, lasts for at least 12 h (Ullman & Svedmyr, 1988), indeed it is possible to achieve a 24 h increase in lung function with twice daily dosing (Ullman *et al.*, 1990). Thus, it may be possible to achieve a 24 h anti-inflammatory effect in man, by administering salmeterol twice daily, which would give it a clear clinical advantage over the short-acting β_2-adrenoceptor agonists currently available for the treatment of asthma. Such properties may account for some of the clinical actions of salmeterol, which are not shared by the shorter-acting compounds, such as inhibition of allergen-induced late-phase responses in asthmatics (Twentyman *et al.*, 1990). The anti-inflammatory actions of salmeterol may be complementary to those of the inhaled steroids and furthermore, may be applicable to the therapy of other inflammatory disorders.

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References

ADAMSKI, S.W., LANGONE, J.J. & GREGA, G.J. (1987). Modulation of macromolecular permeability by immune complexes and a β-adrenoceptor stimulant. *Am. J. Physiol.*, **253**, H1586–H1595.

AOKI, S.W., BOUBEKEUR, K., BURROWS, L., MORLEY, J. & SANJAR, S. (1987). Recruitment of eosinophils by platelet activating factor (PAF) in the guinea-pig lung. *J. Physiol.*, **394**, 130P.

ARCHER, C.B. & MACDONALD, D.M. (1987). Treatment of atopic dermatitis with salbutamol. *Clin. Exp. Dermatol.*, **12**, 323–325.

ARNTZEN, F.C. & BRISEID, K. (1973). Inhibition of carrageenin induced rat paw oedema by catecholamines and amine depleting drugs. *Acta Pharmacol. Toxicol.*, **32**, 179–192.

BAKER, A.J. & FULLER, R.W. (1990). Anti-inflammatory effect of salmeterol on human alveolar macrophages. *Am. Rev. Respir. Dis.*, **141**, A394.

BALL, D.I., BRITTAIN, R.T., COLEMAN, R.A., DENYER, L.H., JACK, D., JOHNSON, M., LUNTS, L.H.C., NIALS, A.T., SHELDRICK, K.E. & SKIDMORE, I.F. (1991). Salmeterol, a novel, long-acting β_2-adrenoceptor agonist: characterization of pharmacological activity *in vitro* and *in vivo*. *Br. J. Pharmacol.*, **104**, 665–671.

BALL, D.I., COLEMAN, R.A. & SHELDRICK, K.E. (1987). Evaluation of some bronchoactive compounds in conscious guinea-pigs. *Br. J. Pharmacol.*, **90**, 151P.

BARNES, P.J., CHUNG, K.F. & PAGE, C.P. (1988). Inflammatory mediators and asthma. *Pharmacol. Rev.*, **40**, 49–84.

BASRAN, G.S., PAUL, W., MORLEY, J. & TURNER-WARWICK, M. (1982). Adrenoceptor agonist inhibition of the histamine induced cutaneous response in man. *Br. J. Dermatol.*, **107** Suppl. 23, 140–142.

BEASLEY, R., ROCHE, W.R., ROBERTS, J.A. & HOLGATE, S.T. (1989). Cellular events in the bronchi in mild asthma and after bronchial provocation. *Am. Rev. Respir. Dis.*, **139**, 806–817.

BONNET, J., LOISEAU, A.M., ORVOEN, M. & BESSIN, P. (1981). Platelet activating factor acether (PAF-acether) involvement in acute inflammatory and pain processes. *Agents Actions*, **11**, 559–562.

BRADSHAW, J., BRITTAIN, R.T., COLEMAN, R.A., JACK, D., KENNEDY, I., LUNTS, L.H.C. & SKIDMORE, I.F. (1987). The design of salmeterol, a long acting selective β_2-adrenoceptor agonist. *Br. J. Pharmacol.*, **92**, 509P.

BUSSE, W.W. & SOSMAN, J.M. (1984). Isoproterenol inhibition of isolated human neutrophil function. *J. Allergy Clin. Immunol.*, **73**, 404–410.

BUTCHERS, P.R., VARDEY, C.J. & JOHNSON, M. (1991). Salmeterol: a potent and long-acting inhibitor of inflammatory mediator release from human lung. *Br. J. Pharmacol.*, **104**, 672–676.

BUTCHERS, P.R., SKIDMORE, I.F., VARDEY, C.J. & WHEELDON, A. (1980). Characterisation of the receptor mediating the anti-anaphylactic effects of β-adrenoceptor agonists in human lung tissue *in vitro*. *Br. J. Pharmacol.*, **71**, 663–667.

COCKROFT, D.W. & MURDOCK, K.Y. (1987). Comparative effects of inhaled salbutamol, sodium cromoglycate and beclomethasone diproprionate on allergen-induced early asthmatic responses, late asthmatic responses, and increased bronchial responsiveness to histamine. *J. Allergy Clin. Immunol.*, **79**, 734–740.

DALY, M.J., FLOOK, J.J. & LEVY, G.P. (1975). The selectivity of β-adrenoceptor antagonists on cardiovascular and bronchodilator responses to isoprenaline in the anaesthetized dog. *Br. J. Pharmacol.*, **53**, 173–181.

DJUKANOVIC, R., ROCHE, W.R., WILSON, J.W., BEASLEY, C.R.W., TWENTYMAN, O.P., HOWARTH, P.H. & HOLGATE, S.T. (1990). Mucosal inflammation in asthma. *Am. Rev. Respir. Dis.*, **142**, 434–457.

DUNNILL, M.S. (1960). The pathology of asthma with special reference to changes in the bronchial mucosa. *J. Clin. Pathol.*, **13**, 27–33.

FOLKERTS, G., HENRICKS, P.A.J. & NIJKAMP, F.P. (1988). Inflammatory reactions in the respiratory airways of the guinea-pig do not necessarily induce bronchial hyperactivity. *Agents Actions*, **23**, 94–96.

GREEN, K.L. (1972). The anti-inflammatory effect of catecholamines in the peritoneal cavity and hind paw of the mouse. *Br. J. Pharmcol.*, **45**, 322–332.

GREEN, K.L., SAPRA, M., GRAHAM, P., HARRIS, J.M. & DARLEY, C.R. (1988). Topical salbutamol in atopic eczema. *Br. J. Clin. Pharmacol.*, **26**, 646P.

GREGA, G.J. & ADAMSKI, S.W. (1988). The role of venular endothelial cells in the regulation of macromolecular permeability. *Microcirc. Endoth. Lymphatics.*, **4**, 143–167.

GREGA, G.J., MACIEJKO, J.J., RAYMOND, R.M. & SAK, D.P. (1980). The interrelationship among histamine, various vasoactive substances and macromolecular permeability in the canine forelimb. *Circ. Res.*, **46**, 264–275.

GUDGEON, J.R. & MARTIN, W. (1989). Modulation of arterial endothelial permeability: studies on an *in vitro* model. *Br. J. Pharmacol.*, **98**, 1267–1274.

HADDY, F.J., SCOTT, J.B. & GREGA, G.J. (1976). Peripheral circulation: fluid transfer across the microvascular membrane. *Int. Rev. Physiol.*, **9**, 63–109.

HOGG, J.C. (1985). The pathology of asthma. In *Glucocorticoids, Inflammation and Bronchial Hyperreactivity*. ed. Hogg, J.C., Ellul-Micallef, R., Brattsand, R. pp. 3–10. Amsterdam, Geneva, Hong Kong, London, Princetown, Sidney, Tokyo: Excerpta Medica.

HORN, B.R., ROBIN, E.D., THEODORE, J. & VAN KESSEL, A. (1975). Total eosinophil counts in the management of bronchial asthma. *New Engl. J. Med.*, **292**, 1152–1155.

HOWARTH, P.H., DURHAM, S.R., LEE, T.H., KAY, A.B., CHURCH, M.K. & HOLGATE, S.T. (1985). Influence of albuterol, cromolyn sodium and ipratropium bromide on the airway and circulating mediator responses to allergen bronchial provocation in asthma. *Am. Rev. Respir. Dis.*, **132**, 986–992.

HURLEY, J.V. (1972). *Acute Inflammation*. Edinburgh, London: Churchill Livingstone.

HUTSON, P.A., HOLGATE, S.T. & CHURCH, M.K. (1988). The effect of cromolyn sodium and albuterol on early and late phase bronchoconstriction and airway leukocyte infiltration after allergen challenge of non-anaesthetised guinea-pigs. *Am. Rev. Respir. Dis.*, **138**, 1157–1163.

JOYNER, W.L., SVENSJO, E. & ARFORS, K.-E. (1979). Simultaneous measurements of macromolecular leakage and arteriolar blood flow as altered by PGE_1 and β_2-receptor stimulant in the hamster cheek pouch. *Microvasc. Res.*, **18**, 301–310.

KELLET, D.N. (1966). On the mechanism of the anti-inflammatory activity of hexadimethrine bromide. *Br. J. Pharmacol.*, **26**, 351–357.

MARTIN, R.J., CICUTTO, L.C., SMITH, H.R., BALLARD, R.D. & SZEFLER, S.J. (1991). Airways inflammation in nocturnal asthma. *Am. Rev. Respir. Dis.*, **143**, 351–357.

PERSSON, C.G.A. (1986). Role of plasma exudation in asthmatic airways. *Lancet*, **ii**, 1126–1128.

PERSSON, C.G.A., EKMAN, M. & ERJEFALT, I. (1978). Terbutaline preventing permeability effects of histamine in the lung. *Acta Pharmacol. Toxicol.*, **42**, 395–397.

PERSSON, C.G.A. & ERJEFALT, I. (1979). Terbutaline and adrenaline inhibit leakage of fluid and protein in guinea-pig lung. *Eur. J. Pharmacol.*, **55**, 199–201.

PERSSON, C.G.A., ERJEFALT, I., GREGA, G.J. & SVENSJO, E. (1982). The role of β-receptor agonists in the inhibition of pulmonary edema. *Ann. N.Y. Acad. Sci.*, **384**, 544–557.

PRASAD, G.M., ADAMSKI, S.W., SVENSJO, E. & GREGA, G.J. (1982). Pharmacological modification of the edema produced by combined infusions of prostaglandin E_1 and bradykinin in canine forelimbs. *J. Pharmacol. Exp. Ther.*, **220**, 293–298.

RAYMOND, R.M., JANDHYALA, B.S. & GREGA, G.J. (1980). The interrelationship among bradykinin, various vasoactive substances and macromolecular permeability in the canine forelimb. *Microvasc. Res.*, **19**, 329–337.

RIPPE, B. & GREGA, G.J. (1978). Effects of isoprenaline and cooling on histamine induced changes of capillary permeability in the rat hindquarter vascular bed. *Acta Physiol. Scand.*, **103**, 252–262.

RYLANDER, R. & HAGLIND, P. (1986). Exposure of cotton workers in an experimental cardroom with reference to airborne endotoxins. *Environ. Health. Perspect.*, **66**, 83–86.

RYLEY, H.C. & BROGAN, T.D. (1968). Variation in the composition of sputum in chronic chest diseases. *Br. J. Exp. Pathol.*, **49**, 625–633.

SANCHIS, J., DOLOVITCH, M., CHALMERS, R. & NEWHOUSE, M. (1972). Quantitation of regional aerosol clearance in the normal human lung. *J. Appl. Physiol.*, **33**, 757–762.

SEEGER, W., STOHR, G., WOLF, H.R.D. & NEUHOF, H. (1985). Alter-

ation of surfactant function due to protein leakage: special interaction with fibrin monomer. *J. Appl. Physiol.*, **58**, 326–338.

SPECTOR, W.G. & WILLOUGHBY, D.A. (1960). The enzymatic inactivation of an adrenaline-like substance in inflammation. *J. Pathol. Bacteriol.*, **80**, 271–279.

SPICER, B.A., BAKER, R.C., HATT, P.A., LAYCOCK, S.M. & SMITH, H. (1990). The effects of drugs on sephadex-induced eosinophilia and lung hyper-responsiveness in the rat. *Br. J. Pharmacol.*, **101**, 821–828.

SVENSJO, E. & GREGA, G.J. (1986). Evidence for endothelial cell-mediated regulation of macromolecular permeability by post-capillary venules. *Fed. Proc.*, **45**, 89–95.

SVENSJO, E., PERSSON, C.G.A. & ARFORS, K.-E. (1976). Effects of bradykinin and terbutalin on macromolecular leakage and its relation to other microvascular effects. *Microvasc. Res.*, **11**, 425.

THOMPSON, A.B., DAUGHTON, D., ROBBINS, R.A., GHAFOURI, M.A., OEHLERKING, M. & RENNARD, S.I. (1989). Intraluminal airway inflammation in chronic bronchitis. *Am. Rev. Respir. Dis.*, **140**, 1527–1537.

TWENTYMAN, O.P., FINNERTY, J.P., HARRIS, A., PALMER, J. & HOLGATE, S.T. (1990). Protection against allergen-induced asthma by salmeterol. *Lancet*, **336**, 1338–1342.

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.

ULLMAN, A., HEDNER, J. & SVEDMYR, N. (1990). Inhaled salmeterol and salbutamol in asthmatic patients. An evaluation of asthma symptoms and the possible development of tachyphylaxis. *Am. Rev. Respir. Dis.*, **142**, 571–575.

VENGE, P. (1985). Eosinophil and neutrophil granulocytes in asthma. In *Glucocorticoids, Inflammation and Bronchial Hyperreactivity*. ed. Hogg, J.C., Ellul-Micallef, R., Brattsand, R. pp. 21–37. Amsterdam, Geneva, Hong Kong, London, Princetown, Sidney, Tokyo: Excerpta Medica.

WANNER, A. (1977). Clinical aspects of mucociliary transport. *Am. Rev. Respir. Dis.*, **116**, 73–125.

WENZEL, S.E., LARSEN, G.L., JOHNSTON, K., VOELKEL, N.F. & WESTCOTT, J.Y. (1990). Elevated levels of leukotriene C_4 in bronchoalveolar lavage fluid from atopic asthmatics after endobronchial allergen challenge. *Am. Rev. Respir. Dis.*, **142**, 112–119.

WENZEL, S.E., WESTCOTT, J.Y. & LARSEN, G.L. (1991). Bronchoalveolar lavage fluid mediator levels 5 minutes after allergen challenge in atopic subjects with asthma: relationship to the development of late asthmatic responses. *J. Allergy Clin. Immunol.*, **87**, 540–548.

WHELAN, C.J. & JOHNSON, M. (1990). The anti-inflammatory effects of inhaled salmeterol and salbutamol in guinea-pig lung. *Br. J. Pharmacol.*, **101**, 528P.

WHELAN, C.J. & JOHNSON, M. (1991a). Salmeterol, but not salbutamol, has anti-inflammatory activity in guinea-pig skin. *Br. J. Pharmacol.*, **102**, 95P.

WHELAN, C.J. & JOHNSON, M. (1991b). Salmeterol inhibits granulocyte accumulation in guinea-pig lung. *Br. J. Pharmacol.*, **102**, 176P.

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