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Investigations into factors determining the duration of action of the β_2-adrenoceptor agonist, salmeterol

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1 This study has explored the mechanism underlying the long duration of action of the β_2-adrenoceptor agonist, salmeterol.

2 Salmeterol, salbutamol and isoprenaline caused a concentration-related inhibition of electrically-induced contractile responses of the guinea-pig superfused trachea preparation. The effects of both isoprenaline and salbutamol were rapid in onset and rapidly reversed upon removal of the agonist. In contrast, the effects of salmeterol were slower in onset and could not be reversed by superfusion of the tissue with agonist-free Krebs solution even for periods of up to 10 h.

3 The effects of salmeterol were, however, readily reversed by a number of β-adrenoceptor blocking drugs, as was the effect of a continuous infusion of isoprenaline. Upon removal of the antagonist, however, the effects of salmeterol and of the isoprenaline infusion were reasserted at a rate which was inversely related to the lipophilicity of a β-adrenoceptor blocking drugs.

4 Salmeterol inhibited the binding of [^{125}I]-(−)-iodopindolol (100 pM) to rat lung membranes (pIC_{50} 7.1), with isoprenaline (pIC_{50} 6.2) and salbutamol (pIC_{50} 5.1) having lower potencies. The inhibition of binding by salmeterol was apparently non-competitive, whereas that produced by salbutamol and isoprenaline was competitive in nature.

5 Isoprenaline and salbutamol rapidly dissociated from their binding sites, whereas in marked contrast, the binding of salmeterol showed no dissociation for periods of up to 1 h.

6 These data are consistent with a mechanism in which salmeterol binds adjacent to the active site of the β_2-adrenoceptor, such that the drug cannot be washed out of the tissue, yet can interact with and activate the receptor. This latter property is susceptible to antagonism by β-adrenoceptor blocking drugs but is reasserted when the antagonists are removed.

Keywords: β-Adrenoceptor agonists; β-adrenoceptor antagonists; salmeterol; guinea-pig trachea; rat lung membrane; duration of action; exosite

Introduction

Salmeterol is a potent and selective, long-acting β_2-adrenoceptor agonist both *in vitro* and *in vivo* (Ball *et al.*, 1987a, b; 1991; Bradshaw *et al.*, 1987; Johnson, 1990). Moreover, clinical studies have shown that, following aerosol administration, salmeterol is also a long-acting bronchodilator in man (Ullman & Svedmyr, 1988; Maconochie *et al.*, 1987) and is effective in the treatment of bronchial asthma (Britton, 1990; Palmer, 1990).

We have previously suggested that the remarkably sustained β_2-adrenoceptor agonist activity of salmeterol is due to binding to an 'exosite' rather than to an irreversible interaction with the β_2-adrenoceptor itself (Bradshaw *et al.*, 1987; Nials & Coleman, 1988; Jack, 1991). This mechanism involves an association of the lipophilic N-substituent phenylalkyloxyalkyl 'tail' of salmeterol with an exosite which holds the molecule in such a position that the phenylethanolamine 'head' can repeatedly interact with, and activate, the β_2-adrenoceptor. This proposal was based mainly upon two observations. Firstly, the long-lasting effects of salmeterol can be competitively antagonized by propranolol (Ball *et al.*, 1987a). Secondly, the smooth muscle relaxant activity of salmeterol, which can be reversed by the β-adrenoceptor antagonist, sotalol, reasserts itself when the sotalol is removed (Ball *et al.*, 1991), implying some form of specific retention of salmeterol adjacent to the active site of the β_2-adrenoceptor.

In this present study, the interaction of salmeterol with β_2-adrenoceptors has been explored further. Specifically, we have investigated the effects of a number of β-adrenoceptor antagonists on the rates of reversal and reassertion of salmeterol-induced smooth muscle relaxation in a superfused, electrically-stimulated guinea-pig trachea preparation. In addition, we have also investigated the interaction of salmeterol with rat lung β_2-adrenoceptors using a radioligand binding assay.

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Methods

Preparation of isolated tracheal strip

Tracheal strips were prepared as described by Coleman & Nials (1989). Briefly, adult, male guinea-pigs (400–550 g) were killed by a blow to the head with subsequent exsanguination. The tracheae were excised and placed in Krebs solution in a Petri dish. The adherent connective tissue was dissected away and the lumen gently flushed with Krebs solution. Tracheae were dissected into rings containing 3–4 cartilage bands (Castillo & DeBeer, 1947; Akcasu, 1959) and the rings opened to form strips by cutting through the cartilage on the side opposite to the smooth muscle band (Coburn & Tomita, 1973). A long cotton thread was attached to the cartilage at one end of the strip for attachment to a strain gauge, and a cotton loop to the other end for anchoring the tissue in the superfusion chamber.

Tissue superfusion

The superfusion apparatus employed in these experiments has been described previously (Coleman *et al.*, 1986; Coleman & Nials, 1989). Preparations were mounted under a

resting tension of 1 g, and superfused at a rate of 2 ml min^{-1} with oxygenated (5% CO_2 in O_2) modified Krebs solution (Apperley *et al.*, 1976) maintained at 37°C. Bipolar platinum electrodes were positioned parallel with and in close proximity to, the superfused tissue.

Determination of agonist activity on electrically-stimulated guinea-pig trachea

For experiments involving electrical stimulation, preparations were taken from guinea-pigs pretreated with 6-hydroxydopamine (200 mg kg^{-1}, i.p.), in order to eliminate any adrenergic component in the electrically-induced response (Coleman *et al.*, 1986; Coleman & Nials, 1989). To measure relaxant activity in the guinea-pig trachea, phasic contractile responses were induced by electrical field stimulation with 10 s trains of square wave pulses of 5 Hz frequency, 0.1 ms duration and just maximal voltage (8–16 V) every 2 min. These electrically-induced responses were highly reproducible for periods of at least 8–10 h of stimulation.

For each preparation, constant responses to the standard agonist, isoprenaline, were first obtained by infusing increasing concentrations in a sequential manner, until sensitivity was constant. A 30 min equilibration period was allowed before a concentration-effect curve to a test agonist was started. The magnitude of each response was measured and calculated as a percentage inhibition of the electrically-induced contractile response.

Potency values for the β-adrenoceptor agonists were expressed in both absolute terms (concentration required to induce 50% inhibition, EC_{50}) and relative to isoprenaline, as an equipotent concentration (EPC, i.e. EC_{50} for the test agonist/EC_{50} for isoprenaline).

Determination of rate of onset of action

The time for onset of action (Ot_{50}) is defined as the time from starting the administration of the test agonist to attainment of 50% of the response maximum for an EC_{50} concentration. Ot_{50} values were determined by interpolation from a plot of % response against time to attainment of 50% of each response maximum.

Determination of rate of offset of action

The time for offset of action (Rt_{50}) is defined as the time from stopping administration of the test agonist to attainment of 50% recovery from an EC_{50} concentration. Rt_{50} values were determined by interpolation from a plot of % response against time to 50% recovery of each response (Rt_{50}).

Interaction of β-adrenoceptor antagonists with salmeterol and isoprenaline

Electrically-induced contractile responses were inhibited by infusion of salmeterol (10–100 nM), and when the inhibitory responses had equilibrated, the infusion was stopped. In control preparations, an infusion of isoprenaline (30–100 nM) was continued throughout the experiment. Some of the preparations were left with no further treatment for the duration of the experiment in order to determine whether any spontaneous recovery from the inhibitory responses of salmeterol and isoprenaline occurred. On other preparations, a β-adrenoceptor blocking drug was infused. When the antagonism of the β-adrenoceptor agonist effect had equilibrated, the antagonist infusion was halted, and the tissue superfused with an antagonist-free Krebs solution. A range of seven β-adrenoceptor blocking drugs of varying potency, lipophilicity and β_1/β_2 adrenoceptor selectivity were used: sotalol, propranolol, atenolol, ICI 118551, labetalol, pindolol and timolol. Each β-adrenoceptor blocking drug was tested at a single concentration which approximated to its pA_{100} (i.e. $\sim 100 \times pA_2$) at β_2-adrenoceptors. For the β_1-adrenoceptor selective antagonist, atenolol, this was not possible due to its low β_2-adrenoceptor antagonist potency, and therefore an approximate pA_{10} concentration was used. We attempted to identify on each preparation a concentration of salmeterol or isoprenaline which caused 70–90% inhibition of electrically-induced contractions. On each preparation, the maximum degree of reversal of the agonist-induced inhibition, the rate of reversal, and the rate at which the agonist effect reasserted itself once the antagonist infusion had been halted (Ra_{50} the time taken for the agonist to reassert to 50% of its pre-antagonist level) were measured.

Radioligand binding assays

The binding of the radioligand [^{125}I]-(−)-iodopindolol ([^{125}I]-PIN) to rat lung membrane preparations was determined by an assay based upon that described by Barovsky & Brooker (1980). [^{125}I]-PIN was either synthesized from (-)-pindolol and Na^{125}I using the chloramine-T oxidation method (Barovsky & Brooker, 1980) or was purchased.

Membrane preparation

All procedures were performed on ice or at 4°C. Lungs were removed from female Wistar rats (200 g, killed by cervical dislocation), scissor-minced and homogenized (3 × 5 s bursts, Polytron homogenizer) in 10 volumes of ice-cold buffer (50 mM Tris-HCl, pH 7.5). The homogenate was centrifuged at 800 *g* for 10 min and the pellet discarded. The supernatant fraction was recentrifuged at 40,000 *g* for 20 min to produce a crude membrane pellet. This was washed by resuspension in ice-cold assay buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 0.1 mg ml^{-1} L-ascorbic acid, pH 7.8) using a loose-fitting glass Dounce homogenizer (Jencons), and recentrifuged at 70,000 *g* for 20 min. The washed membrane pellet was resuspended in ice-cold assay buffer and the protein content, determined by the method of Lowry *et al.* (1951), was adjusted to 1 mg ml^{-1} with assay buffer. This suspension was stored as aliquots at − 60°C for periods of up to 1 month, and was thawed for use as required.

Binding assay

Binding assays were performed in triplicate. Assay mixtures (final volume 0.2 ml), comprising [^{125}I]-PIN (0–400 pM), guanosine-5'-triphosphate (GTP, 100 μM), membrane suspension (50 μg protein) and drugs where appropriate, all prepared in assay buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 0.1 mg ml^{-1} L-ascorbic acid, pH 7.8), were incubated for 30 min at 37°C. Non-specific [^{125}I]-PIN binding was defined as that occurring in the presence of 200 μM (-)-isoprenaline, and constituted ~5% of total binding at a radioligand concentration of 100 pM. Assays were terminated by dilution with ice-cold assay buffer followed by rapid vacuum filtration through glass fibre filters (Whatman GF/B) mounted in a 12-place manifold (Millipore). Assay tubes and filters were washed with ice-cold assay buffer and, when dry, filters were counted in an LKB gamma counter to determine the amount of membrane-bound radioactivity trapped in the filter.

For experiments examining the reversibility of β_2-adrenoceptor agonist binding, aliquots of rat lung membranes (2 ml of a 1 mg ml^{-1} suspension) were pretreated for 30 min at 37°C with assay buffer (controls), salmeterol (10 μM), salbutamol (300 μM) or isoprenaline (100 μM); all solutions were prepared with assay buffer containing 100 μM GTP. The membrane suspensions were then centrifuged for 2 min at 12,000 *g* and the supernatant fractions discarded. The pellets were surface rinsed and resuspended in assay buffer (containing 100 μM GTP) to a membrane protein concentration of 1 mg ml^{-1}. Each pretreated membrane suspension was then divided into two samples which were further diluted (1:4) with assay buffer containing [^{125}I]-PIN (final concentration

Table 1 Superfused, electrically-stimulated guinea-pig trachea: potencies and durations of action of isoprenaline, salbutamol and salmeterol

Agonist	EC_{50} (nM)	*EPC* (Isopren = 1)	*Onset* (Ot_{50}, min)	*Duration* (Rt_{50}, min)	n
Isoprenaline	15.4 (9.3–25.5)	1	<2	>2 <4	8
Salbutamol	40.2 (8.0–208)	3.9 (1.9–8.1)	2.8 (1.9–5.3)	6.4 (3.1–10.7)	5
Salmeterol	4.8 (2.5–9.1)	0.3 (0.26–0.47)	27.6 (21.8–33.7)	>470	6

Data are geometric (EC_{50}, EPC) or arithmetic (onset, duration) means with 95% confidence limits shown in parentheses. EC_{50} is defined as the concentration of agonist required to induce 50% inhibition of the electrically-induced contractile response. Ot_{50} is defined as the time from starting the administration of the agonist to attainment of 50% of the response maximum for an EC_{50} concentration. Rt_{50} is defined as the time from stopping administration of the test agonist to attainment of 50% recovery from an EC_{50} concentration (see Methods). Isopren = isoprenaline.

100 pM) and GTP (100 μM final concentration) with or without isoprenaline (200 μM final concentration) to define non-specific binding.

This procedure results in a 250 to 300 fold dilution of the original drug solutions as estimated from experiments applying the same procedure to solutions of [^{125}I]-PIN. These mixtures were subsequently incubated at 37°C, and at timed intervals, samples (200 μl, 50 μg protein) were removed, diluted with ice-cold buffer and filtered to terminate binding. The time course for specific (i.e. total minus non-specific) [^{125}I]-PIN binding was compared for drug-treated or buffer-treated (control) membranes, and the binding to drug-treated membranes expressed as a percentage of that obtained for binding to control membranes.

Data (counts per minute) were analysed with the computer programmes LIGAND (Munson & Rodbard, 1980) and ALLFIT (DeLean *et al.*, 1978) or by the procedures detailed by Weiland & Molinoff (1981).

Drugs

The drugs used in this study were adrenaline bitartrate (Sigma, U.K.), atenolol (ICI, U.K.), erythro-DL-1 (7-methylindan-4-yloxy)-3-isopropylamino-butan-2-ol (ICI 118551, ICI, U.K.), 6-hydroxydopamine hydrobromide (Sigma, U.K.) indomethacin (Sigma, U.K.), isoprenaline sulphate (Sigma, U.K.), labetalol (Glaxo, U.K.), noradrenaline bitartrate (Sigma, U.K.), pindolol (Sandoz, Switzerland), propranolol hydrochloride (Sigma, U.K.), salbutamol (Glaxo, U.K.), salmeterol 1-hydroxy-2-naphthoate, (Glaxo, U.K.), sotalol hydrochloride (Mead Johnson, U.S.A.), timolol maleate (Merck, U.S.A.).

Sodium [^{125}I]-iodide and [^{125}I]-(−)-iodopindolol were obtained from Amersham International and New England Nuclear respectively.

For the functional studies, isoprenaline, salbutamol and salmeterol were dissolved in a small volume (40 μl) of glacial acetic acid and made up to final volume with phosphate buffer (pH 7.0). Indomethacin was dissolved in 10% (w/v) $NaHCO_3$ and made up to volume with 0.9% (w/v) saline. All other compounds were dissolved in distilled water. Drug dilutions were made up to volume in 0.9% saline. Solutions of the β-adrenoceptor agonists contained ascorbic acid (11 mM). For the binding studies, salmeterol was dissolved in 10% final volume of 1 M acetic acid and diluted in assay buffer to volume. All other drugs were dissolved and diluted in assay buffer.

Results

Potency and duration of action of isoprenaline, salbutamol and salmeterol

All three β-adrenoceptor agonists caused a concentration-related inhibition of electrically-induced contractile responses of the guinea-pig superfused trachea. The results are summarized in Table 1. Salmeterol was approximately 3 fold more potent than isoprenaline, and approximately 12 fold more potent than salbutamol. Marked differences were observed in the rates of onset and offset of action of the agonists (Table 1). Thus, while the onset of action of both

Table 2 Superfused, electrically-stimulated guinea-pig trachea: interaction of a range of β-adrenoceptor blocking drugs with the inhibitory effects of salmeterol (persisting after stopping salmeterol administration) and isoprenaline (continuous infusion)

		vs *Salmeterol* (10–30 nM)				vs *Isoprenaline* (10–100 nM)			
Antagonist	*Conc* (μM)	*Agonist response* (% inhibition of electrically-induced contractile response)	*Antagonist-induced reversal* (% max)	*Onset of antagonist activity* (Ot_{50}, min)	*Agonist reassertion time* (Ra_{50}, min)	*Agonist response* (% inhibition of electrically-induced contractile response)	*Antagonist-induced reversal* (% max)	*Onset of antagonist activity* (Ot_{50}, min)	*Agonist reassertion time* (Ra_{50}, min)
Atenolol	10	85.2 (± 8.7)	47.3 (± 8.0)	2.9 (± 0.4)	4.7 (± 0.9)	93.1 (± 3.1)	77.8 (± 6.9)	5.0 (± 1.3)	10.0 (± 2.2)
Sotalol	10	75.7 (± 9.5)	95.8 (± 2.7)	6.6 (± 0.7)	21.8 (± 3.1)	85.9 (± 2.2)	88.5 (± 3.2)	6.7 (± 1.2)	18.4 (± 3.2)
Pindolol	0.1	69.2 (± 5.2)	86.1 (± 3.8)	10.3 (± 0.6)	53.6 (± 10.2)	89.1 (± 4.2)	93.8 (± 4.1)	3.8 (± 0.4)	47.0 (± 7.9)
Labetalol	0.1	83.7 (± 9.3)	71.2 (± 12.5)	4.6 (± 0.8)	81.7 (± 18.5)	71.9 (± 5.8)	67.4 (± 6.9)	4.4 (± 0.8)	81.5 (± 23.0)
Timolol	0.1	81.6 (± 4.6)	87.9 (± 4.4)	5.2 (± 0.6)	136.4 (± 22.8)	85.8 (± 4.0)	94.3 (± 2.9)	5.0 (± 0.4)	113.5 (± 30.7)
Propranolol	0.1	83.1 (± 4.9)	83.5 (± 4.1)	4.2 (± 1.1)	220.1 (± 39.9)	92.9 (± 2.1)	93.2 (± 2.2)	11.0 (± 2.5)	180.6 (± 18.5)
ICI 118551	0.1	87.2 (± 3.7)	87.3 (± 3.7)	3.4 (± 0.7)	155 ->886	85.7 (± 3.2)	68.8 (± 5.4	2.9 (± 0.3)	179.6 (± 21.9)

Results are arithmetic means ± s.e.mean in parentheses of at least 4 experiments.

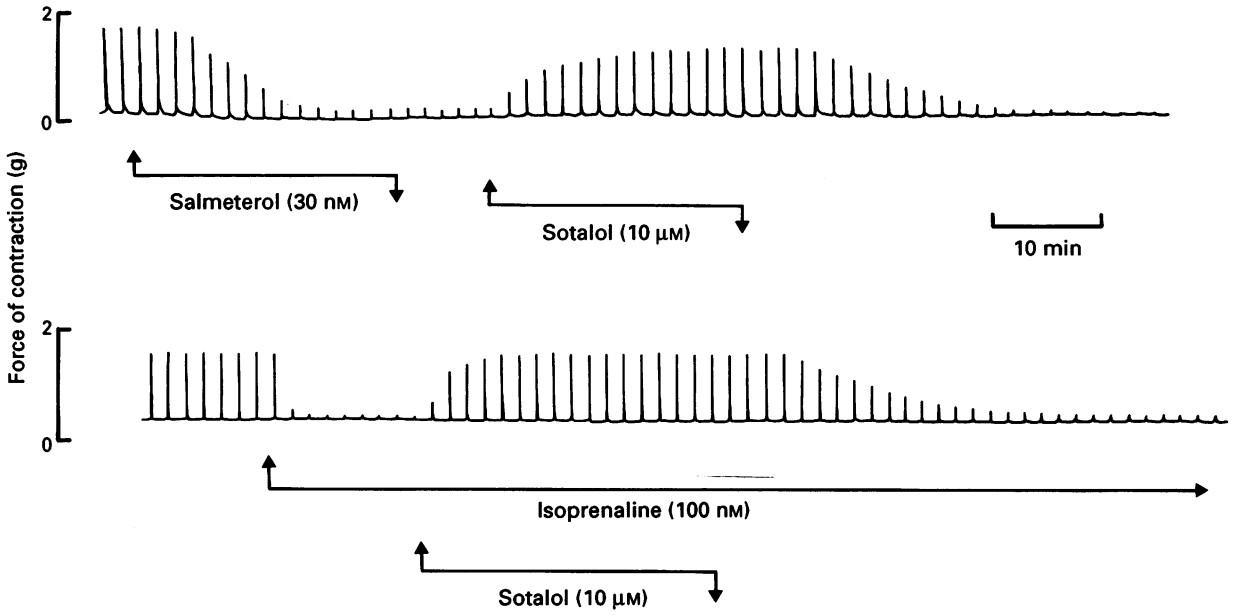


Figure 1 Superfused, electrically-stimulated guinea-pig trachea: typical experiments illustrating the effects of administration and subsequent withdrawal of sotalol (10 μM) on the inhibitory responses to salmeterol (persisting after salmeterol withdrawal) and isoprenaline (continuous infusion).

isoprenaline and salbutamol was rapid ($Ot_{50} < 3$ min), that of salmeterol was slower ($Ot_{50} = 27.6$ min). Isoprenaline and salbutamol were also both short-acting, ($Rt_{50} < 7$ min); however, no recovery was observed from responses to salmeterol even after periods in excess of 8 h, despite continuous superfusion of the tissue with agonist-free Krebs solution.

Interaction of a range of β-adrenoceptor antagonists with salmeterol and isoprenaline

On electrically-stimulated guinea-pig trachea preparations, infusions of sotalol and atenolol (10 μM), and propranolol, ICI 118551, labetalol, pindolol and timolol (0.1 μM) reversed

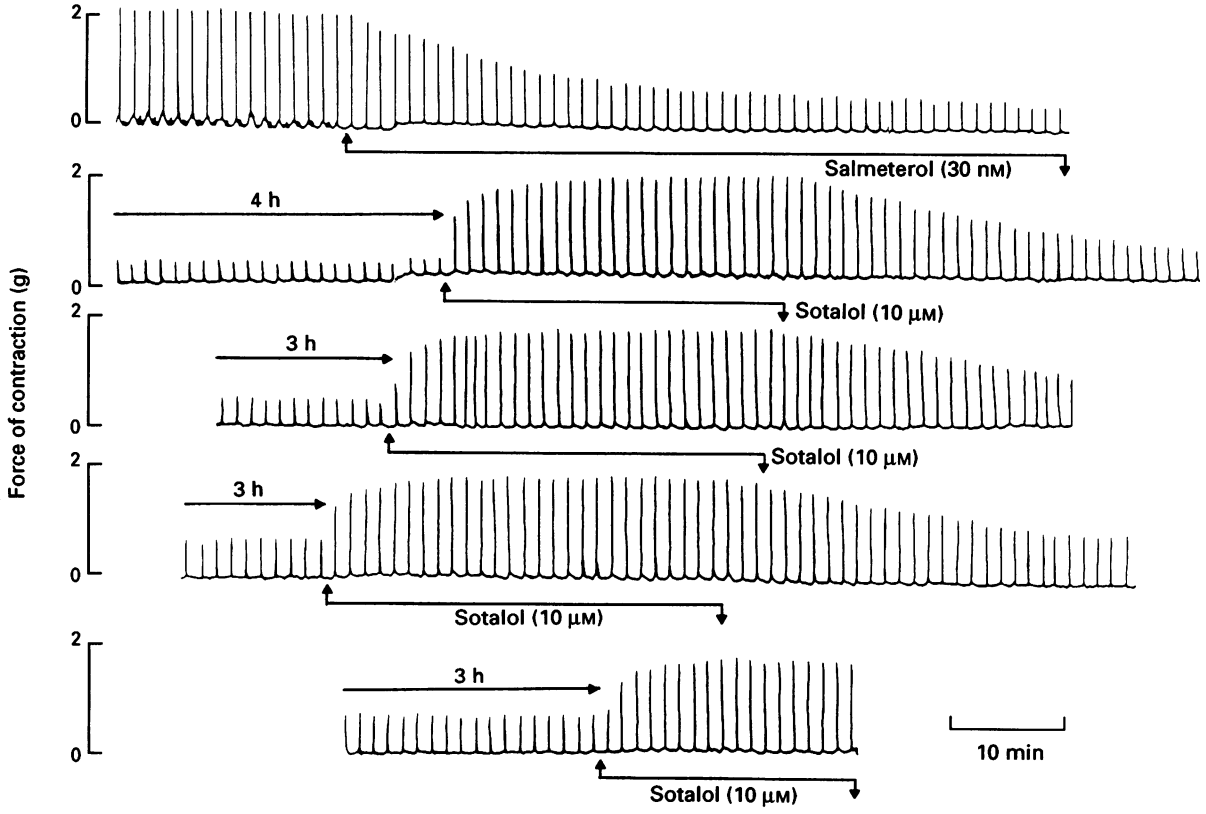
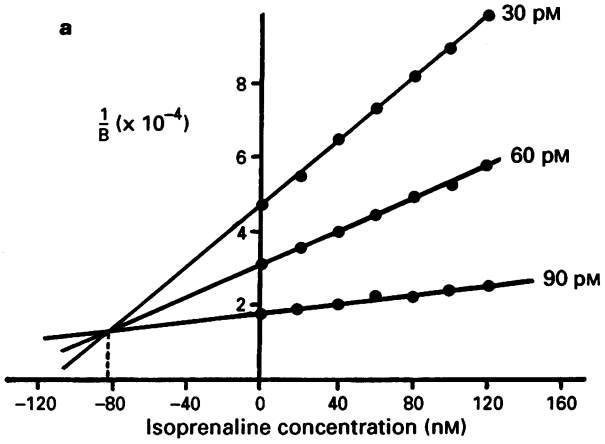
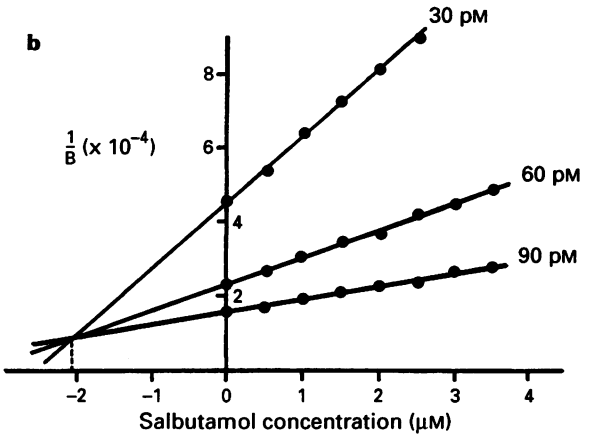


Figure 2 Superfused, electrically-stimulated guinea-pig trachea: repeated administration and subsequent withdrawal of sotalol (10 μM) on the inhibitory responses to salmeterol (persisting after salmeterol withdrawal).





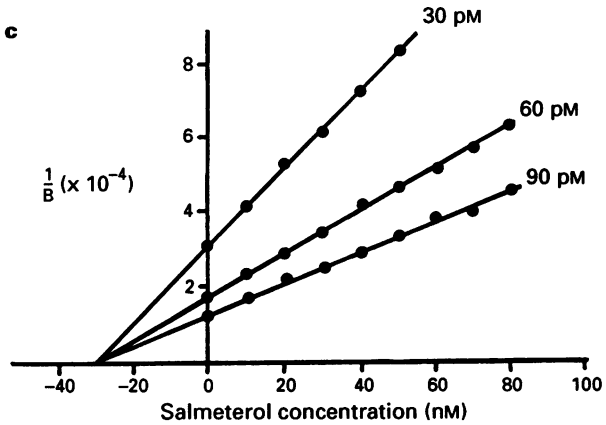


Figure 3 Rat lung membranes: Dixon plots for the inhibition of specific [^{125}I]-(-)-pindolol ([^{125}I]-PIN) binding (B) to rat lung β_2-adrenoceptors by (a) isoprenaline, (b) salbutamol and (c) salmeterol. Inhibition curves were constructed at three radioligand concentrations, 30 pM, 60 pM and 90 pM, and the data plotted according to the method of Dixon (Boeynaems & Dumont, 1980). Note that the profiles of isoprenaline and salbutamol are that of a competitive inhibitor, whereas the inhibition produced by salmeterol is apparently non-competitive. Results are from a single representative experiment which was repeated on at least 3 occasions.

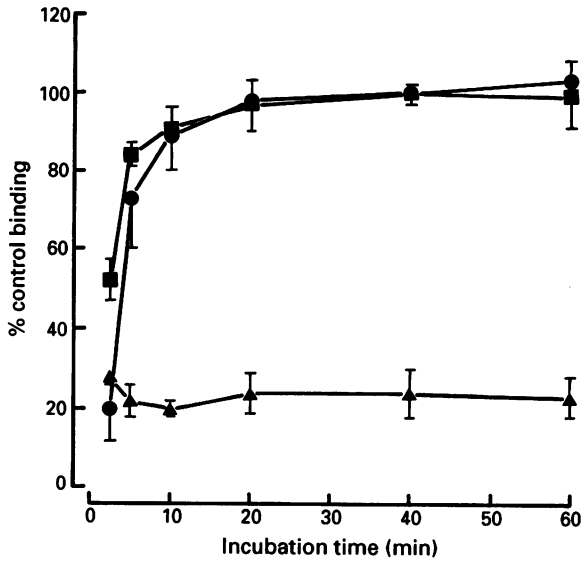


Figure 4 Rat lung membranes: dissociation of β_2-adrenoceptor agonists from rat lung membrane β_2-adrenoceptors. Rat lung membranes were pretreated for 30 min at 37°C with assay buffer (controls), isoprenaline (100 μM, ●), salbutamol (300 μM, ■) or salmeterol (10 μM, ▲) and then diluted 250 to 300 fold with buffer containing [^{125}I]-(-)-pindolol (100 pM) to induce drug dissociation. The time course of radioligand binding was followed and compared in drug-treated and buffer-treated (control) membranes. Results are means ± s.e.mean (vertical bars) of at least 3 separate determinations.

both the persistent salmeterol-induced inhibition and the inhibition resulting from a continuous isoprenaline infusion. The rate of reversal of both salmeterol and isoprenaline-responses by each antagonist was rapid, the time to 50% reversal of agonist activity being 11 min or less (Table 2). With the exception of atenolol and ICI 118551 against salmeterol, and isoprenaline respectively, at least 80% reversal was obtained (Table 2). When the response to the β-adrenoceptor blocking drug had equilibrated, the infusion of the antagonist was halted. In every case, the salmeterol-induced inhibition, or the inhibition resulting from the continuous isoprenaline infusion was reasserted (e.g. Figure 1). However, the rates at which the reassertion of agonist

Table 3 Rat lung membranes: inhibition of [^{125}I]-(-)-pindolol binding to β_2-adrenoceptors

Inhibitor	*pIC_{50}*	*Slope*	n
Agonists			
Salmeterol	7.1 ± 0.1	0.93 ± 0.05	9
Isoprenaline	6.2 ± 0.1	0.78 ± 0.02*	8
Adrenaline	5.8 ± 0.1	0.71 ± 0.15*	3
Salbutamol	5.1 ± 0.2	0.95 ± 0.05	4
Noradrenaline	4.8 ± 0.1	0.70 ± 0.12*	3
Antagonists			
Propranolol	8.7 ± 0.1	0.92 ± 0.02	3
(-)-Pindolol	8.6 ± 0.1	0.96 ± 0.12	3
ICI 118551	8.4 ± 0.2	0.91 ± 0.06	3
Atenolol	4.9 ± 0.1	0.93 ± 0.10	3

Rat lung membranes (50 μg protein) were incubated for 30 min at 37°C with [^{125}I]-PIN (100 pM) and drugs (0.1 nM–0.1 mM) and the IC_{50} and slope of the concentration-effect curve for inhibition of specific [^{125}I]-PIN binding determined. The pIC_{50} value is the negative $\log_{10}$ of the IC_{50} value. Results are means ± s.e.mean of *n* determinations. Significantly different from unity: *$P < 0.05$.

activity occurred differed markedly with the various antagonists (Table 2). Thus, following atenolol, Ra_{50} values of ~5 and 10 min against salmeterol and isoprenaline respectively were obtained. In contrast, with ICI 118551, the corresponding values were at least 2.5 and 4 h. Despite the differences in the rates of agonist reassertion following exposure to the different antagonists, in each case, the rates were of a similar order for both salmeterol and isoprenaline. Thus, the rank order of reassertion rate for salmeterol (persistent) and isoprenaline (continued infusion) responses was: atenolol > sotalol > pindolol > labetalol > timolol > propranolol > ICI 118551.

Repeated blockade and reassertion with sotalol

In one series of experiments, after full reassertion of the response to salmeterol following sotalol (10 μM)-treatment, sotalol was re-administered and the salmeterol response inhibited once again. When this antagonism had equilibrated, the sotalol infusion was stopped a second time and the salmeterol response was again fully reasserted. This process was repeated four times over a period of approximately 15 h, after which the final salmeterol response was little changed from the original response before the first sotalol infusion (see Figure 2).

Binding studies

The radioligand, [^{125}I]-PIN, bound with a high affinity (equilibrium dissociation constant, $K_d = 67 \pm 2$ pM) to a non-interacting (Hill coefficient 1.03 ± 0.05) population of sites (density 111 ± 13 fmol mg^{-1} protein, $n = 3$). This binding was rapid, equilibrating within 30 min at 37°C (association rate constant, $k_{+1} = 2.23 \pm 0.45 \times 10^7$ M^{-1} s^{-1}) and was readily displaced by 200 μM isoprenaline (dissociation rate constant, $k_{-1} = 8.57 \pm 0.76 \times 10^{-4}$ s^{-1}). The kinetically-derived radioligand equilibrium dissociation constant (from $K_d = k_{-1}/k_{+1}$) was 38 pM, compared to the value of 67 pM obtained from saturation studies performed over the radioligand concentration range 0–400 pM. A radioligand concentration of 100 pM was employed in all subsequent experiments.

The binding of [^{125}I]-PIN (100 pM) to rat lung membranes was inhibited, with a similar maximum in each case, by a range of β-adrenoceptor agonists and antagonists (Table 3), but not by agents selective for other receptor types, including 5-hydroxytryptamine, ketanserin, mepyramine, ranitidine, phentolamine or atropine (all at 1 μM). Inhibition curves for isoprenaline, adrenaline and noradrenaline had slopes significantly less than unity (Table 3). All other compounds tested produced inhibition curves with slopes of unity. The rank order of potency of the β-adrenoceptor agonists for inhibiting [^{125}I]-PIN binding (Table 3) was salmeterol > isoprenaline > adrenaline > salbutamol ≥ noradrenaline, a profile in keeping with binding to adrenoceptors of the β_2-subtype. This was confirmed by the greater affinity for the selective β_2-adrenoceptor antagonist, ICI 118551 (Lemoine *et al.*, 1985) than for the selective β_1-antagonist, atenolol (Lemoine *et al.*, 1988) (Table 3). Since the binding of salmeterol was apparently irreversible (see below), estimated IC_{50} values were not converted to equilibrium dissociation constants, since the Cheng & Prusoff transformation (1973) applies only to competitive inhibitors. Accordingly, only IC_{50} values are quoted in Table 3. The non-competitive nature of the interaction of salmeterol with rat lung β_2-adrenoceptors is illustrated by Dixon plots (Boeynaems & Dumont, 1980) of the inhibition data for salmeterol compared with those for salbutamol and isoprenaline (Figure 3). Thus, the Dixon plots of the inhibition of [^{125}I]-PIN binding, measured at three radioligand concentrations, by isoprenaline (Figure 3a) and salbutamol (Figure 3b) clearly show intercepts above the abscissa scale, indicating competitive inhibition. In contrast, the Dixon plot for salmeterol (Figure 3c) shows an intercept on the abscissa, a profile expected for a non-competitive inhibitor (Boeynaems & Dumont, 1980).

In an attempt to investigate the reversibility of the binding of β_2-adrenoceptor agonists, rat lung membranes were pretreated with a concentration of agonist which was estimated to produce at least 80% occupation of binding sites as judged from inhibition curves with these agonists, and the dissociation of the agonist measured indirectly by measuring the association of [^{125}I]-PIN following dilution to induce drug dissociation. Using this approach, it was found that both isoprenaline and salbutamol rapidly dissociated from the membrane binding sites (Figure 4), with estimated $t_{\frac{1}{2}}$ values of 4 min and 2.5 min respectively, whereas salmeterol showed no signs of dissociation, at least over the 60 min time period examined (Figure 4).

Discussion

The results of this study confirm those previously reported on guinea-pig trachea (Ball *et al.*, 1987a,b; 1991), in that salmeterol is slightly more potent as a bronchorelaxant than isoprenaline, and approximately ten fold more potent than the 'prototype' β_2-adrenoceptor agonist, salbutamol. Furthermore, salmeterol proved to be long-acting, despite continuous washing of tissues with agonist-free medium, whereas under similar conditions, the effects of isoprenaline and salbutamol were rapidly lost.

We have previously shown that the β-adrenoceptor blocking drug, sotalol, is capable of inhibiting persistent relaxant responses to salmeterol, but that these responses are fully reasserted when the sotalol is washed from the tissue. Indeed, it proved possible to repeatedly reverse, and on each occasion to fully reassert, the effect of salmeterol on individual preparations over a 15 h period. It was results such as these which led us to propose that salmeterol is held in the vicinity of the β-adrenoceptors in such a way that it is free to interact with the receptors, but is not free to diffuse away. We further proposed the existence of 'exosite' binding to account for these observations (Bradshaw *et al.*, 1987). However, from these original studies, it was not clear whether the salmeterol reassertion phenomenon would be observed with other β-adrenoceptor blocking drugs. It was also not clear whether the reassertion rate after sotalol removal reflected the rate at which sotalol dissociated from the β-adrenoceptors, or the rate at which salmeterol re-engaged with them. In order to address such questions, we undertook the present study, in which we have extended the range of β-adrenoceptor blocking drugs to include compounds of differing potency, β-adrenoceptor subtype selectivity and physico-chemical characteristics. We have also studied isoprenaline, for comparative purposes. Because responses to isoprenaline are rapidly lost on washing, it was necessary to use continuous infusion to mimic, as far as possible, the persistent effects following exposure to salmeterol.

In these experiments, we attempted to standardize both the β-adrenoceptor agonist response and the degree of antagonist-induced reversal. With all of the antagonists except atenolol, we achieved 70–90% inhibition of salmeterol-induced relaxant responses, whereas that obtained with atenolol was only 47%. The rather modest effect of atenolol against salmeterol reflects its low β_2-adrenoceptor blocking potency (pA_2 at β_2-adrenoceptors in guinea-pig trachea of 5.6; O'Donnell & Wanstall, 1979), and thus a concentration of 10 μM would only be expected to cause a rightward shift of the order of 5 fold. This is supported by the observation that it was more effective against isoprenaline (78% inhibition) which interacts with both β_1- and β_2-adrenoceptors in guinea-pig trachea (O'Donnell & Wanstall, 1979). The results with the selective β_2-adrenoceptor blocking drug, ICI 118551, also support this finding, in that it was more effective against the selective β_2-adrenoceptor agonist, salmeterol, (87% inhibition) than against isoprenaline (69% inhibition). The other five antagonists show little or no β_1/β_2-adrenoceptor selectivity (Main, 1990), and produced similar degrees of inhibi-

tion against both salmeterol and isoprenaline.

A number of interesting observations were made in these studies. Most obviously, it was clear that the ability to reverse salmeterol-induced relaxant responses, and to permit salmeterol reassertion on washout is not restricted to sotalol. However, the rate of reassertion of salmeterol activity differs for the different antagonists, with that after atenolol being particularly rapid, occurring within 5 min, whilst that after ICI 118551 was the slowest, with 50% reassertion taking about 2.5 h. It therefore seems unlikely that the rate of reassertion following antagonist washout is a function of re-equilibration of salmeterol with the β-adrenoceptors, but rather the rate at which the antagonist dissociates from the tissue. This being the case, it is perhaps not surprising that the rank order of rate of reassertion after the seven antagonists corresponds to their rank order of lipophilicity (Cruickshank, 1980). However, these data alone do not rule out some influence of salmeterol/receptor re-equilibration in the reassertion process, although bearing in mind the relatively rapid rate of the reassertion following atenolol, it would appear to be a minor contribution. Significantly, there were no differences in the rates of reassertion for salmeterol and isoprenaline after washout of any of the antagonists.

It is interesting to consider what information these results provide towards understanding the mechanism of action underlying the persistent agonist activity of salmeterol. The rates of reversal of β-agonist activity for all of the antagonists were of the same order (3–11 min), and there were no consistent differences in these rates for the various antagonists against salmeterol when compared with those against isoprenaline. These experiments do not prove that salmeterol and isoprenaline dissociate from the active site of the β-adrenoceptors at the same rate, but they do indicate that dissociation is not rate-limiting as far as onset of action of antagonist activity is concerned. Thus, bearing in mind the rapid onset of action of atenolol in particular, both agonists must obviously dissociate within this time period, and slow dissociation from β-adrenoceptors cannot therefore be the mechanism of the long duration of action of salmeterol.

Further insight into the nature of the interaction of salmeterol with β_2-adrenoceptors is provided by studies on rat lung membrane β_2-adrenoceptors labelled with the radioligand,[^{125}I]-PIN (Barovsky & Brooker, 1980; Neve *et al.*, 1986). These binding sites were confirmed as β_2-adrenoceptors by the characteristic catecholamine agonist rank-order of potency (isoprenaline > adrenaline > noradrenaline; Lands *et al.*, 1967) and by the 1000 fold greater affinity for the β_2-adrenoceptor antagonist, ICI 118551 than for the β_1-adrenoceptor antagonist, atenolol (O'Donnell & Wanstall, 1980; Lemoine *et al.*, 1988). Of the agonists examined, salmeterol showed the highest apparent binding affinity. The non-competitive nature of the interaction between salmeterol and the β-adrenoceptor blocking drug, [^{125}I]-PIN reported in rat lung membranes contrasts with that previously reported between salmeterol and propranolol in functional studies in guinea-pig trachea, where a competitive interaction was observed (Ball *et al.*, 1991). However, it is important to appreciate that in the functional studies, we allowed the antagonist to equilibrate with the β-adrenoceptors before adding salmeterol, whereas in the binding studies, salmeterol was allowed to equilibrate before addition of antagonist. When one considers the particular characteristics of the interaction of salmeterol with the β_2-adrenoceptor, it is quite clear that different patterns of competition could result.

Surprisingly, despite the continued presence of GTP to minimize complications such as interconvertible receptor states (DeLean *et al.*, 1980; Abramson & Molinoff, 1984), isoprenaline, adrenaline and noradrenaline consistently produced inhibition curves with shallow slopes (Hill coefficients 0.7–0.8). In contrast, the slopes of the inhibition curves produced by salbutamol, salmeterol and the β-adrenoceptor antagonists were not significantly different from unity. These differences in slope parameters might reflect binding site heterogeneity, since [^{125}I]-PIN recognises β_2-adrenoceptors with only a 3 fold greater affinity than β_1-adrenoceptors (Neve *et al.*, 1986). However, this seems unlikely in view of the monophasic inhibition curves obtained with all other compounds, including β_2-adrenoceptor-selective agonists and β_1 and β_2-selective antagonists. A more plausible explanation is that the concentration of GTP was insufficient to ensure all of the receptor population was in its ground state. If this were so, it is interesting to speculate that the differences seen between slope parameters for the β-adrenoceptor agonists might reflect differences in agonist efficacies. By this token, it would be predicted that salbutamol and salmeterol might be partial (low efficacy) agonists compared with isoprenaline, an observation in keeping with several reports of functional studies (Ball *et al.*, 1991; Dougall *et al.*, 1991; Waldeck & Kallstrom, 1991).

The major finding of this part of the study was the apparent irreversibility of the binding of salmeterol. Unfortunately, the interaction of salmeterol with the β_2-adrenoceptor could not be examined directly because suitably radiolabelled salmeterol with sufficiently high specific activity was not available. Accordingly, the binding of salmeterol was examined indirectly by its ability to inhibit the binding of the radioligand, [^{125}I]-PIN. Using this approach, there was a clear distinction between the apparent dissociation profile (measured as the association of [^{125}I]-PIN to the vacated binding sites) for salmeterol compared with that for isoprenaline or salbutamol. Essentially, both salbutamol and isoprenaline dissociated from the binding sites so rapidly that there was little difference in the rate of [^{125}I]-PIN binding to membranes pretreated with these agonists compared with that to membranes pretreated with vehicle. This contrasted markedly with the apparent lack of dissociation of salmeterol. These differences in dissociation of the β-adrenoceptor agonists therefore produced different inhibition profiles, with salmeterol behaving as an apparently irreversibly bound, non-competitive inhibitor.

The apparent inconsistency between the irreversibility of binding in the rat lung membranes and the functional studies in guinea-pig trachea may result from the nature of the two types of experiment. In the functional studies, high concentrations of antagonist were employed which compete with the persisting local levels of salmeterol for occupancy of the β_2-adrenoceptors. In contrast, in the binding studies, low concentrations of radiolabelled antagonist were employed, which were insufficient to compete with the relatively high local levels of β_2-agonist, but which will bind increasingly to the receptors as agonist is removed from the membranes by dilution. While the concentration of isoprenaline and salbutamol at the β_2-adrenoceptors will be reduced by dilution, allowing progressively more [^{125}I]-PIN to associate with the β_2-adrenoceptors, dilution does not appear to affect the amount of salmeterol in the vicinity of the β_2-adrenoceptors, and thus there is no such increase in [^{125}I]-PIN binding. Indeed, the results of the functional and binding studies lead to exactly the same conclusion, that washing the tissues even for extended periods of time fails to reduce the effective tissue concentration of salmeterol accessible to the β_2-adrenoceptors. In contrast, both techniques demonstrate rapid and complete loss of both isoprenaline and salbutamol. If higher concentrations of [^{125}I]-PIN were used, comparable to those of the antagonists used in the functional studies, then increasing β-antagonist binding would presumably be observed even in the face of maintained tissue levels of salmeterol. Unfortunately, at [^{125}I]-PIN concentrations above nanomolar levels, the degree of non-specific binding would render the assay useless.

These observations are consistent with the 'exosite' hypothesis, originally described by Bradshaw *et al.* (1987) and subsequently developed by Johnson (1990) and Jack (1991). This envisages the 'anchoring' of the lipophilic N-substituent phenylalkyloxyalkyl 'tail' of salmeterol to a site adjacent to or within the β-adrenoceptor, allowing the phenylethanol-

amine head group to interact with the active site of the receptor. This mechanism, described as the 'charnière' (hinge) effect, was first proposed by Rocha e Silva (1969) to explain the slow offset of action of the histamine H_1-receptor blocking drug, diphenhydramine.

Although lipophilic compounds generally tend to be long-acting *in vitro*, they do not normally exhibit the persistence of action associated with salmeterol. In the present study, the duration of action of the β-adrenoceptor blocking drugs tested, correlated with their rank order of lipophilicity (Cruikshank, 1980; Conway *et al.*, 1987). The most lipophilic of the antagonists, ICI 118551, has a calculated log P (octanol: water) of 3.85 (Medchem Data Base), which is similar to that of salmeterol (4.05, Johnson *et al.*, 1992). However, while the agonist activity of salmeterol persists apparently unchanged for periods of at least 10 h, there was obvious, albeit slow, recovery from the effects of ICI 118551, resulting in 50% recovery usually within 3 h.

Although, lipophilicity might be expected to hinder the molecule leaving the lipid environment of the cell membrane and re-entering the aqueous phase, it would not be expected to prevent it. Under the conditions of these superfusion experiments, there would effectively be an infinite concentration gradient from the hydrophobic membrane lipid phase, to the extracellular aqueous phase, and any molecule within the membrane would slowly re-enter the aqueous phase. This clearly does not happen to salmeterol, which *in vitro* at least, shows persistence of action for periods of 10 h or more, far greater than the time over which substantial loss of activity was observed for the isolipophilic β-adrenoceptor antagonist, ICI 118551. These results argue strongly for 'anchoring' of the salmeterol molecule in the vicinity of the active site of the receptor and this we believe is affected by the postulated exosite. If this is so, then what and where is the exosite? It is now well established that the β-adrenoceptor protein is arranged in the cell membrane in a series of 7 transmembrane domains (Dohlman *et al.*, 1987; Lefkowitz & Caron, 1988; O'Dowd *et al.*, 1989). There is now considerable knowledge, based on deletion experiments, of the amino acids essential for β_2-agonist binding, these being Asp113, Ser204 and Ser207, located in the IIIrd and Vth domains (Strader *et al.*, 1988; 1989). To interact with the appropriate residues, the agonist molecule must sit within the central pore where it can span the appropriate binding sites on the receptor protein (Dixon *et al.*, 1987). The binding of β-adrenoceptor agonists within the β-adrenoceptor has been modelled (Jack, 1991), and it has been suggested that the phenylethanolamine head of salmeterol binds in similar fashion to the natural agonist, adrenaline, to the appropriate Asp and Ser residues (Strader *et al.*, 1989), and that the N-substituent is orientated 'downwards' in an intracellular direction. However, such modelling cannot be regarded as definitive, and the N-substituent may equally be orientated 'upwards', in an extracellular direction, or even laterally. It is not clear how the phenylalkyloxyalkyl chain would interact with the receptor protein if it assumed these different orientations, or if it did, with which amino acid residues the flexible N-substituent of salmeterol could interact (Anderson, 1991). In the case of the lateral orientation, it is unlikely that such a long chain could be accommodated in the receptor pore at all, and perhaps the most likely arrangement would involve a protrusion between the transmembrane helices, into the membrane itself. It is important to appreciate that whereas hydrophilic agonists such as isoprenaline and salbutamol almost certainly access the active site of the receptor directly from the extracellular aqueous phase, more lipophilic compounds rapidly accumulate in the membrane lipid (Herbette *et al.*, 1988), and would almost certainly access the receptor from the cell membrane. To achieve this, it would be necessary for the phenylethanolamine end of the molecule to enter the receptor pore between the transmembrane helices. If this is so, the postulated exosite may not be a part of the receptor protein itself, but rather the 'peri-receptor' lipid, defined as that lipid around and between the receptor transmembrane domains. Salmeterol has a fully extended length of 25 angstroms, which is sufficient in theory to allow the phenylethanolamine head to interact with the active site, yet for the lipophilic phenylalkyloxyalkyl side-chain to protrude from the receptor into the 'peri-receptor' lipid. The possibility that the 'peri-receptor' lipid or a hydrophobic domain of the receptor protein itself is the postulated exosite is currently being investigated.

In conclusion, although the theory of 'exosite' binding to explain the persistent action of salmeterol is not proven, the data presented in the present report are all consistent with it. Indeed, in view of the lack of evidence from functional studies for avid binding of salmeterol to the β_2-adrenoceptors themselves, it is difficult to envisage another explanation for the pharmacological profile of this compound. In the light of this, we have speculated that the postulated exosite may be the 'peri-receptor' lipid or a hydrophobic domain of the receptor itself. However, there is scope for further experimental work aimed at investigating the validity of the 'exosite' hypothesis. Increased understanding of the mechanism may enable it it be used in extending the duration of action of other classes of therapeutic agent, where this cannot be achieved by more conventional approaches.

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