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The duration of action of non-β_2-adrenoceptor mediated responses to salmeterol

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1 To investigate further the mechanism of the long duration of action of the selective β_2-adrenoceptor agonist, salmeterol, we have determined the duration of action of some responses to salmeterol which are not mediated through β_2-adrenoceptors.

2 In the presence of propranolol (1 μM), salmeterol (1–30 μM) caused concentration-related relaxation of superfused, pre-contracted strips of guinea-pig gastric fundus. On washing the tissues, these relaxant responses were rapidly lost, the time to 50% recovery being approximately 30 min.

3 In human neutrophils, salmeterol (1–100 μM) caused concentration-related inhibition of FMLP-induced O_2^- release. Propranolol (1 μM) had little or no effect on the inhibitory activity of salmeterol. Washing the cells twice over a 40 min period caused a marked reduction of the inhibitory activity of salmeterol.

4 In guinea-pig superfused trachea, in the absence of propranolol, infusions of (**RS**)-salmeterol (10–30 nM) and the less potent (**S**)-enantiomer of salmeterol (300–3000 nM) inhibited electrically-induced contractile responses. When the infusion was stopped, there was no recovery from the inhibitory responses within 200 min. In the presence of propranolol (1 μM), infusions of (**RS**)-salmeterol (10 μM) and (**S**)-salmeterol (10–100 μM) also inhibited the contractile responses, but, in contrast, on stopping the infusions differences were observed in recovery times. Thus no appreciable recovery was observed from the responses to (**RS**)-salmeterol, whereas a rapid loss of inhibition was observed on stopping the infusion of (**S**)-salmeterol, the time to 50% recovery being 30–35 min.

5 These relatively short-lasting effects of salmeterol which are not mediated through β_2-adrenoceptors, contrast with the persistence of the responses which are mediated through β_2-adrenoceptors seen in a variety of tissues, but are similar to the rate of dissociation of salmeterol observed from artificial membranes. These observations suggest that the sustained agonist activity of salmeterol is peculiar to responses mediated by β_2-adrenoceptors.

Keywords: Salmeterol; non-β-adrenoceptor; duration of action; guinea-pig gastric fundus; human neutrophils; guinea-pig trachea

Introduction

The effects of salmeterol mediated through β_2-adrenoceptors in a variety of cell types are long-lasting (Ball *et al*., 1991; Butchers *et al*., 1991; Johnson *et al*., 1993). The mechanism of the sustained agonist activity of salmeterol is not fully understood, although it has been suggested that the lipophilic N-substituent aralkyloxyalkyl side-chain binds to a specific exo-site which holds the molecule in the vicinity of the active site of the β_2-adrenoceptor (Bradshaw *et al*., 1987; Nials *et al*., 1993). Alternatively, the long duration of action may be determined by the physicochemical interactions of the drug with the membrane lipid bilayers (plasmalemma diffusion microkinetic model, Anderson *et al*., 1994). In short, the lipophilic nature of the salmeterol molecule allows it to be concentrated into the membrane lipid, from where the molecules slowly access the β_2-adrenoceptor.

At high concentrations, salmeterol also has effects which are not mediated by β_2-adrenoceptors, e.g. inhibition of thromboxane B_2 release from alveolar macrophages (Baker & Fuller, 1990), relaxation of guinea-pig gastric fundus (Barker *et al*., 1992) and inhibition of electrically-induced contractions in ferret trachea (Bergendal *et al*., 1992). However, the mechanism or mechanisms underlying these non-β_2-adrenoceptor mediated responses has yet to be established.

If high lipophilicity and membrane solubility were solely responsible for the persistence of responses to salmeterol mediated through β_2-adrenoceptors, then actions not mediated through β_2-adrenoceptors should also be sustained.

In the present study, we have demonstrated that in two isolated smooth muscle preparations, guinea-pig gastric fundus and guinea-pig trachea, and in human isolated neutrophils, salmeterol can induce responses which are not mediated through β_2-adrenoceptors. All these non-β_2-adrenoceptor-mediated responses were short-lasting, suggesting that the sustained effects of salmeterol are peculiar to those mediated through β_2-adrenoceptors.

Preliminary accounts of this work have been communicated to the American Thoracic Society (Nials *et al*., 1994a; Coleman *et al*., 1994; Vardey *et al*., 1994).

Methods

Guinea-pig gastric fundus

Preparation Dunkin Hartley guinea-pigs (450–650 g) were killed by a blow to the head and subsequent exsanguination. The abdominal cavity was opened and the stomach exposed. The gastric fundus was dissected out and placed in a petri dish containing modified Krebs solution (Apperley *et al*., 1976) at room temperature. The adherent connective tissue was dissected away and the stomach contents gently removed. The fundus was opened and cut into strips (4–6 mm wide, 10–

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15 mm long) running parallel to the longitudinal smooth muscle fibres. The gastric mucosa was carefully removed from each strip by peeling it away from the muscle layer with forceps.

Strips were mounted under a resting tension of 1 g, maintained at 37°C, and superfused at a rate of 2 ml min^{-1} with oxygenated (5% CO_2 in O_2) modified Krebs solution containing indomethacin (2.8 μM) to inhibit endogenous prostanoid synthesis, atropine (0.4 μM), to block any endogenous cholinergic activity, phenoxybenzamine (1 μM), to block catecholamine uptake and α-adrenoceptors and propranolol (1 μM), to block β_1- and β_2-adrenoceptors.

Agonist activity on guinea-pig gastric fundus Resting tone was elevated by adding a submaximally-effective concentration of prostaglandin (PG) $F_{2\alpha}$ (1–3 μM) to the organ bath. Cumulative concentration-effect curves to isoprenaline (0.01–30 μM) were repeated until constant sensitivity was attained. A concentration-effect curve to salmeterol was then constructed. Relaxant responses were expressed as a percentage of the maximum relaxation of tone obtained when the tissues were washed with $PGF_{2\alpha}$-free Krebs solution. Potency values for the β-adrenoceptor agonists were expressed in both absolute terms (EC_{50}, i.e. the concentration required to induce 50% of the maximum response to each agonist) and relative to isoprenaline, as an equieffective concentration (i.e. EC_{50} for the test agonist/EC_{50} for isoprenaline). An equieffective concentration of less than unity indicates greater potency than isoprenaline, and an equieffective concentration of greater than unity, a lower potency than isoprenaline.

Antagonist potency of alprenolol The experimental protocol was similar to that described above. Briefly, tone was elevated with $PGF_{2\alpha}$, and constant tissue sensitivity was obtained to isoprenaline by repeating relaxant concentration-effect curves. One tissue was then employed as a control (incubated with vehicle only), and the others were incubated with different concentrations of alprenolol (0.1–10 μM) for at least 45 min. A further concentration-effect curve to the test agonist was then repeated on each preparation. Agonist potency was calculated (EC_{50}) in the presence and absence of the antagonist. The antagonist potencies were calculated by comparing the potencies of the agonists in the absence and presence of the antagonist to give the concentration-ratio (CR) defined as:-

$$CR = \frac{\text{Agonist } EC_{50} \text{ (+ antagonist)}}{\text{Agonist } EC_{50} \text{ (− antagonist)}}$$

Antagonist potencies (pA_2) were then calculated according to the method of Arunlakshana & Schild (1959).

Duration of action Strips of guinea-pig fundus were mounted in superfusion chambers and superfused with modified Krebs solution containing indomethacin (2.8 μM), atropine (0.4 μM), phenoxybenzamine (1 μM), propranolol (1 μM) and $PGF_{2\alpha}$ (3 μM). A sequential concentration-effect curve to isoprenaline was constructed. Each concentration of isoprenaline was infused until the response had equilibrated, at which time the infusion was halted. The tissue was then allowed to recover before the infusion of the next, higher concentration was started. The magnitude of each response was determined as a percentage of the maximum effect, where maximum is defined as the relaxation in the presence of $PGF_{2\alpha}$-free Krebs solution. The times for attainment of half-maximal responses and half-maximal recovery from each concentration were measured. Onset times (Ot_{50}) and recovery times (Rt_{50}) were calculated according to the method of Coleman & Nials (1989). Ot_{50} was expressed as the time from administration of an EC_{50} concentration of an agonist to attainment of 50% maximal response. Rt_{50} was expressed as the time from stopping the administration of the agonist to attainment of 50% recovery from the response to an EC_{50} concentration. Ot_{50} and Rt_{50} values were determined from a plot of % response against time to attainment of 50% of each response (for Ot_{50}), or of 50% recovery from the response (for Rt_{50}).

Human neutrophils

Isolation of neutrophils from human blood Human neutrophils were isolated as described by Wheeldon & Vardey (1993). Human blood was removed by venepuncture and collected into citrate buffer (sodium citrate 2.83 mg ml^{-1}, glucose 2.92 mg ml^{-1}, citric acid monohydrate 1.04 mg ml^{-1}, 0.15 × blood volume) to prevent coagulation. The citrated blood was overlaid onto prewarmed (37°C) methyl cellulose (0.6%) hypaque (13%) (2 × blood volume) and allowed to settle for 30–40 min at room temperature. The supernatant containing the white cells was then removed and underlayed with prewarmed (37°C) Ficoll solution (0.25 × supernatant volume) and centrifuged at 400 *g* for 20 min at 20°C. The cells at the interface were discarded, and the granulocyte pellet was washed twice in Hanks buffered salt solution (HBSS, $-Ca^{2+}-Mg^{2+}$) buffer by gently resuspending and centrifuging at 400 *g* for 4 min at 20°C. The granulocytes were then suspended in a known volume of HBSS buffer ($+Ca^{2+}$, $+Mg^{2+}$).

O_2^- generation by human neutrophils The reduction of ferricytochrome C with a consequent increase in absorption at 550 nm was used to assay the release of superoxide anion. Salmeterol or corresponding solvent control (50 μl), was pipetted into tubes and warmed to 37°C. The cells were resuspended in buffer containing ferricytochrome C (0.2 mM), and prewarmed to 37°C for 20 min. The cells (400 μl) were added to each tube (containing salmeterol or solvent control) and incubated for a further 20 min at 37°C before challenge with the neutrophil stimulant formyl-methionyl-leucyl-phenylalanine (fMLP, final concentration 200 nM), or corresponding solvent control (to estimate spontaneous release), and incubated for a further 20 min at 37°C.

The cells were then pelleted by centrifugation (400 *g*) for 5 min at 5°C. The supernatants were aspirated into 96-well flat-bottomed microtitre plates, and optical density (550 nm) read in a plate reader. The percentage inhibition of O_2^- generation by the β-adrenoceptor agonists was calculated.

$$\% \text{ inhibition} = \frac{\text{mean OD (−salmeterol)} - \text{OD (+salmeterol)}}{\text{mean OD (−salmeterol)} - \text{mean OD (spontaneous release)}} \times 100$$

Duration of action In these experiments, the cells were incubated with the salmeterol as described above. However, the cells were then subjected to two further washes (over 30 min) in HBSS at 37°C before stimulation witn fMLP.

Guinea-pig trachea

Preparation For experiments involving electrical stimulation, preparations were taken from guinea-pigs (male, Dunkin-Hartley, 350–500 g) pretreated intraperitoneally with 6-hydroxydopamine (200 mg kg^{-1}) in order to eliminate any adrenergic component in the electrically-induced response (Coleman & Nials, 1989). The guinea-pigs were killed by a blow to the head and subsequent exsanguination. The tracheal preparations were cut into rings and then opened by cutting the cartilage opposite the smooth muscle (Coburn & Tomita, 1973). A cotton thread was tied to one end for attachment to a strain gauge, and a cotton loop to the other end for anchoring the tissue in the superfusion chamber (Coleman & Nials, 1989).

The tracheal strips were mounted under a resting tension of 1 g, maintained at 37°C, and superfused at a rate of 2 ml min^{-1} with oxygenated (5%CO_2 in O_2) modified Krebs solution containing indomethacin (2.8 μM) to inhibit endogenous prostanoid synthesis.

Agonist activity on guinea-pig trachea Phasic contractile responses were induced by electrical stimulation with 10 s trains of square wave pulses of 5 Hz frequency, 0.1 ms duration and just maximal voltage (8–14 V) every 2 min. Increasing concentrations of the standard agonist, isoprenaline, were infused in a sequential manner onto each preparation. Following this, single concentrations of the test agonists were then infused in a similar fashion. Responses to agonists were measured as percentage reduction (inhibition) of the control twitch height.

Duration of action A single concentration-effect curve to isoprenaline was constructed by infusing increasing concentrations in a sequential manner as described above. Test compounds were then infused in a similar fashion. In experiments with (**RS**)-salmeterol, in which there was no recovery from the inhibitory responses, the experimental protocol was altered; thus salmeterol was evaluated on paired preparations, a single concentration being added to each preparation. The concentrations of salmeterol chosen were those which caused responses approximately 20–40% of maximum on one preparation, and 60–80% of the maximum on the other preparation. A composite, two point concentration-effect plot spanning the EC_{50} was then constructed. Onset and recovery times were calculated as described above.

Materials

The following compounds were used: alprenolol hydrochloride (Sigma, U.K.) ascorbic acid (BDH Chemicals, U.K.), atropine sulphate (Sigma, U.K.), Ficoll-Paque (Pharmacia, U.K.), formyl-methionine-leucine-phenylalanine (fMLP, Sigma, U.K.), indomethacin (Sigma, U.K.), isoprenaline sulphate (Sigma, U.K.), methyl cellulose (Sigma, U.K.), phenoxybenzamine (Smith, Kline-French, U.K.), propranolol hydrochloride (Sigma, U.K.), prostaglandin $F_{2\alpha}$ tromethamine salt (Lutalyse, Upjohn) and salmeterol (GRD, U.K.).

Salmeterol was dissolved in glacial acetic acid and diluted to stock concentrations with phosphate buffer (pH 7.0). Prostaglandin $F_{2\alpha}$ was supplied as the tromethamine salt in aqueous solution containing 5 mg ml^{-1} free acid. Indomethacin was dissolved in 1% sodium bicarbonate in 0.9% saline. All of the other compounds were dissolved in distilled water. All dilutions of stock solutions were made in 0.9% saline. To prevent the auto-oxidation of isoprenaline and to maintain identical vehicle constituents, all solutions of β-adrenoceptor agonists contained ascorbic acid (11 μM).

The Krebs solution used had the following composition (g l^{-1}): NaCl 6.92, $NaHCO_3$ 2.1, KCl 0.35, $MgSO_4.7H_2O$ 0.15, KH_2PO_4 0.16, glucose 2.0 and $CaCl_2.6H_2O$ 0.28.

Results

Guinea-pig gastric fundus

Potencies of β-adrenoceptor agonists In the presence of propranolol (1 μM), isoprenaline and salmeterol caused concentration-related inhibition of $PGF_{2\alpha}$-induced tone (Figure 1). A representative trace, showing typical relaxant concentration-effect curves to isoprenaline and salmeterol is shown in Figure 2. There were no marked differences in the response maxima of these agonists. Isoprenaline (EC_{50} 1.8 μM) was apparently more potent than salmeterol (EC_{50} 6.2 μM), although this difference was not statistically significant ($P>0.05$, Table 1).

Antagonist potency of alprenolol In the presence of propranolol (1 μM), alprenolol (0.1–10 μM) caused concentration-related rightward shifts of concentration-effect curves to isoprenaline. By use of the method of Arunlukshana & Schild (1959), a pA_2 value of 6.0 (5.6–6.5, $n=6$) was calculated with a slope value of 0.9 (0.6–1.2) which was not significantly different ($P>0.05$) from unity. In contrast, alprenolol (0.1–10 μM) failed to antagonize the relaxant responses to salmeterol, the mean concentration-ratio at 10 μM being 1.4 (0.6–3.5, $n=4$).

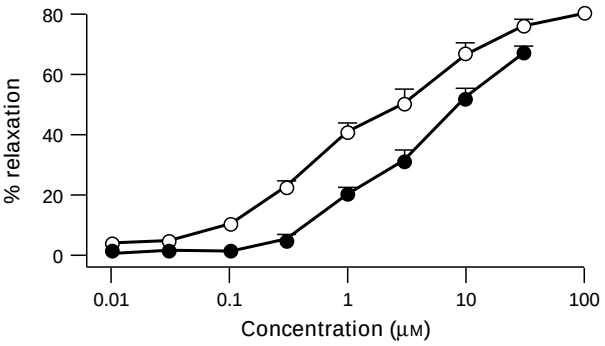

Figure 1 $PGF_{2\alpha}$-contracted guinea-pig gastric fundus. Mean relaxant concentration-effect curves for isoprenaline (○) and salmeterol (●). Each point is the arithmetic mean of at least 6 determinations; vertical lines show s.e.mean.

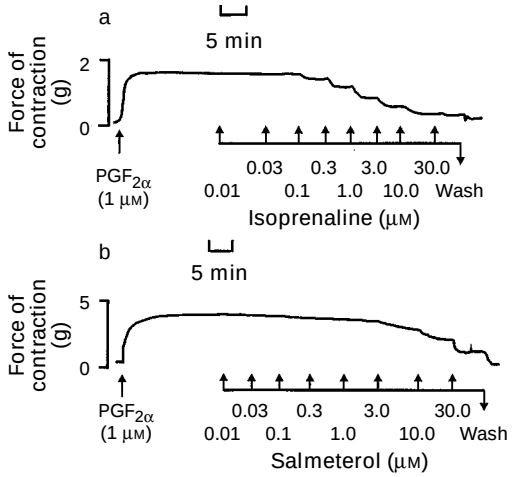

Figure 2 $PGF_{2\alpha}$-contracted guinea-pig gastric fundus. Representative experiment illustrating relaxant responses to increasing concentrations of (a) isoprenaline and (b) salmeterol.

Table 1 $PGF_{2\alpha}$-contracted guinea-pig gastric fundus: relaxant potencies of isoprenaline and salmeterol

Agonist	*Absolute potency* EC_{50} (μM)	*Relative potency* *EEC* (Iso = 1)	n
Isoprenaline	1.8 (1.4–2.5)	1.0	40
Salmeterol	6.2 (2.0–18.8)	5.4 (1.9–15.7)	6

Results are geometric means with 95% confidence limits in parentheses.

Duration of action of β-adrenoceptor agonists When infusions of the β-adrenoceptor agonists were stopped, marked recovery from their relaxant responses was observed. Thus the Rt_{50} value for isoprenaline (3 μM) was 1.1 (0.9–1.3, $n=12$) min. In contrast, the time taken for the relaxant response to salmeterol (3 μM) to recover was substantially longer with an Rt_{50} of 26.9 (14.2–45.8, $n=6$) min, but nevertheless, as with isoprenaline, virtually complete recovery was observed. Representative responses are illustrated in Figure 3.

Further experiments with a greater range of concentrations of salmeterol suggest that its duration of action was concentration-related. Thus, as the concentration of salmeterol was increased, so the duration of action of the resulting re-

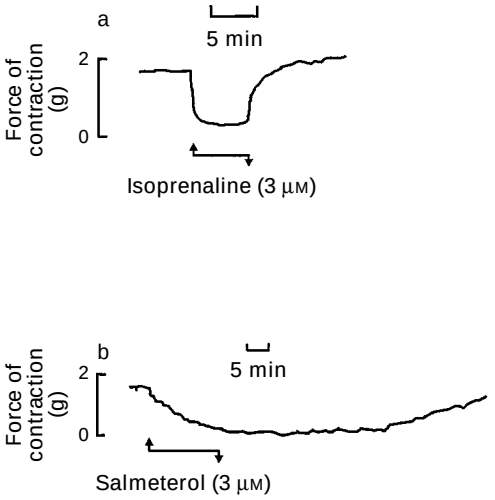

Figure 3 $PGF_{2\alpha}$-contracted superfused guinea-pig gastric fundus. Representative experiment illustrating relaxant responses to infusions of (a) isoprenaline and (b) salmeterol and, on stopping the infusion, the subsequent recoveries from those relaxant responses.

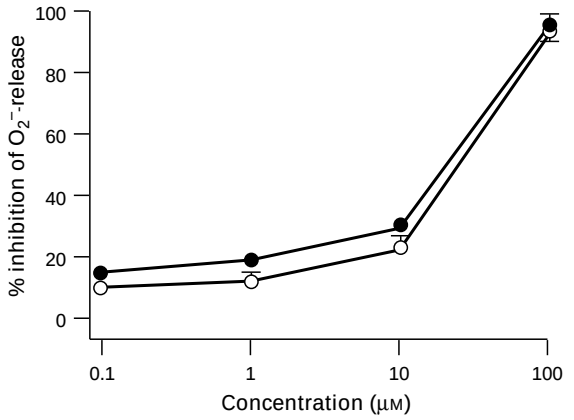

Figure 4 fMLP-stimulated human isolated neutrophils. Inhibition of O_2^- release by salmeterol in the absence (○) and presence (●) of propranolol (1 μM). Results are arithmetic means of 4 experiments, vertical lines represent s.e.mean.

laxant responses became more prolonged. From 4 separate experiments, for salmeterol, at concentrations of 0.1, 1.0 and 10 μM, the mean relaxant responses were 12.3 (2.9–21.7), 47.3 (13.7–80.9) and 75.9 (63.6–86.2)%, respectively. The times to 50% recovery from these responses were 9.3 (5–23.6), 36.3 (19–89.4) and 67.6–>140 min respectively.

In a further series of experiments, in order to ensure that recovery from the effects of salmeterol was not associated with desensitization of the tissues, consecutive infusions of the same concentration of salmeterol were performed. Thus, when substantial recovery from the relaxant response to an initial infusion of salmeterol (3 μM) was obtained, a second infusion of the same concentration was applied. In each case, the magnitude of the relaxant response to this second infusion was of a similar order to that obtained to the initial infusion. Thus, the mean % relaxation (95% CL) to the first infusion was 65.9 (50.1–81.7, $n=4$)%, and that to the subsequent infusion following recovery was 60.0 (47.4–72.4, $n=4$)%.

Human neutrophils

Inhibitory potency Salmeterol (10–100 μM) caused concentration-related inhibition of fMLP-induced O_2^- release (EC_{50} (95% CL) = 22.7 (19.0–27.2) μM, $n=4$). Pretreatment of the cells with propranolol (1 μM), had no effect on the inhibitory potency of salmeterol (EC_{50} = 18.1 (6.3–52.2) μM, $n=4$). The mean inhibitory concentration-effect curves to salmeterol in the absence and presence of propranolol are shown in Figure 4.

Duration of action At a concentration of 100 μM, salmeterol caused 95.8 (89.3–102.2)% inhibition of O_2^- release. However, this inhibitory activity was significantly ($P<0.01$) reduced by washing the cells. Thus, within 60 min of washing the cells, the inhibitory activity was reduced to 22.9 (13.0–32.9)%.

Electrically-stimulated guinea-pig trachea

*Duration of the inhibitory responses to (**RS**)- and (**S**)-salmeterol* When infused onto superfused preparations of electrically-stimulated guinea-pig trachea, (**RS**)- (10–30 nM) and (**S**)- (300–3000 nM) salmeterol inhibited the electrically-induced contractile responses in a concentration-related manner (Table 2). The onset of these inhibitory responses was slow (>25 min). When the infusions of (**RS**)- and (**S**)-salmeterol were stopped, there was no appreciable recovery from the inhibitory responses over periods of up to 200 min. However, this inhibition was rapidly and fully reversed by infusion of the β-adrenoceptor blocking drug, propranolol (0.1 μM), suggesting that the responses were mediated by β-adrenoceptors. A representative trace is shown in Figure 5.

Table 2 Electrically-stimulated, superfused guinea-pig trachea: inhibitory responses and durations of action for (**RS**)- and (**S**)-salmeterol

Agonist	*Concentration* (nM)	*Mean % inhibition of twitch response*	Rt_{50} (min)	n
(**RS**)-salmeterol	10	64.4 (42.7–88.0)	>200	4
(**RS**)-salmeterol	30	89.7 (83.9–95.4)	>200	5
(**S**)-salmeterol	300	60.0 (27.7–92.2)	>200	4
(**S**)-salmeterol	1000	80.0 (68.0–90.0)	>200	5
(**S**)-salmeterol	3000	97.1 (59.6–134.5)	>200	3

Results are arithmetic means with 95% confidence limits in parentheses.

In the continual presence of propranolol (1 μM), higher concentrations of (**RS**)- (10 μM) and (**S**)- (10–100 μM) salmeterol also inhibited the twitch responses (Table 3). As with the experiments performed in the absence of propranolol, the onset of the inhibitory responses to (**RS**)-salmeterol were slow (Ot_{50}>25 min) and their duration of action sustained (Rt_{50}>180 min, Table 3). However, in contrast, both the onset of action and duration of action of the inhibitory responses to (**S**)-salmeterol were shorter in the presence of propranolol. Thus the onset time (Ot_{50}) was less than 12 min, and the recovery time (Rt_{50}) was less than 60 min (Figure 5).

This recovery from responses to (**S**)-salmeterol (30 μM) was not due to desensitization, as following recovery from the initial inhibitory response, an inhibitory response of a similar magnitude was observed to subsequent infusions of the same concentration of the agonist. Thus the mean % inhibitory response (95% CL) to the initial infusions was 70.6 (51.9–84.1, $n=3$)%, and that to a subsequent infusion was 65.3 (50.8–81.1, $n=3$)%.

Discussion

The aim of this series of experiments was to investigate the duration of action of responses to salmeterol which are not mediated by β_2-adrenoceptors. The experiments were designed to investigate further whether the mechanism by which sal-

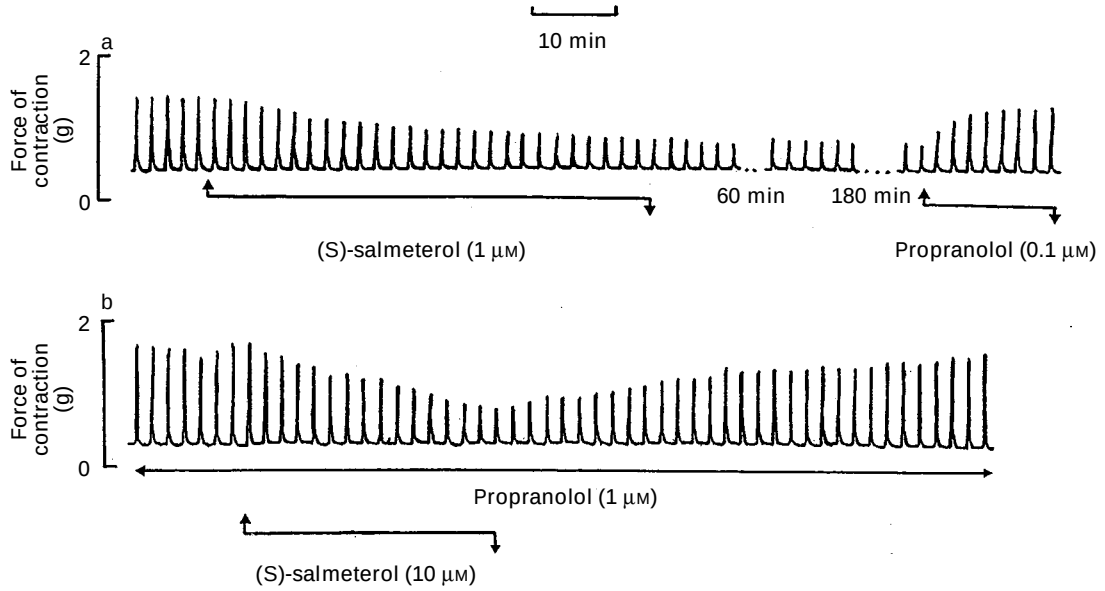

Figure 5 Electrically-stimulated, superfused guinea-pig trachea. Representative experiment illustrating the duration of action of inhibitory responses to (**S**)-salmeterol, in the absence (a) and presence (b) of propranolol (1 μM).

Table 3 Electrically-stimulated, superfused guinea-pig trachea: inhibitory responses and durations of action for (**RS**)- and (**S**)-salmeterol in the presence of propranolol

Agonist	*Concentration* (μM)	*Mean inhibition of twitch response*	Rt_{50} (min)	n
(**RS**)-salmeterol	10	73.3 (45.7–99.1)	>180	4
(**S**)-salmeterol	10	46.0 (25.0–67.0)	34.0 (20.0–48.0)	4
(**S**)-salmeterol	100	83.3 (61.6–105.0)	57.3 (22.9–91.7)	3

Results are arithmetic means with 95% confidence limits in parentheses.

meterol is retained in the vicinity of β_2-adrenoceptors to cause sustained agonist activity is in some way specific to these receptors.

We (Bradshaw *et al*., 1987; Nials *et al*., 1993) have postulated and presented indirect evidence for the existence of a specific exosite which binds the molecule through interaction with its lipophilic side chain in the vicinity of the active site of the β_2-adrenoceptor. An irreversible binding of salmeterol molecules to the active site of the β_2-adrenoceptor, as described by Standifer *et al*. (1989) for some carbostyril-based β_2-adrenoceptor agonists, has been effectively discounted by experiments demonstrating the rapid and complete reversal of its effects by β-adrenoceptor blocking drugs (Nials & Coleman, 1988; Ball *et al*., 1991; Nials *et al*., 1993). The plasmalemma microkinetic diffusion theory (Anderson, 1992; Anderson *et al*., 1994) argues for a non-specific mechanism, with the membrane acting as a reservoir for the highly lipophilic salmeterol molecules. If this is true, then all responses to salmeterol should be equally long-lasting, irrespective of whether they are mediated by β_2-adrenoceptors.

Our data show clearly that at least some non-β_2-adrenoceptor-mediated responses to salmeterol are short-lasting. However, an important consideration is the observation that the concentrations required to cause non-β_2-adrenoceptor-mediated responses are much higher than those to cause responses mediated by β_2-adrenoceptors. It is possible that this concentration difference may have some influence on the respective durations of action of salmeterol. In particular, low concentrations of salmeterol may be efficiently retained by the membrane whereas in contrast, high concentrations of salmeterol may be less efficiently retained (possibly due to the highly lipophilic salmeterol molecules altering the characteristics of the membrane), and thus fall below threshold concentrations during washing, resulting in a loss of the response. However, we believe that the difference in concentration is unlikely to matter. If the responses follow first order kinetics, the functional $t_{1/2}$ should be independent of concentration, as long as the responses measured are not caused by supramaximally effective concentrations of agonist. Furthermore, our experiments in guinea-pig trachea with salmeterol in the presence of propranolol and with the less active enantiomer, (**S**)-salmeterol have shown that β_2-adrenoceptor-mediated responses induced by high concentrations of salmeterol are still persistent.

The first series of experiments in the present study investigated the relaxant activity of salmeterol and isoprenaline in $PGF_{2\alpha}$-contracted guinea-pig gastric fundus. The β-adrenoceptors mediating relaxation of this tissue have previously been characterized as being of the β_3-subtype (Coleman *et al*., 1987). Our experiments with the putative β_3-adrenoceptor blocking drug, alprenolol (Blue *et al*., 1990), have shown that the relaxant responses to isoprenaline are, at least in part, mediated by β_3-adrenoceptors. However, in contrast, the mechanism of the relaxant activity of salmeterol on guinea-pig fundus does not appear to involve a substantial contribution from activation of β_3-adrenoceptors, and it is possible that it results from the lipophilic nature of the salmeterol molecule, causing relaxation by 'membrane stabilization'. Previous studies have shown a good correlation between lipophilicity and the ability of a drug to cause membrane stabilization (Ijzerman *et al*., 1987). However, whatever the mechanism of action of salmeterol's relaxant activity, its effects were short-lasting when compared with responses mediated in other preparations through β_2-adrenoceptors. Thus, the mechanism(s) responsible for retaining salmeterol in the vicinity of the β_2-adrenoceptor does not appear to be involved in the relaxant response on the guinea-pig fundus.

We also investigated the duration of action of inhibitory responses in isolated neutrophils. Although salmeterol has previously been shown to cause sustained inhibitory activity on human lung mast cells through activation of β_2-adrenoceptors (Butchers *et al*., 1991), in other cells there is evidence for non-β-adrenoceptor-mediated inhibitory responses. Thus, for example the work of Baker & Fuller (1990), has shown that the inhibition of thromboxane B_2 release in human alveolar mac-

rophages by salmeterol is not blocked by propranolol. However, they did not determine the duration of action of salmeterol in these cells. The inhibitory effects of salmeterol on human neutrophils, like those in human alveolar macrophages, are insensitive to propranolol, again suggesting a non-β-adrenoceptor-mediated mechanism. Furthermore, if the cells are washed after incubation with salmeterol, this inhibitory activity is lost within an hour. The possibility that this 'recovery' was a result of desensitization was not investigated. The mechanism of the inhibitory effects of salmeterol is not understood, but membrane stabilization is once again a possibility.

The final series of experiments on guinea-pig trachea were designed to demonstrate differences in the duration of action of β_2- and non-β_2-adrenoceptor-mediated responses within the same tissue. The aims, therefore, were threefold. Firstly, to determine whether, in the presence of propranolol, a non-β-adrenoceptor induced inhibitory response could be obtained. Secondly, to determine the duration of action of this response and finally to determine the persistence of this response compared with that of a β_2-adrenoceptor induced response on the same tissue. Initial experiments showed that it was not possible to perform these studies with (**RS**)-salmeterol itself, because at the concentrations required to obtain a non-β-adrenoceptor-mediated response (>3 μM), the effects of propranolol (1 μM) were overcome; thus a component of the response was also β_2-adrenoceptor-mediated and long-lasting. When the concentration of propranolol was increased to block the β_2-adrenoceptor effects of salmeterol, propranolol itself was found to have inhibitory effects. This was presumably due to non-specific membrane stabilizing activity of propranolol, which is also a lipophilic compound.

Ideally, this experiment would best be performed with a potent β-adrenoceptor antagonist with little or no membrane stabilising activity. However, we were unable to identify a suitable compound. Alternatively, the experiment could be performed in an isolated tissue system where contractions are induced by a method other than electrical stimulation (e.g. intermittent administration of a contractile agent). However, the responses induced by the contractile agent would have to be sufficiently reproducible over a period of time (4–6 h) in order to allow duration of action to be evaluated. In our experience, transient contractile responses induced by agents such as histamine or carbachol tend to fade with time and only electrically-induced contractile responses are sufficiently stable.

These problems were, in part, overcome by using the (**S**)-enantiomer of salmeterol. (**S**)-Salmeterol is about 40 fold less potent than (**RS**)-salmeterol, but otherwise retains similar characteristics (Nials *et al*., 1994b). As a result of its lower β_2-adrenoceptor potency, it was possible to achieve the concentrations required to produce the non-β-adrenoceptor mediated agonist activity, without overcoming the propranolol-induced β-adrenoceptor blockade.

The experiments with (**S**)-salmeterol have demonstrated marked differences in the duration of action of β_2- and non-β_2-adrenoceptor-mediated responses. Thus, as previously described with (**RS**)-salmeterol, the inhibitory responses to (**S**)-salmeterol at β_2-adrenoceptors persisted for at least 3 h, while in contrast the non-β_2-adrenoceptor-responses were relatively short-lived (30–60 min). It is also interesting to note the difference in the rates of onset of action of the β_2- and non-β_2-adrenoceptor-mediated responses to (**S**)-salmeterol, the non-β_2-adrenoceptor-mediated relaxant responses were markedly more rapid in onset.

The mechanism of the non-β_2-adrenoceptor-induced inhibitory responses on the guinea-pig trachea has not been established, but may again be due to a non-specific membrane stabilising effect as already suggested for guinea-pig gastric fundus.

A consistent observation from these experiments on the guinea-pig fundus, trachea and human neutrophils is that, with respect to the sustained duration of action of salmeterol at β_2-adrenoceptors, there is a relatively rapid loss of effect within one hour, and indeed, almost complete recovery within 2 h. The duration of action (30 min–1 h) of the non-β-adrenoceptor-mediated responses to salmeterol is consistent with the rate of loss of salmeterol from synthetic membrane systems, as shown by Rhodes *et al*. (1992). They showed that on washing, there is a marked reduction in the concentration of salmeterol within 1–3 h, depending on the conditions of the experiment. Thus, the duration of action of salmeterol in guinea-pig fundus, human neutrophils and guinea-pig trachea, in the presence of propranolol, may reflect the period of retention of the salmeterol molecules within the membrane. If the mechanism of non-β-adrenoceptor mediated inhibitory activity is indeed related to the physicochemical characteristics of the molecule (i.e. lipophilicity), and a result of the molecule being taken up into the cell membrane, then the loss of the effect probably reflects the loss of the molecule from the membrane.

A previous study (Swales & Paterson, 1990) has addressed this question. These workers investigated the ability of the agonist activity of salmeterol to reassert following reversal with sotalol on β_1-adrenoceptor (guinea-pig ileum and perfused heart) and β_2-adrenoceptor (guinea-pig trachea, rat uterus and bovine sphincter pupillae) containing preparations. Reassertion of the agonist activity of salmeterol was observed on the β_2-, but not on the β_1-adrenoceptor-containing preparations. Thus they concluded that the long duration of action of salmeterol was only observed at β_2-adrenoceptors. While the results of their studies on β_2-adrenoceptors are in agreement with ours, it is surprising that they were able to demonstrate any marked activity at all with salmeterol at β_1-adrenoceptors, as in our hands, salmeterol has little or no activity at β_1-adrenoceptors.

Thus, our observatons seem to refute the hypothesis (plasmalemma microkinetic diffusion theory) proposed by Anderson and colleagues (1994) that physicochemical interactions with the membrane lipid are solely responsible for the long duration of action of salmeterol. Our experiments suggest that there is considerable loss of the molecule from the lipid phase, and it is only β_2-mediated responses that are sustained. Thus, one would have to argue for a lipid environment that is specific to the β_2-adrenoceptor, and if this is so, it could well be the postulated exosite.

In conclusion, our studies have shown that not all responses to salmeterol are long-lasting. Indeed, long duration of action appears to be specific to responses mediated through β_2-adrenoceptors. That duration of action is unrelated to the different concentrations of salmeterol required for the two types of response is provided by a number of different observations: (1) in guinea-pig trachea in the presence of propranolol, very much higher concentrations of salmeterol ($\sim$1000 fold) were required to achieve a β-mediated relaxation, but the duration was identical to that in the absence of propranolol. (2) In guinea-pig trachea, in this particular study, the concentration of the **S**-enantiomer of salmeterol to cause β-mediated effects was 30–100 fold higher than that of the racemate, but the durations of those β-mediated effects were identical. (3) With the **S**-enantiomer of salmeterol, the concentrations required for β-mediated and non-β-mediated effects were only 10 fold different, but the durations of action were not only quantitatively, but qualitatively distinct. (4) When we determined the durations of action of a range of different concentrations of salmeterol on guinea-pig fundus, there was no inverse relationship between concentration and duration, if anything, the reverse appeared to be true.

Furthermore, the results tend to argue against a general membrane lipid reservoir mechanism, since even in guinea-pig trachea, salmeterol was capable of being washed out of membranes over a much shorter time period than that for which its β_2-adrenoceptor-mediated responses persisted, however, further studies are required to identify the non-β_2-adrenoceptor-mediated mechanism of the relaxant/inhibitory effects of salmeterol and close analogues in guinea-pig trachea, guinea-pig fundus and human neutrophils.

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