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Effects of β-adrenoceptor agonists in human bronchial smooth muscle

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1 We have investigated the potency and duration of action of isoprenaline and a range of β-adrenoceptor agonists as relaxants of inherent tone in human superfused, isolated bronchial smooth muscle, a tissue reported to contain a homogeneous population of β_2-adrenoceptors.

2 All of the β-adrenoceptor agonists caused concentration-related inhibition of inherent tone, with isoprenaline having an EC_{50} of 27 nM. The rank order of agonist potency was: formoterol ⩾ salmeterol ⩾ clenbuterol > fenoterol = isoprenaline > terbutaline ⩾ salbutamol > quinprenaline.

3 Relaxant responses to salmeterol were fully reversed by the selective β_2-adrenoceptor blocking drug, ICI 118551, demonstrating the involvement of β_2-adrenoceptors.

4 Rt_{50}, i.e. the time taken for 50% recovery from the effects of an EC_{50} concentration of agonist, differed considerably between the different β_2-adrenoceptor agonists. Most agonists were short-acting, having Rt_{50} values less than 13 min. Quinprenaline was of moderate duration, with an Rt_{50} value of ⩾ 20 min. In contrast, salmeterol was extremely long-acting, with no sign of recovery within 4 h.

5 Estimates of relative potency and duration of action were similar to those previously determined for these agonists in the guinea-pig isolated trachea. These results suggest, therefore, that guinea-pig trachea is a suitable alternative to human bronchus for the evaluation of the actions of β-adrenoceptor agonists on airways smooth muscle.

Keywords: β-Adrenoceptor agonists; salmeterol; formoterol; human bronchus; inherent tone; relaxation; duration of action

Introduction

The guinea-pig isolated trachea has been widely used to evaluate the actions of β-adrenoceptor agonists on airways smooth muscle *in vitro* (Castillo & DeBeer, 1947; Foster, 1966; Coleman & Nials, 1986). We have previously used the electrically-stimulated superfused preparation to identify the potent and long-acting β_2-adrenoceptor agonist, salmeterol (Ball *et al.*, 1991) and subsequently, to investigate its mechanism of action (Nials *et al.*, 1993).

More recently, we have attempted to carry out similar studies with β-adrenoceptor agonists on electrically-stimulated human superfused bronchial smooth muscle (Coleman *et al.*, 1993), a tissue which has previously been shown to contain a homogeneous population of β_2-adrenoceptors which mediate relaxation (Goldie *et al.*, 1984). However, the presence of inherent tone in this tissue complicates the evaluation of the relaxant activity of β-adrenoceptor agonists. On the other hand, such inherent tone in human bronchial smooth muscle is well-maintained, resistant to the effects of indomethacin, atropine and phenoxybenzamine (Rabe *et al.*, 1992a; Coleman *et al.*, 1993) and stable for many hours. Inherent tone may, therefore, be suitable for the study of both the potency and the duration of action of spasmolytic agents.

We now report the potency and duration of action of a range of β-adrenoceptor agonists as relaxants of inherent tone in human bronchial smooth muscle.

Methods

Tissue preparation

Samples of human bronchus were obtained from patients undergoing surgical resection of the lung. No account was taken of previous drug therapy. Bronchial tissue was carefully dissected clear of lung parenchyma and vascular tissue and placed in a modified Krebs solution. The composition of the Krebs solutions was as described by Apperley *et al.* (1976). Bronchus of lumen diameter 2–4 mm was cut into rings of 2–3 mm width, which were then opened to form strips. The preparations were either used immediately or stored overnight in oxygenated (5% CO_2 in O_2) Krebs solution at 4°C.

The preparations were mounted in superfusion chambers by tying a cotton thread to one end for attachment to a strain gauge, and a cotton loop to the other end for anchoring the tissue within the chamber. The superfusion apparatus employed in these experiments has been described previously (Coleman *et al.*, 1986; Coleman & Nials, 1989). The 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 maintained at 37°C and containing indomethacin (2.8 μM) to inhibit endogenous prostanoid synthesis. In order to demonstrate the presence of inherent tone, a single concentration of isoprenaline (100 nM) was infused on to each preparation. Experiments were carried out on preparations which underwent changes in tension of greater than 0.2 g in response to the isoprenaline infusion.

Determination of agonist potency

The relaxant activities of β-adrenoceptor agonists were measured against inherent tone. For each preparation, two cumulative concentration-effect curves to the standard β-adrenoceptor agonist, isoprenaline, were obtained by infusing increasing concentrations onto the tissue until the response to each concentration was maximal. Tissues were superfused with agonist-free Krebs solution for 30 min between curves. A cumulative concentration-effect curve to a test agonist was then constructed in an identical fashion. The magnitude of each response was measured and calculated as a percentage

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of the maximum isoprenaline response obtained in the final control curve.

Potency values for the β-adrenoceptor agonists were expressed in both absolute terms (concentration required to induce 50% of the maximum response to each agonist, EC_{50}) and relative to isoprenaline, as an equieffective concentration (EEC, i.e. EC_{50} for the test agonist/EC_{50} for isoprenaline). An EEC of less than unity indicates greater potency than isoprenaline, and an EEC greater than unity, a lower potency than isoprenaline.

Determination of the times for onset and offset of action

A single concentration-effect curve to isoprenaline was constructed by infusing increasing concentrations onto the tissue preparations in a sequential fashion. This was achieved by infusing each concentration until peak effect was attained, the infusion then being stopped and the tissues allowed to recover before the next concentration was administered. A curve to the test agonist was then constructed in a similar fashion. However, if no recovery was observed from the relaxant responses to the test agonist, the experimental protocol was altered. Thus, long-acting β_2-agonists (i.e. salmeterol and quinprenaline) were evaluated on paired preparations, in each case a sequential concentration-effect curve to isoprenaline first being constructed. Following this, a single concentration was added to each preparation. The concentration of test agonist chosen was that which caused a response approximately 20–40% of maximum on one preparation and 60–80% of maximum on the other. A composite, two point concentration-effect plot spanning the EC_{50} was then constructed.

Onset time (Ot_{50}) is defined as the time from administration of an EC_{50} of an agonist to attainment of 50% maximal response. Recovery (Rt_{50}) is defined as the time from stopping administration of the test agonist to attainment of 50% recovery from the EC_{50}. Ot_{50} and Rt_{50} values were determined by interpolation 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}; Coleman & Nials, 1989).

Drugs used

The following compounds were used: ascorbic acid (BDH Chemicals, UK), atropine sulphate (Sigma, UK), clenbuterol (Glaxo Group Research, UK), fenoterol (Sigma, UK), formoterol (Glaxo Group Research, UK), ICI 118551 (erythro-DL-1 (7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol, ICI Pharmaceuticals, UK), indomethacin (Sigma, UK), isoprenaline sulphate (Sigma, UK), mepyramine maleate (May & Baker, UK), papaverine (Sigma, UK), propranolol hydrochloride (Sigma, UK), quinprenaline (Pfizer, USA), salbutamol (Glaxo Group Research, UK), terbutaline (Glaxo Group Research, UK).

β-Adrenoceptor agonists were dissolved in 2 drops of glacial acetic acid and diluted to stock concentrations with phosphate buffer (pH 7.0). Dilutions of stock concentrations were made with 0.9% w/v saline. To prevent the autooxidation of isoprenaline and to maintain identical vehicle constituents, all solutions of β-adrenoceptor agonists contained ascorbic acid (11 μM).

Results

Agonist potency

Isoprenaline (1–300 nM), formoterol (0.3–100 nM), salbutamol (10–3000 nM) and salmeterol (0.3–30 nM), caused concentration-related relaxations of inherent tone in human bronchial smooth muscle. The results are summarized in Table 1, and composite cumulative concentration-effect curves are shown in Figure 1. There were no marked differences between the maximum responses obtained to isoprenaline, formoterol and salbutamol. Maximum responses were not achieved by salmeterol over the concentration-range tested, and relaxations at the highest concentration (30 nM) used were consistently lower than the isoprenaline maximum at 300 nM. The rank order of potency was: formoterol ≥ salmeterol > isoprenaline > salbutamol.

Potency, and times for onset and offset of action

In these experiments, two concentrations of each agonist (one causing < 50% relaxation, the other causing > 50% relaxation) were infused on one of each pair of tissues and potency, time for onset of action and time for offset (duration) of action were determined. The results are summarized in Table 2. Although this method is less precise for determination of agonist potency than that described in the previous section, the rank order of potency for the agonists was similar. Thus, under these conditions, formoterol and salmeterol were 20 and 11 fold more potent than isoprenaline, whilst salbutamol was nine fold less potent than isoprenaline. Quinprenaline was a partial agonist of low potency, being at least 140 fold less active than isoprenaline and achieving only 40–50% of the isoprenaline maximum response. In one experiment, the relaxant response to quinprenaline was biphasic in nature, in that a second phase of relaxation was observed following termination of the infusion of the agonist.

Onset of action

Relaxant responses to isoprenaline, salbutamol, terbutaline, clenbuterol, fenoterol and formoterol were all rapid, Ot_{50} values ranging from 1.5–5.3 min (Table 2). However, relaxant responses to salmeterol were significantly slower, with a mean Ot_{50} value of ~36 min. A precise Ot_{50} value for quinprenaline could not be determined, mainly because of the shallow nature of the responses.

Table 1 Human isolated bronchus; relaxant potencies of a range of β-adrenoceptor agonists

Agonist	*Equieffective concentration* (Isoprenaline = 1)	n
Isoprenaline	1.0 (EC_{50} = 24.4 (17.7–35.6) nM)	8
Formoterol	0.08 (0.06–0.09)	7
Salbutamol	9.3 (4.1–21.2)	6
Salmeterol	0.15 (0.04–0.48)	4

Values are expressed as geometric means of (*n*) individual experiments (with 95% confidence limits).

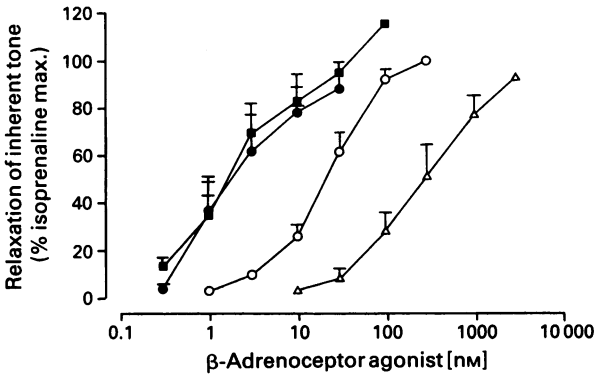

Figure 1 Human isolated, superfused bronchus; mean cumulative relaxant concentration-effect curves to isoprenaline (○), salmeterol (●), salbutamol (△) and formoterol (■). Each point is the mean ± s.e.mean of the least 4 determinations.

Table 2 Human isolated bronchus: relaxant potencies, onset of action and duration of action of a range of β-adrenoceptor agonists

Agonist	*Equieffective concentration* (Isoprenaline = 1)	*Onset* (Ot_{50} min)	*Recovery* (Rt_{50} min)	n
Isoprenaline	1.0 (EC_{50} = 26.6 (18.9–36.4) nM)	1.5 (± 0.1)	2.2 (± 0.3)	14
Clenbuterol	0.2 (0.02–1.36)	2.3 (± 0.9)	12.7 (± 5.4)	5
Fenoterol	0.9 (0.1–11.3)	2.1 (± 0.4)	4.6 (± 0.9)	4
Formoterol	0.05 (0.02–0.10)	5.3 (± 1.4)	6.6 (± 1.3)	5
Quinprenaline	141 [55–235]	–	⩾ 20 [20–>180]	3
Salbutamol	9 (2 – 44)	3.3 (± 0.3)	6.8 (± 1.3)	5
Salmeterol	0.09 (0.06 – 0.13)	35.6 (± 4.6)	>275	8
Terbutaline	5.4 (3.9 – 7.6)	3.0 (± 0.4)	6.9 (± 1.9)	4

Potency values are expressed as geometric means (with 95% confidence limits) or [range]. Onset and duration values are expressed as arithmetic means (± s.e.mean) or [range].

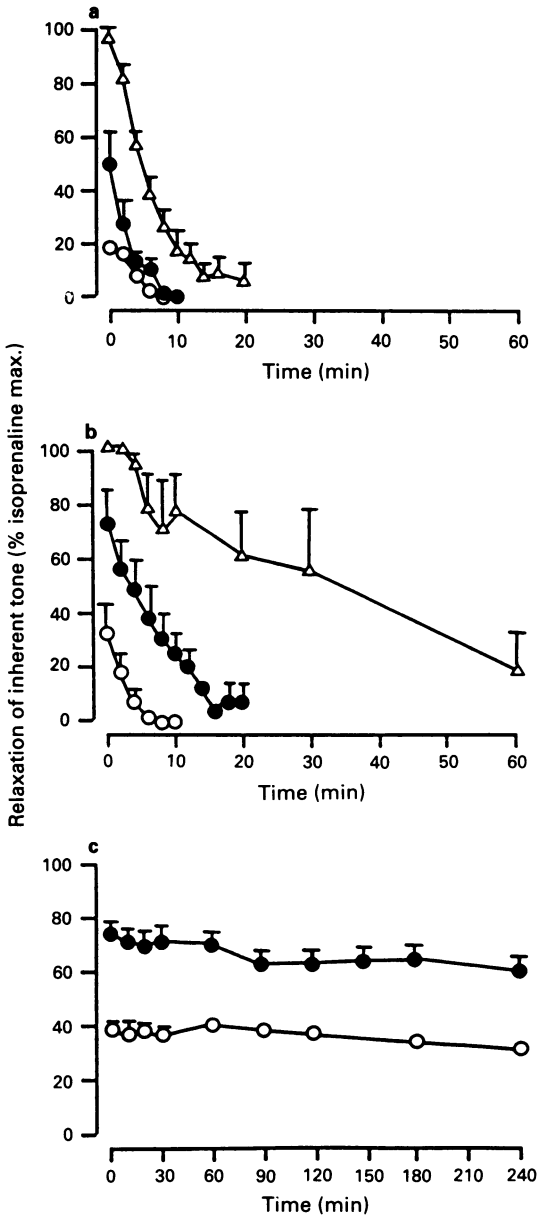

Figure 2 Human, isolated, superfused bronchus: relationship between duration of action of relaxant responses and concentration of agonist (a) salbutamol (10 nM, ○; 100 nM, ●; 1 000 nM, △); (b) formoterol (1 nM, ○; 10 nM, ●; 100 nM, △); and (c) salmeterol (1 nM, ○; 10 nM, ●) Data are expressed as arithmetic means ± s.e.mean.

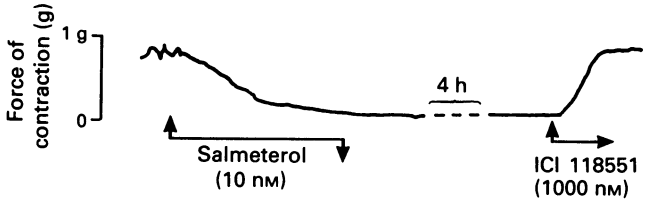

Figure 3 Human, isolated, superfused bronchus: a typical relaxant response to an infusion of salmeterol and its subsequent reversal by infusion of the β_2-adrenoceptor blocking drug, ICI 118551.

Duration of action

The relaxant responses to isoprenaline, salbutamol, terbutaline, clenbuterol, fenoterol and formoterol were short-lived, with Rt_{50} values ranging from 2.2–12.7 min. The duration of action of quinprenaline was somewhat longer, with an Rt_{50} value of ⩾ 20 min. In contrast, the effects of salmeterol were long-lasting, with an Rt_{50} value of >275 min, no appreciable recovery being observed during the time course of the experiment (Figures 2c and 3).

The durations of action of both salbutamol and formoterol were concentration-related (Figure 2a and b). At a concentration of 10 nM, relaxant responses to formoterol ranged from 70–85% of the isoprenaline maximum, and were of 4–13 min duration following wash out. As the concentration was increased ten fold, responses were all ⩾ 100%, and the duration increased to 54–196 min. In contrast, the duration of action of salmeterol was not concentration-related, as little or no recovery was obtained for at least 240 min from concentrations (1, 10 nM), producing approximately 40% and 75% relaxation respectively (Figure 2c).

The relaxant responses to salmeterol were, however, fully reversed by infusions of the β-adrenoceptor blocking drugs, (±)-propranolol (0.1–1 μM) or ICI 118551 (0.1–1 μM; Figure 3).

Discussion

The aim of these experiments was to evaluate the potency, onset and duration of action of a range of β-adrenoceptor agonists in human bronchial smooth muscle *in vitro*, and to compare the results with those previously obtained in guinea-pig trachea. The β-adrenoceptors mediating relaxation of human bronchial smooth muscle have previously been shown to be of the β_2-subtype (Goldie *et al.*, 1984).

The guinea-pig trachea has been extensively used to study the potency and duration of action of a wide range of β-adrenoceptor agonists (Castillo & DeBeer, 1947; Foster, 1966; Coleman & Nials, 1986), and was particularly impor-

tant in the identification of novel, long-acting β_2-adrenoceptor agonists such as salmeterol (Ball *et al.*, 1991). Guinea-pig tracheal smooth muscle exhibits a pronounced level of inherent tone, which is mediated almost exclusively by prostanoids. The evaluation of β-adrenoceptor agonists against this tone in guinea-pig trachea is complicated by the fact that they can enhance prostanoid release in this preparation (Coleman & Farmer, 1971; Farmer *et al.*, 1972) which would tend to offset their relaxant activity. For the evaluation of long-acting β_2-adrenoceptor agonists, such an effect could make the results difficult to interpret. Spasmogen-induced contractions of guinea-pig trachea preparations, in the presence of a cyclo-oxygenase inhibitor such as indomethacin to prevent endogenous prostanoid release, are poorly maintained for periods in excess of 30 min, and are therefore similarly unsuitable for the evaluation of long-acting β_2-adrenoceptor agonists. For this reason, we developed the electrically-stimulated preparation on which the potencies and durations of action of such agonists can be determined for periods of at least 7 h (Ball *et al.*, 1991).

In order, therefore, to compare the profiles of β-adrenoceptor agonists in human bronchus with those in guinea-pig trachea, we attempted to use electrical stimulation. Initial experiments showed, however, that despite the inclusion of indomethacin in the bathing solution, human bronchial preparations still exhibit marked inherent tone and as a result, β-adrenoceptor agonists not only inhibited the electrically-induced contractile responses, but also relaxed the inherent tone. Furthermore, the inherent tone was more sensitive to the relaxant effects of β-adrenoceptor agonists, and at low concentrations, while an inhibitory effect on inherent tone was observed; this was associated with an apparent enhancement of electrically-induced contractions (Coleman *et al.*, 1993). This same phenomenon has previously been observed in guinea-pig trachea, but inherent tone in this tissue can be abolished by indomethacin thereby enabling this complication to be overcome (Coleman & Farmer, 1971; Farmer *et al.*, 1972).

The nature of inherent tone in human bronchial smooth muscle is unknown, although it has variously been reported to be due to prostanoids, histamine and 5-lipoxygenase products (Ito *et al.*, 1989). In our studies, although we included the cyclo-oxygenase inhibitor, indomethacin, and also phenoxybenzamine, which among its range of effects, is a histamine H_1-receptor blocking drug, we failed to inhibit the inherent tone. We concluded, therefore, that the electrically-stimulated preparation of human bronchus is not suitable for the evaluation of the airway relaxant effects of β-adrenoceptor agonists. However, we and others (Goldie *et al.*, 1984; Chideckel *et al.*, 1987; Ito *et al.*, 1990; Rabe *et al.*, 1992a), have found that inherent tone in the human airway is particularly stable and therefore permits the evaluation of both the potency and duration of action of spasmolytic drugs. Therefore, in the present study, we have used inherent tone of the human bronchus to evaluate the relaxant activity of β-adrenoceptor agonists.

Although we have evaluated β-adrenoceptor agonists against electrically-induced contractions in guinea-pig trachea, and against inherent tone in human bronchus, the results obtained are similar. Thus, as on guinea-pig trachea, all of the β-adrenoceptor agonists relaxed human bronchus in a concentration-related manner, and the rank order of agonist potency is the same in both preparations, with formoterol, salmeterol and clenbuterol being more potent than isoprenaline, fenoterol being approximately equipotent with isoprenaline, and the other agonists all being weaker. As in guinea-pig trachea, quinprenaline was more than 100 fold weaker than isoprenaline. This rank order of agonist potency and the observation that relaxant responses to salmeterol were reversed by the β_2-adrenoceptor blocking drug, ICI 118551, support the conclusion that relaxation of human bronchial smooth muscle is mediated by β_2-adrenoceptors (Goldie *et al.*, 1984). Interestingly, in one preparation, quinprenaline produced a biphasic effect, as previously reported for guinea-pig airways (Nials *et al.*, 1991), with only partial relaxation being observed on addition of the agonist, but a further complete relaxation developing following termination of infusion. This is the first report of the occurrence of this phenomenon in human airways. The explanation for such biphasic activity is not clear, but it has been suggested that quinprenaline induces a permanently-activated conformation of the β_2-adrenoceptor protein, which only manifests itself after removal of the agonist from the biophase (Jack, 1991). However, there is as yet no conclusive evidence that this actually occurs.

In addition to the similarity in both the absolute and relative agonist potencies of the various β-adrenoceptor agonists in guinea-pig and human tissue, there is also a similarity in their respective durations of action. As in guinea-pig tissues, salmeterol is by far the longest-acting agonist tested, although we did not attempt to follow the duration for such an extended period as previously carried out (Ball *et al.*, 1991). Nevertheless, it is clear that responses to salmeterol in human bronchial smooth muscle persist without decline for periods in excess of 4.5 h, despite continuous washing with agonist-free medium. Although quinprenaline is approximately 1000 fold weaker than salmeterol, it also exhibits an extended duration of action, responses persisting for periods of at least 20 min. This result is similar to that previously reported by Nials *et al.* (1990) on guinea-pig trachea. All of the other β-adrenoceptor agonists tested were of short duration, none having an Rt_{50} in excess of 13 min. The fact that these results closely resemble those previously obtained in guinea-pig trachea, both qualitatively and quantitatively, is encouraging in that it suggests that guinea-pig trachea may be predictive of the human bronchus in terms of *in vitro* responses to β-adrenoceptor agonists. However, the question remains of the utility of either preparation for the prediction of the *in vivo* bronchodilator activity of β_2-adrenoceptor agonists in man.

As far as salmeterol is concerned, both isolated preparations suggest that its effects at β_2-adrenoceptors are highly persistent, and it is now well established that salmeterol (50 μg) has a long duration of action after inhalation in man (Ullman & Svedmyr, 1988). Indeed, the duration of action after a single administration to asthmatic patients has been reported to be as long as 20 h (Rabe *et al.*, 1992b). Similarly, the *in vitro* data in both preparations with isoprenaline, salbutamol, clenbuterol, fenoterol and terbutaline are all consistent with their known relatively short durations of action in the clinic. One apparent inconsistency concerns formoterol. Although others have reported that formoterol has persistent relaxant effects in human bronchial smooth muscle (Advenier *et al.*, 1991), we have found this compound to be clearly short-acting *in vitro*, in both guinea-pig trachea (Nials *et al.*, 1990) and now in human bronchus (present study). There are two likely explanations for the persistent effects observed by Advenier *et al.* (1991): the first is that very high concentrations of formoterol were tested, and the other is that the authors used an immersion rather than a superfusion technique. The importance of the concentration used is apparent in the results of the present study, where we found that at low concentrations, there was little difference in the durations of action of salbutamol and formoterol, whereas at higher concentrations, the recovery time for formoterol was considerably extended when compared with that for salbutamol. The relevance of immersion rather than superfusion is that the washing of the tissues is likely to have been substantially less rigorous than that used in our studies; as formoterol is more lipophilic than salbutamol (but less than salmeterol), it is likely to wash out from superfused preparations rather more slowly than salbutamol, particularly with only intermittent washing as was used in the immersion experiments. Unlike salbutamol and formoterol, salmeterol is long-acting under both immersed and superfused conditions, irrespective of the concentration tested. No attempt was

made to determine the rates of recovery from the effects of the shorter-acting compounds, from which the kinetics of the process could possibly be derived, as rate is likely to be a complex function. A number of factors could contribute towards recovery rate, including receptor affinity and efficacy, and physico-chemical factors, such as membrane: water partition coefficients. Indeed the influence of concentration on the pattern of recovery from the responses to formoterol illustrate this, as the recovery from the highest concentration tested (100 nM), clearly does not follow first-order kinetics.

Formoterol is also relatively short-acting *in vivo* when administered by aerosol to conscious guinea-pigs, and in man, when administered by the oral route (Lofdahl & Svedmyr, 1989). However, it is also clear that after inhalation in man, formoterol exhibits a prolonged duration of action (Lofdahl & Svedmyr, 1989; Derom *et al.*, 1989; Larsson *et al.*, 1990). The explanation for the extended duration of effect, associated with a particular route of administration, almost certainly lies with the high local concentrations achieved in the lung after inhalation of the recommended therapeutic doses (12–24 μg). This would explain why we have demonstrated only a short duration of action in guinea-pigs after administration by the inhaled route, since these experiments were carried out using only threshold effective doses. The evidence suggests that the mechanism of the extended duration of action of formoterol is different from that of salmeterol, which exhibits a long duration of action, both *in vitro* and *in vivo* irrespective of dose and of route of administration.

In conclusion, therefore, we have shown that reduction in inherent tone of human superfused bronchial smooth muscle can be used to evaluate the potency and duration of action of β-adrenoceptor agonists. The results obtained with salmeterol are similar, both qualitatively and quantitatively, to those previously obtained in electrically-stimulated, superfused guinea-pig trachea and support its well-established clinical profile as a potent, long-acting bronchodilator (Ullman & Svedmyr, 1988). The similarity in the data with β-adrenoceptor agonists in the human and guinea-pig airways preparations suggests that the latter may be used instead of human bronchus in the *in vitro* evaluation of this class of compounds.

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