British Journal of Pharmacology (1996) 117, 1387–1394

Anti-inflammatory, membrane-stabilizing interactions of salmeterol with human neutrophils *in vitro*

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1 We have investigated the effects of salmeterol (0.3–50 μM) on several pro-inflammatory activities of human neutrophils *in vitro*.

2 Oxidant production by FMLP- and calcium ionophore (A23187)-activated neutrophils was particularly sensitive to inhibition by low concentrations (0.3–3 μM) of salmeterol, while the responses of phorbol myristate acetate- and opsonised zymosan-stimulated cells were affected only by higher concentrations (3–50 μM) of the drug. At these concentrations salmeterol is not cytotoxic, nor does it act as a scavenger of superoxide.

3 These anti-oxidative interactions of salmeterol with neutrophils were insensitive to propranolol but could be eliminated by washing the cells, or by pretreatment with low concentrations (1–2 μM) of the pro-oxidative, membrane-destabilizing phospholipids, lysophosphatidylcholine (LPC), platelet activating factor (PAF) and lysoPAF (LPAF).

4 At concentrations of 6.25–50 μM salmeterol interfered with several other activities of stimulated neutrophils, including intracellular calcium fluxes, phospholipase A_2 activity and synthesis of PAF.

5 In an assay of membrane-stabilizing activity, salmeterol (25 and 50 μM) neutralized the haemolytic action of LPC, PAF and LPAF.

6 Of the other commonly used β_2-adrenoceptor agonists, fenoterol, and formoterol, but not salbutamol, caused moderate inhibition of neutrophil oxidant generation by a superoxide-scavenging mechanism. However, unlike salmeterol, these agents possessed only weak membrane stabilizing properties.

7 We conclude that salmeterol antagonizes the pro-inflammatory, pro-oxidative activity of several bioactive lipids implicated in the pathogenesis of bronchial asthma, by a mechanism related to the membrane-stabilizing, rather than to the β_2-agonist properties of this agent.

Keywords: Salmeterol; neutrophils; oxidants; lysophospholipids; platelet activating factor; membrane-stabilization

Introduction

The commonly-used short- and long-acting β_2-adrenoceptor agonists possess anti-inflammatory activities some of which are consequent to their interactions with β_2-adrenoceptors on vascular endothelium, respiratory epithelium and mast cells (Fugner, 1989; Erjefalt & Persson, 1991; Advenier *et al.*, 1992; Whelan *et al.*, 1993; Kanthakumar *et al.*, 1994). These agents, particularly the long-acting β_2-agonists, formoterol and salmeterol, have also been reported to inhibit both the early and late phases of allergen-induced bronchoconstriction and bronchial hyperresponsiveness in atopic asthmatic patients by anti-inflammatory mechanisms which are distinct from those involving β_2-adrenoceptors (Twentyman *et al.*, 1990; Palmqvist *et al.*, 1992). These activities seem to be directed at various types of inflammatory cell, such as eosinophils (Eda *et al.*, 1993), macrophages (Baker & Fuller, 1990) and neutrophils (Ramage *et al.*, 1993; Vardey *et al.*, 1994), and may, at least in the case of salmeterol, enable reductions in the dose of inhaled corticosteroids (Greening *et al.*, 1994). Although clinically important, little is known about the biochemical mechanisms of these β_2-adrenoceptor-independent interactions of β_2-agonists with inflammatory cells.

The primary objective of the present study was to indentify the sites and mechanisms of the anti-oxidative, anti-inflammatory interactions of salmeterol, and to a lesser extent those of fenoterol, formoterol and salbutamol, with human neutrophils *in vitro*.

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Methods

Neutrophils

Human neutrophils were obtained from heparinised (5 units of preservative-free heparin ml^{-1}) venous blood of healthy adult volunteers and separated from mononuclear leucocytes by centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden)-metrizoate (Nyegaard, Oslo, Norway) cushions at 400 *g* for 25 min at room temperature. The resultant pellet was suspended in phosphate-buffered saline (PBS, 0.15 M, pH 7.4) and sedimented with 3% gelatin for 15 min at 37°C to remove most of the erythrocytes. After centrifugation, erythrocytes were removed by selective lysis with 0.84% ammonium chloride at 4°C for 10 min. The neutrophils, which were routinely of high purity (>90%) and viability (>95%), were resuspended to 1×10^7 ml^{-1} in PBS and held on ice until used.

Superoxide generation

This was measured using a lucigenin (*bis*-N-methylacridinium nitrate)-enhanced chemiluminescence (LECL) method (Minkenberg & Ferber, 1984). Neutrophils were pre-incubated for 15 min at 37°C in 900 μl HBSS containing 0.2 mM lucigenin in the presence and absence of the test agents (0.3–50 μM). Spontaneous and stimulus-activated LECL responses were then recorded in an LKB Wallac 1251 chemiluminometer (Turku, Finland) after the addition of 100 μl of the following stimuli of neutrophil membrane-associated oxidative metabolism: the synthetic chemotactic tripeptide N-formyl-L-leucyl-L-phenylalanine (FMLP; 1 μM), the calcium ionophore A23187,

(1 μM); phorbol 12-myristate 13-acetate (PMA; 25 ng ml^{-1}) and opsonised zymosan (OZ; 0.5 mg ml^{-1}). LECL readings were integrated for 5 s intervals and recorded as mV × seconds^{-1} (mV s^{-1}). Additional experiments were performed to investigate the following: (i) the duration of pre-incubation of neutrophils at 37°C with salmeterol (6.25–50 μM) on PMA-activated LECL; (ii) the durability of the effects of salmeterol on the FMLP- and PMA-activated LECL responses of neutrophils following washing (twice) of cells which had been exposed to the drug for 15 and 30 min at 37°C; (iii) the effects of propranolol (12.5 μM, this being the highest concentration of this agent which did not affect LECL) on the LECL responses of salmeterol-treated (25 μM) PMA-activated neutrophils; (iv) the effects of low, non-cytotoxic concentrations (1–2 μM) of the membrane-destabilizing phospholipids, lysophosphatidylcholine (LPC), platelet activating factor (PAF) and lysoPAF (LPAF) added 1 min before salmeterol, on salmeterol (0.3–2.5 μM) interactions with FMLP-activated neutrophils; (v) superoxide-scavenging activity of the test agents using a cell-free hypoxanthine (1 mM)-xanthine oxidase (17 milliunits ml^{-1}) superoxide-generating system.

Oxygen consumption

A 3-channel, Clark-type oxygen electrode (model DW1, Hansatech Ltd, King's Lynn, Norfolk) was used to measure the effects of salmeterol (25 and 50 μM) on O_2 consumption by PMA-activated neutrophils (2×10^6 ml^{-1}). The reduction in Po_2 was monitored for 15 min after the addition of PMA.

NADPH-oxidase activity

Neutrophils (5×10^6 ml^{-1}) were pre-incubated for 30 min at 37°C in the presence and absence of salmeterol (25 and 50 μM) followed by addition of PMA. After 10 min incubation the cells were centrifuged at 4°C and the pellets resuspended in 0.34 M sucrose supplemented with 0.5 mM phenylmethyl-sulphonyl fluoride (Calbiochem Corp., La Jolla, California, U.S.A.) and disrupted by sonication. Cellular debris was removed by centrifugation and the membrane fractions in the supernatants harvested following centrifugation at 70 000 *g* for 30 min. The membrane pellets were dispersed in 1 ml sucrose and assayed for NADPH-oxidase activity using LECL. Reaction mixtures (1 ml) contained lucigenin, membrane fractions (200 μl) and NADPH (2 mM), which was added last to initiate superoxide generation. In an additonal series of experiments, designed to investigate the effects of salmeterol on fully-assembled NADPH-oxidase, the drug (25 and 50 μM) was pre-incubated for 10 min at 37°C with purified membrane fractions prepared from control PMA-activated neutrophils, followed by addition of NADPH and measurement of LECL.

Intracellular calcium fluxes

These were measured spectrofluorometrically with fura 2 (Calbiochem) as the calcium-sensitive indicator (Grynkiewicz *et al.*, 1985). Neutrophils (1×10^7 ml^{-1}) were pre-loaded with fura 2 (2 μM) for 30 min at room temperature in HBSS, washed twice and resuspended in HBSS. The fura 2-loaded cells (1×10^6 ml^{-1}) were then pre-incubated with salmeterol (0.3–50 μM) for 5–10 min at 37°C then activated with 1 μM FMLP or 30 μM arachidonic acid (Naccache *et al.*, 1989) in the thermoregulated cuvette of a Hitachi 650-10S fluorescence spectrophotometer with excitation and emission wavelengths set at 340 nm and 500 nm respectively. Intracellular calcium concentrations ($[Ca^{2+}]_i$) were calculated by procedures which have been described previously (Grynkiewicz *et al.*, 1985).

PAF synthesis and phospholipase A_2 activity

For these experiments, cell numbers, incubation times and stimulant concentration were established in a series of preliminary experiments. In both assays the cells were pre-incubated with salmeterol for 15 min at 37°C. PAF was assayed in the supernatants of control and salmeterol-treated, calcium ionophore (2.5 μM)-activated neutrophils by use of a commercially-available radioimmunoassay (Du Pont NEN, Boston, MA, U.S.A.) after 10 min incubation at 37°C. In additional experiments designed to control for possible interactions of salmeterol with PAF, salmeterol was added retrospectively to PAF-containing supernatants from control, calcium ionophore-activated neutrophils. The mixtures were incubated for 15 min and the PAF quantitated by radioimmunoassay. In a similar series of experiments the immunoreactivity of reagent PAF (6.25–100 ng ml^{-1}) was measured following a 15 min exposure to salmeterol (12.5 μM).

Phospholipase A_2 activity was measured according to the extent of release of radiolabelled arachidonate ([^{3}H]-AA) from calcium ionophore-activated neutrophils (Anderson *et al.*, 1989). Briefly, neutrophils (1×10^7 ml) were coincubated with 5 μCi ml^{-1} [^{3}H]-AA [5,6,8,9,11,12,14,15-^{3}H](N), 100 Ci $mmol^{-1}$, Du Pont NEN) for 30 min at 37°C in Ca^{2+}-free HBSS, washed twice and resuspended to 1×10^7 ml^{-1} in HBSS. Following pre-incubation with salmeterol for 15 min at 37°C, the cells (5×10^6 in 2 ml) were activated with 2.5 μM calcium ionophore. After 5 min incubation at 37°C the reactions were terminated and the lipids extracted by addition of 5 ml of *n*-hexane/isopropanolol/concentrated HCl (300 : 200 : 4, v/v/v). The upper organic phase was separated, dried under a stream of nitrogen, reconstituted in 100 μl of hexane/isopropanolol (3 : 2, v/v) and [^{3}H]-AA quantitated by radiometric thin layer chromatography (t.l.c.) (Anderson *et al.*, 1989).

Protein kinase C activity

The effects of salmeterol (50 μM) on the activity of purified protein kinase C (PKC) from rat brain (Boehringer Mannheim, Germany) were measured with a commercial, colourimetric PKC assay kit (Pierce, Rockford, IL, U.S.A.). Briefly, salmeterol was coincubated with PKC (1.6 mu ml^{-1}) for 15 min at 30°C, after which an aliquot of the mixture (10 μl) was transferred to vials containing 8 μg of dye-labelled PKC substrate (glucose synthase peptide), 20 mM Tris assay buffer containing 2 mM ATP, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$ and phosphatidyl-L-serine (5 μg) as activator to give a final reaction volume of 25 μl. Vials were incubated for 30 min at 30°C after which phosphorylated and non-phosphorylated peptide was separated by centrifugation through a SpinZyme affinity separation unit (Pierce). Following elution, the amount of phosphorylated peptide was determined spectrophotometrically at 570 nm.

Cellular ATP levels

After 30 min incubation at 37°C in HBSS, neutrophil ATP levels were measured in the lysates of control and salmeterol-treated cells (10^6) using a luciferin/luciferase chemiluminescence method (Holmsen *et al.*, 1972).

Membrane stabilization

The membrane-stabilising potential of the test agents was measured by a haemolytic assay. Sheep erythrocytes were washed 3 times and resuspended to 5% in HBSS. The erythrocytes (final concentration of 0.5%) were then coincubated with the various test agents (25 and 50 μM) for 30 min at 37°C followed by the addition of LPC, PAF and LPAF at concentrations (5–8 μM) which caused incomplete haemolysis. After 5 min, intact erythrocytes were removed by centrifugation and the supernatants assayed spectrophotometrically at 415 nm for haemoglobin content.

Interactions of salmeterol with LPC and PAF

The ultraviolet absorption spectra of mixtures of salmeterol (250 μM) and LPC or PAF (both at 2000 μM) relative to

identical concentrations of the individual agents were measured using a Pye Unicam SP 1700 double-beam u.v. spectrophotometer.

Drugs and reagents

Three of the β_2-agonists used in this study (salbutamol, fenoterol and salmeterol) are the most commonly prescribed in South Africa, while formoterol was included because other than salmeterol, it is the only other clinically available long-acting β_2-agonist in this country. Salmeterol and salbutamol were provided by Glaxo Group Research, while fenoterol hydrobromide and formoterol fumarate were supplied by Boehringer Ingelheim KG (Germany) and Ciba-Geigy Ltd (Switzerland) respectively. These agents were dissolved in 0.05 N HCl to give stock solutions of 10 mM and diluted thereafter in indicator-free, Hanks' balanced salt solution (HBSS), pH 7.4, and used at a final concentration range of 0.3–50 μM. Following the 1/200 dilution in HBSS the pH of systems containing 50 μM of the test agents was not detectably different from that of control systems (pH 7.4). Unless indicated, all other chemicals and reagents were purchased from Sigma Chemical Co., St Louis, MO, U.S.A.

Statistical analysis

The results of each series of experiments are expressed as the mean values ± s.e. mean. Where appropriate, levels of statistical significance were calculated by Student's *t* test.

Results

Superoxide generation

These results are shown in Figure 1. Salmeterol caused dose-related inhibition of the LECL responses of neutrophils activated with all 4 stimuli of membrane-associated oxidative metabolism. The responses of FMLP- and calcium ionophore-activated neutrophils were more sensitive to salmeterol than those of opsonised zymosan and PMA with statistically significant inhibition ($P<0.01-P<0.0001$) observed at concentrations of 0.3 μM and upwards. In the case of opsonised zymosan and PMA, statistically significant inhibition of neutrophil LECL responses was observed at concentrations of 3 μM ($P<0.04$) and 12.5 μM ($P<0.01$) respectively. The duration of exposure (15–90 min) of neutrophils to salmeterol (25 μM) did not affect the magnitude of inhibition of the LECL responses of PMA-activated neutrophils.

Washing of neutrophils that had been coincubated with salmeterol (25 and 50 μM) was associated with either complete or partial loss of inhibition of PMA-activated LECL. For neutrophils continuously exposed to salmeterol for 30 min prior to activation, the mean percentages PMA-stimulated LECL of the drug-free control system were 59 ± 15 ($P<0.02$) and 6 ± 5 ($P<0.002$) for the cells treated with 25 μM and 50 μM salmeterol respectively. The corresponding responses of washed, matched neutrophils were 121 ± 15 (NS) and 81 ± 3 ($P<0.04$). These are the results of 4 separate experiments.

Coincubation of neutrophils with propranolol (12.5 μM) for 15 min prior to addition of salmeterol did not reduce the level of salmeterol (25 μM)-mediated inhibition of the LECL responses of PMA-activated neutrophils. The mean percentages LECL of the drug-free control system, of neutrophils exposed to propranolol only, salmeterol only or to the combination of propranolol and salmeterol were 97 ± 3, 46 ± 3 and 40 ± 3 respectively.

The effects of pretreatment (1 min) of neutrophils with low, non-cytotoxic concentrations (1 and 2 μM) of LPC, on salmeterol-mediated inhibition of FMLP-activated LECL responses are shown in Figure 2. At all concentrations tested (0.3–2.5 μM) salmeterol caused significant ($P<0.005$) inhibition of the LECL responses of FMLP-activated neutrophils, while LPC at both concentrations significantly ($P<0.05-P<0.0005$) antagonized the inhibitory effects of salmeterol. PAF and LPAF, at both concentrations tested, also caused statistically significant protection ($P<0.05-P<0.005$) of the LECL responses of FMLP-activated neutrophils against the inhibitory effects of all 4 concentrations of salmeterol (not shown). At concentrations of 2 μM, but not at 1 μM, both LPC and LPAF significantly increased the FMLP-activated responses of neutrophils to 124 ± 5% ($P<0.025$) and 127 ± 7% ($P<0.05$) of control values respectively. The corresponding values for 1 μM of LPC and LPAF were 103 ± 2% and 105 ± 4. PAF at 1 μM and 2 μM significantly increased the FMLP-activated responses of neutrophils to 143 ± 13% ($P<0.05$) and 171 ± 6% ($P<0.025$) of the control value respectively. Because of these sensitizing effects, the levels of antagonism were cal-

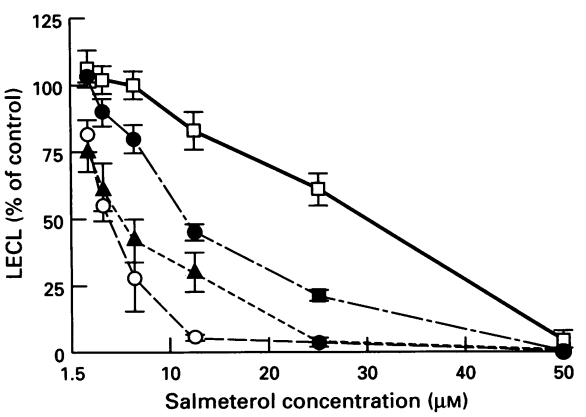

Figure 1 The effects of salmeterol on the peak lucigenin-enhanced chemiluminescence (LECL) responses of neutrophils activated with FMLP (▲), calcium ionophore (○), opsonized zymosan (●) and PMA (□). The results of 6–14 experiments are expressed as the mean percentage ± s.e. mean of the corresponding drug-free control systems. The absolute peak LECL values for the drug-free control systems activated with FMLP, calcium ionophore, opsonized zymosan and PMA were 1020 ± 180, 1434 ± 215, 6369 ± 570 and 4237 ± 262 mV s^{-1} respectively. The peak LECL responses were reached after 4 min, 15 min, 14 min and 9 min for FMLP, calcium ionophore, opsonized zymosan and PMA respectively.

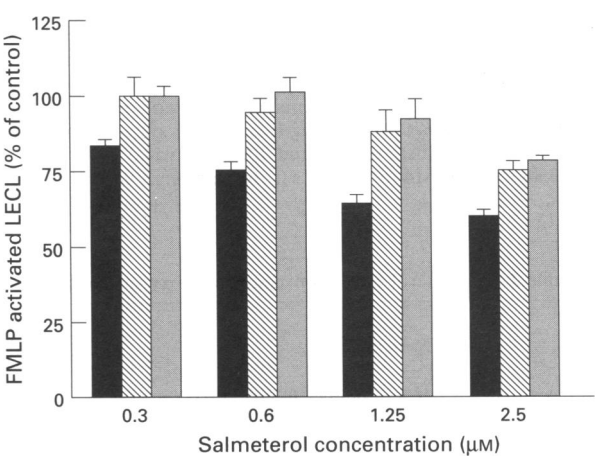

Figure 2 The effects of salmeterol on the lucigenin-enhanced chemiluminescence (LECL) responses of FMLP-activated neutrophils in the absence (solid columns) and in the presence of 1 μM (hatched columns) and 2 μM (stippled columns) of LPC. The results of 5 experiments are presented as the mean percentage of the corresponding drug-free control systems ± s.e. mean. The absolute peak LECL values for control, unstimulated neutrophils and for FMLP-activated neutrophils were 174 ± 2 mV s^{-1} and 1163 ± 148 respectively.

culated by comparing the values obtained for cells treated with the combination of salmeterol + phospholipid with the corresponding phospholipid-treated, salmeterol-free systems. At concentrations of 1 and 2 μM, none of the phospholipids affected the spontaneous LECL responses of neutrophils in the absence of a stimulant, while at concentrations >2.5 μM the lysophospholipids and PAF were progressively cytotoxic.

The comparative effects of salmeterol, salbutamol, fenoterol and formoterol (6.25–50 μM) on neutrophil PMA-activated LECL responses are shown in Figure 3. These responses were unaffected by salbutamol, but were significantly reduced by fenoterol at concentrations of 12.5–50 μM ($P<0.05-P<0.01$) and formoterol at the same concentrations ($P<0.004-P<0.001$), with respective ED_{50} values of 26 μM, >50 μM and >50 μM. These inhibitory effects of fenoterol and formoterol were: (a) less than those of salmeterol (b) insensitive to propranolol and (c) completely eliminated by washing the cells. Fenoterol and formoterol also caused dose-related inhibition of the LECL reponses of neutrophils activated with FMLP and calcium ionophore. The inhibitory effects of these agents on FMLP-activated LECL could not however, be prevented by pretreatment of neutrophils with LPC (not shown).

Superoxide scavenging

Using the cell-free xanthine/xanthine oxidase superoxide-generating system, neither salmeterol nor salbutamol at concentrations of up to 50 μM were found to possess superoxide-scavenging properties. However, both fenoterol and formoterol caused dose-related inhibition of superoxide generation by this system. These results are shown in Table 1 for a time-point (3 min) at which the reaction was linear, as well as for the peak responses which were reached at 6–10 min. Both agents inhibited LECL generated by the xanthine-xanthine oxidase system. The inhibitory effects of formoterol were clearly dose-related, while those of fenoterol were fairly similar for the three concentrations tested. Sodium fumarate at concentrations of 25 and 50 μM did not affect superoxide generation by the xanthine-xanthine oxidase system, demonstrating that the inhibitory effects of formoterol cannot be attributed to the fumarate moiety of the molecule.

Since consumption of O_2 (measured with an O_2 electrode) by the xanthine-xanthine oxidase system was unaffected by fenoterol and formoterol (50 μM), it appears that these agents are scavengers of superoxide rather than inhibitors of xanthine oxidase.

O_2 consumption and NADPH-oxidase activity

The effects of salmeterol (25 and 50 μM) on O_2 consumption by PMA-activated neutrophils and on the activity of NADPH-oxidase in purified membrane fractions prepared from these cells are shown in Table 2. Both activities were significantly inhibited by salmeterol at these concentrations, but not at lower concentrations. Addition of salmeterol (25 and 50 μM) to purified membrane fractions prepared from PMA-stimulated, control neutrophils did not inhibit the activity of NADPH-oxidase, demonstrating that this agent at these concentrations primarily affects the assembly rather than the activity of the oxidase complex. Neither fenoterol nor formoterol at concentrations of

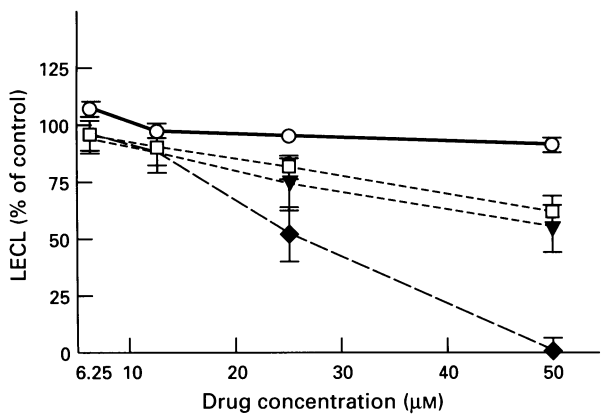

Figure 3 Comparative effects of salmeterol (◆), salbutamol (○), fenoterol (▼) and formoterol (□) on the peak lucigenin-enhanced chemiluminescence (LECL) responses of PMA-activated neutrophils. The results of 3–5 experiments are presented as the mean percentage ± s.e. mean of the drug-free control system. The absolute LECL values for unstimulated and PMA-activated control systems were 350 ± 56 and 5140 ± 205 mV s^{-1} respectively.

Table 1 Effects of fenoterol and formoterol on superoxide generation by the xanthine/xanthine oxidase system

System	*LECL (mV s^{-1}) generated by the xanthine/xanthine oxidase system after:* 3 min/37°C	6–10 min/37°C
Control	330 ± 22	417 ± 45
Fenoterol 12.5 μM	134 ± 21	324 ± 41
Fenoterol 25 μM	130 ± 28	318 ± 25
Fenoterol 50 μM	121 ± 3	307 ± 22
Formoterol 12.5 μM	92 ± 24	243 ± 17
Formoterol 25 μM	61 ± 18	128 ± 21
Formoterol 50 μM	48 ± 16	52 ± 13

The results of 3 experiments are expressed as the mean values ± s.e.means. The time-course of the experiments was 15 min and the results shown are for an early time point (3 min), when the reaction was linear, and for the peak lucigenin-enhanced chemiluminescence (LECL) responses, which occurred 6–10 min after the reactions were initiated. Both agents, at all 3 concentrations tested, caused statistically significant inhibition of LECL ($P<0.04-P<0.0006$).

Table 2 Salmeterol effects on neutrophil oxygen consumption and NADPH-oxidase activity

System	*O_2 consumption* (μM per 10^6 cells 15 min^{-1}) ($n=3$)	*NADPH-oxidase activity* (mV s^{-1})($n=8$)
Unstimulated neutrophils	–	44 ± 11
PMA-activated neutrophils in the absence of salmeterol	82 ± 20	2304 ± 178
PMA-activated neutrophils + salmeterol 25 μM	62 ± 18	1724 ± 170°
PMA-activated neutrophils + salmeterol 50 μM	17 ± 3	347 ± 21°

Results are expressed as the mean values ± s.e.mean of *3 and †8 experiments. O_2 consumption was monitored for the 15 min period following addition of PMA (25 ng ml^{-1}) to neutrophils when uptake was linear. NADPH-oxidase activity was measured in purified membrane fractions prepared from PMA-activated, control or salmeterol-treated neutrophils.
°$P<0.03-P<0.0001$.

25 and 50 μM affected utilisation of O_2 by PMA-activated neutrophils. The mean percentages O_2 consumption for neutrophils treated with 50 μM fenoterol or formoterol, relative to the drug-free control system were 97 ± 1 and 101 ± 3 respectively.

Intracellular calcium fluxes, PAF synthesis and phospholipase A_2 activity

The effects of salmeterol on intracellular calcium fluxes in FMLP-activated neutrophils and on PAF synthesis by calcium ionophore-activated neutrophils are shown in Figure 4. At concentrations of 6.25 μM and upwards salmeterol caused statistically significant ($P<0.05-P<0.001$) inhibition of both fura-2 fluorescence and synthesis of PAF. The inhibitory effects of salmeterol on the fura-2 fluorescence responses of neutrophils were also observed when arachidonic acid was used as stimulant (results not shown). The inhibitory effects of salmeterol on PAF synthesis by neutrophils appeared to be real since (a) addition of this agent to PAF-containing supernatants from activated neutrophils did not affect the reactivity of the phospholipid in the RIA and (b) the immunoreactivity of reagent PAF was unaffected by coincubation with salmeterol.

Salmeterol also caused dose-related inhibition of the phospholipase A_2 activity of calcium ionophore-activated neutrophils, which achieved statistical significance ($P<0.005$) only at the highest concentration tested (50 μM). Following correction for background values, the respective values for generation of [^{3}H]-arachidonate by calcium ionophore-activated control and salmeterol-treated neutrophils were 46836 ± 3912 and 26322 ± 5088 c.p.m. per 10^7 neutrophils (results of 5 different experiments).

PKC activity

Coincubation of PKC with salmeterol (50 μM) did not affect enzyme activity. The activity of salmeterol-treated PKC was $100 \pm 7\%$ that of the corresponding drug-free control system (results of 3 experiments).

ATP levels

Coincubation of neutrophils with salmeterol at concentrations up to 25 μM for 30 min at 37°C and with fenoterol and formoterol at concentrations of up up 25 μM did not affect cellular ATP levels. However, treatment of neutrophils with 50 μM salmeterol significantly ($P<0.003$) increased cellular ATP levels. The respective values for control neutrophils and for those treated with 25 and 50 μM salmeterol were 8.6 ± 0.4, 10.3 ± 2.3 and 17.7 ± 0.9 nmol ATP per 10^7 neutrophils.

Membrane stabilizing activity

Salmeterol at concentrations of 25 and 50 μM protected sheep erythrocytes against the haemolytic effects of LPC, LPAF and PAF ($P<0.004-P<0.0005$). These results are shown in Figure 5. However, the haemolytic activity of these phospholipids was unaffected by salbutamol, fenoterol and formoterol at concentrations of up to 50 μM.

In a single experiment, the effects of all four β_2-agonists at a fixed concentration of 100 μM on LPC-mediated haemolysis were investigated. In this experiment LPC was used at concentrations of 6, 7 and 8 μM, which caused varying degrees of haemolysis (34%–77%). These results are shown in Table 3. Salmeterol protected the erythrocytes against the haemolytic effects of all three concentrations of LPC, while the other three β_2-agonists showed modest protective activity against the lower concentrations of this lysophospholipid, the order of potency being formoterol > salbutamol ≥ fenoterol.

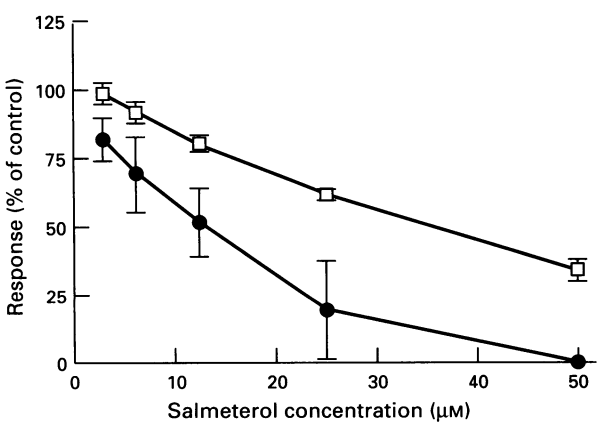

Figure 4 Effects of salmeterol on peak intracellular calcium levels (□) in FMLP-activated neutrophils (6–8 experiments) and on synthesis of PAF (●) by calcium ionophore-stimulated neutrophils (3–6 experiments). The results are expressed as the mean percentage ± s.e. mean of the drug-free control systems. The mean absolute peak value for $[Ca^{2+}]_i$ in control systems was 858 ± 190 nM, while the corresponding value for PAF synthesis was 18.5 ± 4.6 ng PAF per 10^7 cells. PAF could not be detected in the supernatants of resting neutrophils.

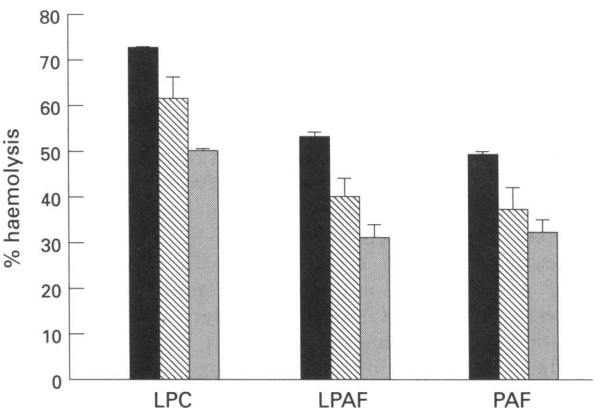

Figure 5 Haemolysis of sheep erythrocytes mediated by LPC, LPAF and PAF (5–8 μM) in the absence (solid columns) and presence of 25 μM (hatched columns) and 50 μM salmeterol (stippled columns). The results of 4 experiments are presented as the mean percentage haemolysis ± s.e. mean.

Table 3 Effects of the β_2-agonists (100 μM) on LPC-mediated haemolysis of sheep erythrocytes

	Haemolysis (%) mediated by LPC at concentrations of:		
Agent	6 μM	7 μM	8 μM
None (control)	34 ± 1	53 ± 1	77 ± 2
Salmeterol (100 μM)	2 ± 0.5*	13 ± 0.3*	31 ± 1*
Formoterol (100 μM)	23 ± 1*	44 ± 1*	78 ± 2
Salbutamol (100 μM)	29 ± 1*	49 ± 1*	82 ± 1
Fenoterol (100 μM)	30 ± 0.2*	57 ± 1	85 ± 2

The results of a single experiment with 6 replicates for each agent are expressed as the mean values ± s.e.mean.
*Denotes statistically significant protection ($P<0.05-P<0.0001$) against LPC-mediated haemolysis.

Interactions of salmeterol with LPC and PAF

Spectrophotometric analysis of mixtures of salmeterol with LPC or PAF did not reveal any interactions between these agents.

Discussion

Of the four β_2-agonists investigated in the present study, salmeterol and to a lesser extent fenoterol and formoterol, but not salbutamol, suppressed the generation of superoxide by activated neutrophils. The anti-oxidative activity of fenoterol and formoterol was mediated by superoxide-scavenging mechanisms rather than by direct effects of these agents on the neutrophil. This conclusion is based on observations that fenoterol and formoterol decreased the LECL responses of the cell-free, xanthine/xanthine oxidase system, without affecting utilization of oxygen either by this system or by activated neutrophils. Although the superoxide-scavenging activities of fenoterol have been described previously (Gillissen *et al.*, 1994; 1995), the aspects of molecular structure which confer this property on fenoterol, as well as on formoterol, have not been identified. Salmeterol did not possess superoxide-scavenging properties and appeared to function as a true inhibitor of neutrophil superoxide production by β_2-adrenoceptor-independent mechanisms. This contention is supported by observations that the inhibitory effects of salmeterol on oxidant production by activated neutrophils were insensitive to propranolol and almost completely eliminated by washing the cells. These observations confirm previous studies in which salmeterol has been reported to inhibit oxidant generation by FMLP- and opsonized zymosan-activated neutrophils by undefined, β_2-adrenoceptor-independent mechanisms (Ramage *et al.*, 1993; Vardey *et al.*, 1994).

The inhibitory effects of salmeterol on neutrophil superoxide generation were dose-related and observed with all four stimuli of membrane-associated oxidative metabolism, the intensity of their pro-oxidative interactions with these cells being an apparent determinant of sensitivity to salmeterol. The responses of neutrophils activated with the two weak stimuli, FMLP and calcium ionophore, were more sensitive to the suppressive effects of salmeterol and were observed with concentrations of this β_2-agonist (0.3–3 μM) which did not appear to influence transductional mechanisms involved in the activation of NADPH-oxidase. At higher concentrations (>3 μM), salmeterol also caused progressive inhibition of oxygen consumption, NADPH-oxidase activity and generation of oxidants by PMA- and opsonized zymosan-activated neutrophils. Addition of salmeterol to purified membrane fractions prepared from control, PMA-activated neutrophils did not affect the activity of fully-assembled NADPH-oxidase, indicating that this agent, at these concentrations, affects the activation rather than the activity of NADPH-oxidase. In this respect it is noteworthy that inhibition of NADPH-oxidase activity coincided with, and was probably a consequence of, salmeterol-mediated interference with neutrophil intracellular calcium fluxes, synthesis of PAF and activity of phospholipase A_2. These effects on neutrophils could not be attributed to non-specific cytotoxicity since cellular ATP levels were unaffected at concentrations of up to 25 μM of salmeterol, and were significantly increased at higher concentrations of this agent (50 μM).

The lipophilic nature of salmeterol relative to the other β_2-agonists used in this study (Johnson, 1995), together with its wide-ranging inhibitory effects on membrane-associated activities of stimulated neutrophils, suggested that the plasma membrane was the probable site of anti-inflammatory action of this agent. Lipophilicity is an important physico-chemical property of salmeterol, which distinguishes this agent from other β_2-agonists. The relative lipophilicity values of salmeterol, formoterol and salbutamol are 3200, 8 and 1 respectively (Johnson, 1995). Consequently, salmeterol is rapidly accumulated by cells, and within 1 min there are >20000 molecules in the cell membrane for each molecule that remains in the extracellular phase (Johnson, 1995). Using a haemolytic assay of membrane stabilizing/destabilizing activity, we have shown that this remarkably high level of intra-membrane accumulation of salmeterol is accompanied by alterations in membrane stability. Pretreatment of sheep erythrocytes with salmeterol, but not with fenoterol, formoterol or salbutamol, at concentrations of up to 50 μM increased the resistance of these cells to the haemolytic effects of the bioactive phospholipids, LPC, PAF and LPAF. The concentrations of salmeterol required to counter the membrane-destabilizing properties of the bioactive phospholipids were somewhat higher than those required to inhibit superoxide generation by activated neutrophils. This can probably be explained by the use of high, cytolytic concentrations (5–8 μM) of LPC, LPAF and PAF in the haemolytic assay of membrane-destabilization with a consequent requirement for relatively high concentrations of salmeterol in this system. We were unable to detect complex-forming interactions between salmeterol and these lytic agents, suggesting that the observed protective effects of the β_2-agonist are mediated exclusively by membrane-stabilizing mechanisms. Modest protective activity was evident with the other β_2-agonists when their concentrations were raised to 100 μM, with membrane-stabilizing potency reflecting relative lipophilicity (formoterol > salbutamol $\geqslant$ fenoterol).

We also used LPC, LPAF and PAF to investigate possible relationships between the membrane-stabilizing and anti-inflammatory properties of salmeterol. Pretreatment of neutrophils with these phospholipids (1–2 μM) prevented the inhibitory effects of salmeterol (0.3–2.5 μM) on generation of superoxide by FMLP-activated neutrophils. We did not include data from similar experiments with PMA-stimulated neutrophils since at the high concentrations (>6.25 μM) of salmeterol required to inhibit these responses, protection can be achieved only by levels of the bioactive phospholipids that are cytotoxic for drug-free, control systems. Neutralization of salmeterol-mediated inhibition of superoxide-generation by FMLP-activated neutrophils pretreated with LPC, PAF or LPAF clearly links the membrane-stabilizing properties of this agent to its anti-oxidative activity. This proposed relationship is supported by previous studies which have demonstrated that optimum activity of NADPH-oxidase is dependent on lateral mobility of the neutrophil membrane (Kusner *et al.*, 1991; Wiles *et al.*, 1994). Our results suggest that these membrane-stabilizing interactions of salmeterol with neutrophils may antagonize the pro-oxidative activities of endogenously-generated lysophosphatides and PAF. These bioactive phospholipids are generated during exposure of neutrophils to stimuli of membrane-associated oxidative metabolism (Zimmerman *et al.*, 1992; Dana *et al.*, 1994) and potentiate the activity of NADPH-oxidase (Oishi *et al.*, 1988; Dana *et al.*, 1994). Addition of exogenous, reagent lysophospholipids and PAF to neutrophils does not activate superoxide generation, but, as also observed in the present study, induces a state of hyperresponsiveness (priming, sensitization) on subsequent exposure of the cells to stimuli of membrane-associated oxidative metabolism (Engelberger *et al.*, 1987; Ginsburg *et al.*, 1989). Importantly, these sensitizing effects of lysophosphatides on the pro-oxidative activities of neutrophils are highly correlated with their membrane-disruptive properties (Ginsburg *et al.*, 1989).

Salmeterol-mediated antagonism of the pro-oxidative activities of lysophosphatides and PAF apparently occurs by two concentration-dependent mechanisms, both involving membrane stabilization. At low concentrations (<6.25 μM), salmeterol appears to neutralize the pro-oxidative interactions of these bioactive phospholipids without affecting their synthesis by neutrophils, while at higher concentrations, intense membrane stabilization affects cellular calcium mobilization and activity of enzymes involved in the synthesis of lysophosphatides and PAF. High level membrane stabilization may

also inhibit the activity of membrane ATPases, which would explain the increased cellular ATP levels of neutrophils exposed to high concentrations of salmeterol.

These membrane-stabilizing, anti-inflammatory properties of salmeterol are unlikely to be restricted to neutrophils since PAF and LPC, both of which are thought to play key roles in the pathogenesis of bronchial asthma and rhinitis (Barnes, 1989; Mehta *et al.*, 1990; Zimmerman *et al.*, 1990), are potent mediators of other inflammatory and cytotoxic processes. Apart from its pro-oxidative and cytotoxic properties, which are shared by PAF and LPAF, LPC also induces mast cell secretion by promoting influx of extracellular calcium (Marquardt & Walker, 1991), while exposure of respiratory epithelium to lysophospholipids and PAF is accompanied by ciliary dysfunction, cytotoxicity and impaired mucociliary clearance (Ganbo *et al.*, 1991; Nieminen *et al.*, 1991; Feldman *et al.*, 1995). Although many of the pro-inflammatory activities of PAF are clearly receptor-mediated (Barnes, 1989; Zimmerman *et al.*, 1992), some activities of this bioactive phospholipid, as well as those of LPC and LPAF, appear to be mediated by receptor-independent, membrane-disruptive mechanisms which may be amenable to pharmacological control by membrane-stabilizing agents.

In conclusion, salmeterol, unlike the other commonly-used short- and long-acting β_2-agonists, is a potent membrane-stabilizing agent, which regulates human neutrophil function, probably by antagonizing the pro-oxidative activities of several bioactive lipids implicated in the pathogenesis of bronchial asthma. These are clearly secondary effects of this agent which are evident at concentrations somewhat higher (0.3–0.6 μM) than those (<0.1 μM) required for β_2-agonist effects (Johnson, 1995). Nevertheless, if operative *in vivo*, they may contribute to the therapeutic activity of this β_2-agonist.

References

ADVENIER, C., QIAN, Y., LAW KOUNE, J.-D., MOLIMARD, M., CANDENAS, M.-L & NALINE, E. (1992). Formoterol and salmeterol inhibit bradykinin and histamine induced airway microvascular leakage in guinea-pig. *Br. J. Pharmacol.*, **105,** 792–798.

ANDERSON, R., BEYERS, A.D., SAVAGE, J.E. & NEL, A.E. (1988). Apparent involvement of phospholipase A_2, but not protein kinase C, in the pro-oxidative interactions of clofazimine with human phagocytes. *Biochem. Pharmacol.*, **37,** 4635–4641.

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

BARNES, P.J. (1989). New concepts in the pathogenesis of bronchial hyperresponsiveness and asthma. *J. Allergy Clin. Immunol.*, **83,** 1013–1026.

DANA, R., MALECH, H.L. & LEVY, R. (1994). The requirement for phospholipase A_2 for activation of the assembled NADPH-oxidase in human neutrophils. *Biochem. J.*, **297,** 217–223.

EDA, R., SUGIYAMA, H., HOPP, R.J., OKADA, C., BEWTRA, A.K. & TOWNLEY, R.G. (1993). Inhibitory effects of formoterol on platelet-activating factor induced eosinophil chemotaxis and degranulation. *Int. Arch. Allergy Appl. Immunol.*, **102,** 391–398.

ENGELBERGER, W., BITTER-SUERMANN, D. & HADDING, U. (1987). Influence of lysophospholipids and PAF on the oxidative burst. *Int. J. Immunopharmacol.*, **9,** 275–282.

ERJEFALT, I & PERRSON, C.G.A. (1991). Long duration and high potency of antiexudative effects of formoterol in guinea-pig tracheobronchial airways. *Am. Rev. Resp. Dis.* **144,** 788–791.

FELDMAN, C., ANDERSON, R., THERON, A., COLE, P.J. & WILSON, R. (1995). The effects of platelet activating factor (PAF) and lysophosphatidylcholine (LPC) on human ciliated epithelium *in vitro*. *Am. J. Resp. Crit. Care Med.*, **151,** A65.

FUGNER, A. (1989). Formation of oedema and accumulation of eosinophils in the guinea-pig lung. Inhibition by beta-stimulants. *Int. Arch. Allergy Appl. Immunol.*, **88,** 225–227.

GANBO, T., HISAMATSU, K., NAKAZAWA, T., KAMJO, A. & MURAKAMI, Y. (1991). Platelet activating factor (PAF) effects on ciliary activity of human paranasal sinus mucosa *in vitro*. *Rhinology*, **29,** 231–237.

GILLISSEN, A., JAWORSKA, M., VAN ZWOLL, D., BARGON, I. & SCHULTZE-WERNINGHAUS, G. (1995). β_2-Agonists scavenge H_2O_2 and O_2 which is related to phenol rings within the molecular structure. *Am. J. Resp. Crit. Care Med.*, **151,** A270.

GILLISSEN, A., SCHÄRLING, B. & SCHULTZE-WERNINGHAUS, G. (1994). The potency of fenoterol, terbutalin, isopreterenol, and prednisolon to inhibit O_2 and H_2O_2 *in vitro*. *Am. J. Resp. Crit. Care Med.*, **149,** A805.

GINSBURG, I., WARD, P.A. & VARANI, J. (1989). Lysophosphatides enhance superoxide responses of stimulated human neutrophils. *Inflammation*, **13,** 163–174.

GREENING, A.P., IND, P.W., NORTHFIELD, M. & SHAW, G. (1994). Added salmeterol versus higher-dose corticosteroid in asthma patients with symptoms on existing inhaled corticosteroid. *Lancet*, **344,** 219–224.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260,** 3440–3450.

HOLMSEN, H.E., STORM, E. & DAY, H.J. (1972). Determination of ATP and ADP in blood platelets: a modification of the firefly luciferase assay for plasma. *Anal. Biochem.*, **46,** 481–502.

JOHNSON, M. (1995). Approaches to the design of long-acting agonists at β-adrenoceptors. In *β-Adrenoceptor Agonists and the Airways*. ed. Johnson, M. & Small, R. pp. 13–27. London: Royal Society of Medicine Press Ltd.

KANTHAKUMAR, K., CUNDELL, D.R., JOHNSON, M., WILLS, P.J., TAYLOR, G.W., COLE, P.J. & WILSON, R. (1994). Effect of salmeterol on human nasal epithelial cell ciliary beating: inhibition of the ciliotoxin, pyocyanin. *Br. J. Pharmacol.*, **112,** 493–498.

KUSNER, D.J., AUCOTT, J.N., FRANCESCHI, D., SARASUA, M.M., SPAGNUOLO, P.J. & KING, C.H. (1991). Protease priming of neutrophil superoxide production: effects on membrane lipid order and lateral mobility. *J. Biol. Chem.*, **266,** 16465–16471.

MARQUARDT, D. & WALKER, L.L. (1991). Lysophosphatidylcholine induces mast cell secretion and protein kinase C activation. *J. Allergy Clin. Immunol.*, **88,** 721–730.

MEHTA, D., GUPTA, S., GAUR, S.N., GANGAL, S.V. & AGRAWAL, K.P. (1990). Increased leukocyte phospholipase A_2 activity and plasma lysophosphatidylcholine levels in asthma and rhinitis and their relationship to airway sensitivity to histamine. *Am. Rev. Resp. Dis.*, **142,** 157–161.

MINKENBERG, I. & FERBER, E. (1984). Lucigenin-dependent chemiluminescence as a new assay for NADPH-oxidase activity in particulate fractions of human polymorphonuclear leukocytes. *J. Immunol. Methods*, **71,** 61–67.

NACCACHE, P.H., MCCOLL, S.R., CAON, A.C. & BORGEAT, P. (1989). Arachidonic acid-induced mobilisation of calcium in human neutrophils: evidence for a multicomponent mechanism of action. *Br. J. Pharmacol.*, **97,** 461–468.

NIEMINEN, M.M., MOILANEN, E.K., NYHOLM, J.-E.J., KOSKINEN, M.O., KARVONEN, J.I., METSÄ-KETELÄ, T.J. & VAPAATALO, H. (1991). Platelet-activating factor impairs mucociliary transport and increases plasma leukotriene B_4 in man. *Eur. Respir. J.*, **4,** 551–560.

OISHI, K., RAYNOR, R.L., CHARP, P.A. & KUO, J.F. (1988). Regulation of protein kinase C by lysophospholipids. *J. Biol. Chem.*, **263,** 6865–6871.

PALMQVIST, M., BALDER, B., LOWHAGEN, O., MELANDER, B., SVEDMYR, N & WAHLANDER, L. (1992). Late asthmatic reaction decreased after pretreatment with salbutamol and formoterol, a new long-acting β_2-agonist. *J. Allergy Clin. Immunol.*, **89,** 844–849.

RAMAGE, L., BLAIR, A., CREE, I.A. & DHILLON, D.P. (1993). Effect of salmeterol on polymorphonuclear leukocyte (PMNL) chemiluminescence *in vitro*. *J. Biolumin. Chemilumin.*, **8,** 247–252.

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.

VARDEY, C.J., NIALS, A.T. & COLEMAN, R.A. (1994). Inhibitory effects of salmeterol in human neutrophils are short-lasting. *Am. J. Resp. Crit. Care Med.*, **149,** A361.

WHELAN, C.J., JOHNSON, M. & VARDEY, C.J. (1993). Comparison of the anti-inflammatory properties of formoterol, salbutamol and salmeterol in guinea-pig skin and lung. *Br. J. Pharmacol.*, **110,** 613–618.

WILES, M.E., DYKENS, J.A. & WRIGHT, C.D. (1994). Regulation of polymorphonuclear leukocyte membrane fluidity: effect of cytoskeletal modification. *J. Leuk. Biol.*, **56,** 192–199.

ZIMMERMAN, G.A., PRESCOTT, S.M. & MCINTYRE, T.M. (1992). Platelet-activating factor: a fluid phase and cell-associated mediator of inflammation. In *Inflammation: Basic Principles and Clinical Correlates.* ed. Gallin, J.I., Goldstein, I.M. & Snyderman, R. pp. 149–176. New York: Raven Press Ltd.

(Received August 7, 1995
Revised November 13, 1995
Accepted November 27, 1995)