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Desensitisation of mast cell β_2-adrenoceptor-mediated responses by salmeterol and formoterol

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1 The long-acting β_2-adrenoceptor agonist formoterol (10^{-10}–10^{-6} M) inhibited the IgE-dependent release of histamine from human lung mast cells in a concentration-dependent manner. Formoterol was more potent and a full agonist relative to the nonselective β-adrenoceptor agonist isoprenaline. By contrast, the long-acting β_2-adrenoceptor agonist salmeterol (10^{-10}–10^{-6} M) was about two-thirds less efficacious than either formoterol or isoprenaline as an inhibitor of histamine release.

2 Isoprenaline, formoterol and salmeterol (all at 10^{-5} M) increased total cell cAMP levels in mast cells over basal by 361 ± 90 ($P<0.05$), 321 ± 89 ($P<0.05$) and $64 \pm 24\%$ ($P>0.05$), respectively.

3 Long-term (24 h) incubation of mast cells with formoterol (10^{-6} M) or salmeterol (10^{-6} M) essentially abolished the subsequent ability of isoprenaline to inhibit histamine release. Both formoterol and salmeterol were more effective at inducing the functional desensitisation than isoprenaline (10^{-6} M) or the short-acting β_2-adrenoceptor agonist salbutamol (10^{-6} M).

4 The desensitisation induced by long-term treatments with salmeterol and formoterol was specific for β_2-adrenoceptor-mediated inhibition of histamine release as the inhibitory effects of alternative cAMP-elevating compounds, prostaglandin E_2, a receptor-mediated activator of adenylate cyclase, and forskolin, a direct activator of adenylate cyclase, were unaffected by desensitising treatments.

5 Radioligand binding studies were performed to determine β_2-adrenoceptor density in cell membranes after pretreatment (24 h) of cells with agonists. Isoprenaline, formoterol and salmeterol (all at 10^{-6} M) reduced β_2-adrenoceptor density by 13 ± 5 ($P>0.05$), 49 ± 13 ($P<0.05$) and $35 \pm 17\%$ ($P>0.05$), respectively.

6 These data indicate that long-term exposure of mast cells to both salmeterol and formoterol can cause substantial levels of desensitisation to β_2-adrenoceptor-mediated responses in mast cells.
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Keywords: Mast cells; β_2-adrenoceptor; formoterol; salmeterol

Abbreviations: [^{125}I]CYP, [^{125}I]-cyanopindolol; E_{max}, maximal response; PGE_2, prostaglandin E_2; pD_2, negative logarithm of the EC_{50}; PBS, phosphate-buffered saline

Introduction

The development of the β_2-adrenoceptor agonists, salmeterol and formoterol, constitutes an important advance in the therapeutic management of asthma (Waldeck, 2002). As bronchodilators, both drugs have been shown to provide symptomatic relief for longer periods ($\sim$12 h) compared to older generation bronchodilators, such as salbutamol and terbutaline, whose duration of action ($\sim$4 h) is shorter (Dahl, 1995; Lötvall, 2001). Salmeterol and formoterol are useful, therefore, in situations where control of symptoms is desirable for longer periods, such as in nocturnal asthma.

The mechanism by which salmeterol and formoterol exert long-lasting effects has been the topic of some debate. One suggestion, to explain the long-lasting actions of salmeterol, is the 'exosite' hypothesis wherein the aliphatic tail of salmeterol engages an exosite, which may or may not be part of the β_2-adrenoceptor itself, permitting the active portion of the molecule, potentially, to interact repeatedly with the β_2-adrenoceptor (Coleman *et al*., 1996). This concept has not been accepted universally and alternative explanations include the suggestion that the lipophilicity of both salmeterol and formoterol allows their accumulation within lipid membranes and, thereby, a more sustained availability of these drugs for interaction with β_2-adrenoceptors either by diffusion or by gradual leaching of the drugs from the membrane (Anderson *et al*., 1994).

Despite the benefits that bronchodilators afford, continued exposure of asthmatics to bronchodilators can lead to tolerance (Svedmyr, 1990). Both short-acting and long-acting agonists have been shown to cause tolerance (O'Connor *et al*., 1992; Cockcroft *et al*., 1993; Yates *et al*., 1997; Van der Woude *et al*., 2001). At the molecular level, tolerance may reflect β_2-adrenoceptor desensitisation. Desensitisation is a complex, time-dependent process that may involve the uncoupling, sequestration and degradation of the receptor (Lefkowitz, 1998). Other processes that may contribute to the development

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of tolerance include the downregulation of $G_{s\alpha}$, the protein coupling the receptor to adenylate cyclase, and the upregulation of phosphodiesterase, the enzyme involved in degrading cAMP (Giembycz, 1996; Finney *et al*., 2001).

Although the main action of β_2-adrenoceptor agonists is to relax airways smooth muscle, additional effects may include the stabilisation of inflammatory cell activity and, in this regard, inhibitory effects on the pulmonary mast cell may be important (Barnes, 1999). Indeed, a large number of studies have shown that β-adrenoceptor agonists are very effective inhibitors of mediator release from lung mast cells (Butchers *et al*., 1980; 1991; Church & Hiroi, 1987; Lau *et al*., 1994; Nials *et al*., 1994). However, both *in vivo* and *in vitro* studies show that mast cells can become tolerant to the effects of β-adrenoceptor agonists (Van der Heijden *et al*., 1984; O'Connor *et al*., 1992; Cockcroft *et al*., 1993; Chong & Peachell, 1999). Our own studies show that long-term exposure of human lung mast cells to short-acting β-adrenoceptor agonists attenuates the inhibitory effects of β-adrenoceptor agonists on mast cells (Chong *et al*., 1995; 2000; 2003; Drury *et al*., 1998; Chong & Peachell, 1999). The aim of the present study was to determine whether the long-acting β_2-adrenoceptor agonists, salmeterol and formoterol, are also capable of inducing desensitisation to β-adrenoceptor agonists in mast cells.

Methods

Buffers

Tyrode's buffer contained (mM): NaCl 137, HEPES 1.2, KCl 2.7, $NaH_2PO_4 \cdot H_2O$ 0.04, glucose 5.6. Tyrode's-BSA was Tyrode's buffer that additionally contained: $CaCl_2 \cdot 2H_2O$ 0.5 mM, $MgCl_2 \cdot 6H_2O$ 1 mM, BSA 1 mg ml^{-1}, DNase 15 μg ml^{-1}. Phosphate-buffered saline (PBS) contained (mM): NaCl 137, $Na_2HPO_4 \cdot 12H_2O$ 8, KCl 2.7, KH_2PO_4 1.5, $CaCl_2 \cdot 2H_2O$ 1, $MgCl_2 \cdot 6H_2O$ 1, glucose 5.6, HSA 30 μg ml^{-1}. The pH of Tyrode's buffers and PBS was titrated to 7.3. Tris buffer contained (mM): Tris 50, NaCl 154, $MgCl_2 \cdot 6H_2O$ 10, EDTA 2. The pH of Tris buffer was titrated to 7.4.

Preparation of compounds

Stock solutions (10 mM) of (−)-isoprenaline bitartrate were prepared daily in 0.05% sodium metabisulphite (dissolved in 0.9% NaCl). Salbutamol hemisulphate was prepared daily as stock solutions (10 mM) in buffer. Salmeterol, the aliphatic tail of salmeterol (phenyl-butoxy-hexylamine; for structure see Bergendal *et al*., 1996) and formoterol were prepared as stock solutions (100 mM) in dimethyl sulphoxide (DMSO). Lyophilised polyclonal goat anti-human IgE antibody was reconstituted in distilled water and stored at 4°C.

Lung tissue

Background, nonlesional tissue from lung resections of patients was obtained following surgery. Most of the patients were undergoing surgery for carcinoma. The male to female split was 70–30% and 90% of the patients were white caucasians. The provision of lung tissue and the use of the tissue in this study were approved by the Local Research Ethics' Committee.

Isolation of mast cells

Mast cells were isolated from human lung tissue by a modification of the method described (Ali & Pearce, 1985). The tissue was stripped of its pleura and chopped vigorously for 15 min with scissors in a small volume of Tyrode's buffer. The chopped tissue was washed over a nylon mesh (100 μm pore size; Cadisch and Sons, London, U.K.) with 0.5–1 l of Tyrode's buffer to remove lung macrophages. The tissue was reconstituted in Tyrode's-BSA (10 ml per g of tissue) containing collagenase Ia (350 U per ml of Tyrode's-BSA) and agitated by using a water-driven magnetic stirrer immersed in a water bath set at 37°C. The supernatant (containing some mast cells) was separated from the tissue by filtration over nylon mesh. The collagenase-treated tissue was then reconstituted in a small volume of Tyrode's-BSA buffer and disrupted mechanically with a syringe. The disrupted tissue was then washed over nylon gauze with Tyrode's-BSA (300–600 ml). The pooled filtrates were sedimented (120 × *g*, room temperature, 8 min), the supernatant discarded and the pellets reconstituted in Tyrode's-BSA (100 ml). The pellet was washed a further two times. The dispersion procedure generated 0.2 to 1×10^6 mast cells per g of lung tissue at 5–20% purity as assessed by alcian blue staining (Gilbert & Ornstein, 1975). These cell preparations were used in histamine release experiments. Mast cell-enriched preparations (>30% purity) were generated by countercurrent elutriation (Beckman J6B centrifuge, JE-5.0 elutriator head) and further purification (⩾85%) was achieved by flotation of mast cell-enriched preparations over Percoll density gradients using slight modifications of the methods that have been described in detail elsewhere (Schulman *et al*., 1982; Ishizaka *et al*., 1983). Purified mast cells were used in cAMP assays.

Histamine release

Histamine release experiments were performed in PBS. Histamine release from mast cells was initiated immunologically with an optimal releasing concentration of anti-IgE (1 : 300). Secretion was allowed to proceed for 25 min at 37°C after which time the cells were pelleted by centrifugation (400 × *g*, room temperature, 3 min). Histamine released into the supernatant was determined by a modification of the automated fluorometric method of Siraganian (1974). When β-adrenoceptor agonists or alternative cAMP-active compounds were employed, cells were incubated with agonist for 10 min at 37°C before the addition of stimulus and then samples were processed as indicated above. Total histamine content was determined by lysing aliquots of the cells with 1.6% perchloric acid. Cells incubated in buffer alone served as a measure of spontaneous histamine release (<6%). Histamine release was thus expressed as a percentage of the total histamine content after subtracting the spontaneous histamine release.

When long-term incubations were performed, RPMI 1640 buffer supplemented with penicillin/streptomycin (10 μg ml^{-1}) and gentamicin (50 μg ml^{-1}) was employed. Cells were incubated (24 h) at a density of 0.1×10^6 mast cells per ml in six-well plates with, usually, 0.5×10^6 mast cells per condition with or without a β-adrenoceptor agonist. After completion of the incubations, the cells were washed three times with PBS and reconstituted in the same buffer for mediator release experiments. Incubations of mast cells with the β-adrenoceptor

agonists had no effect on either the total number of mast cells recovered, the total histamine content or the spontaneous histamine release compared to mast cells incubated in buffer. In preliminary experiments, the effect of long-term incubation (24 h) of mast cells with the vehicle DMSO (0.001%), which was used to prepare some of the drugs (salmeterol, formoterol), on the isoprenaline inhibition of histamine release was determined. Following this treatment with DMSO, there was no difference in the potency (control pD_2 (negative logarithm of the EC_{50}), 8.4 ± 0.2; pD_2 after DMSO, 8.6 ± 0.3) or efficacy (control E_{max}, $36 \pm 6\%$ inhibition; E_{max} after DMSO, $32 \pm 3\%$ inhibition) of isoprenaline compared to control ($n = 7$).

Assays for cAMP

Total cell cAMP levels were monitored according to methods that have been described in more detail elsewhere (Chong *et al*., 1998). Purified cells were incubated (10 min) without or with a β-adrenoceptor agonist and the reaction terminated by the addition of ice-cold acidified ethanol and snap-freezing of samples in liquid nitrogen. After thawing, samples were pelleted by centrifugation, supernatants saved and the ethanol evaporated using a rotary evaporator. Samples were reconstituted in assay buffer and cAMP levels were determined using commercially available EIA kits.

Radioligand binding

Mast cell-enriched (33–67% purity) preparations were employed for radioligand binding studies. Highly purified mast cell preparations were not used in these binding experiments as the numbers of cells required could have only been generated on a very occasional basis. However, previous studies of ours have shown that these mast cell-enriched populations reasonably parallel the behaviour of purified mast cell preparations in the context of β-adrenoceptor expression following exposure to β-adrenoceptor agonists (Chong *et al*., 2003). Mast cell-enriched (Chong *et al*., 2003) preparations, like lung mast cells (Chong *et al*., 2002), express a homogeneous population of β_2-adrenoceptors.

Membrane fractions were prepared from mast cell-enriched preparations ($\geqslant 3 \times 10^6$ cells per condition) after treatment (24 h) with buffer or a given β-adrenoceptor agonist. Membranes were prepared by homogenising in ice-cold Tris buffer using an Ultra Turrax homogeniser for 20 s followed by four strokes ($\times 4$) of a Teflon homogeniser. The homogenate was centrifuged ($500 \times g$, 40 min), the supernatant was harvested and subjected to further centrifugation ($40{,}000 \times g$, 15 min) in an ultracentrifuge (L80, Beckman). The pellet was washed and the high-speed centrifugation step repeated. The pellet was resuspended in Tris buffer and used in receptor binding assays. All procedures were carried out at 4°C. In saturation binding assays, the membrane preparations were assayed for β-adrenoceptor binding sites using [^{125}I]-cyanopindolol ([^{125}I]CYP). Membrane suspensions (100 μl) were incubated (1 h, 37°C) using a range of radioligand concentrations (0.03125–2 nM) in a total volume of 250 μl. Nonspecific binding was determined by displacement with propranolol (1 μM). Specific binding, expressed as a percentage of the total binding, ranged from 67 to 76% at a [^{125}I]CYP concentration of 0.0625 nM. Additions of ice-cold Tris buffer were used to terminate the reactions followed by rapid filtration through Whatman GF/B glass fibre filters. The filters were rapidly washed four times with 3 ml ice-cold buffer and the radioactivity remaining on filters measured in a Packard Cobra auto-gamma counter. All binding experiments were performed in duplicate. Protein content of the membranes was determined by the method of Lowry *et al*. (1951).

Materials

The following were purchased from the sources indicated; anti-human IgE, BSA, collagenase, DNase, HSA, Percoll, isoprenaline, salbutamol, propranolol (all Sigma, Poole, U.K.); [^{125}I]CYP (New England Nuclear, Stevenage, U.K.); cAMP EIA kits (Amersham, Little Chalfont, U.K.). Salmeterol and the aliphatic tail of salmeterol (GSK, Stevenage, U.K.) and formoterol (Yamanouchi, Ibaraki, Japan) were all kindly provided as gifts.

Data analysis

Maximal responses (E_{max}) and potencies (pD_2) were determined by nonlinear regression analysis (GraphPad Prism, version 2). Receptor densities (B_{max}) and radioligand affinity (K_D) were determined by nonlinear regression analysis of saturation curves (GraphPad Prism). To determine whether there was any difference in the responses of mast cells after treatments, either paired *t*-tests or repeated measures ANOVA was performed.

Results

Effects of formoterol and salmeterol on mast cells

The effects of isoprenaline, formoterol and salmeterol (all at 10^{-10}–10^{-5} M) on the IgE-mediated release of histamine from human lung mast cells were investigated (Figure 1). Isoprenaline and formoterol inhibited histamine release in a concentration-dependent manner. Formoterol was a full agonist and about 10-fold more potent than isoprenaline (Table 1). The effects of salmeterol, however, were more complex. Up to 10^{-6} M, salmeterol inhibited IgE-mediated histamine release

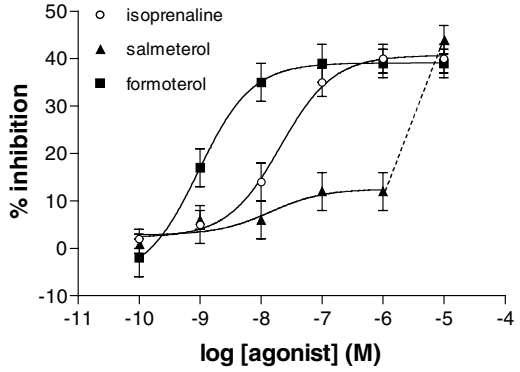

Figure 1 Inhibition of histamine release by β-adrenoceptor agonists. Mast cells were incubated for 10 min without or with either isoprenaline, salmeterol or formoterol before challenge with anti-IgE (1 : 300) for 25 min for histamine release. Values are expressed as the % inhibition of the control histamine release which was $24 \pm 3\%$. Values are means ± s.e.m. from 13 experiments.

weakly (Figure 1) being about two-thirds less efficacious than either isoprenaline or formoterol (Table 1). At the highest concentration employed (10^{-5} M), salmeterol was an effective inhibitor of histamine release (Figure 1). The inhibitory activity of salmeterol, at the highest concentration used (10^{-5} M), was probably due to the aliphatic side chain of salmeterol as salmeterol (10^{-5} M) and the aliphatic tail (10^{-5} M) inhibited histamine release by 30±7 and 32±3%, respectively (data not shown, $n=5$).

Over the course of this study, salmeterol, up to concentrations of 10^{-6} M, displayed variable inhibitory activity among mast cell preparations displaying weak effects in many preparations. This variable inhibitory activity of salmeterol is illustrated in Figure 2.

The effects of isoprenaline, formoterol, salbutamol and salmeterol (all at 10^{-5} M) on cAMP generation in mast cells were evaluated (Figure 3). The extent of cAMP generated by these β-adrenoceptor agonists was isoprenaline ≥ formoterol > salbutamol > salmeterol.

Desensitisation induced by formoterol and salmeterol

We have previously reported that long-term (24 h) treatment with isoprenaline reduces the subsequent effectiveness of isoprenaline to inhibit histamine release (Chong *et al*., 1995). In the present study, we determined the effects of long-term (24 h) treatment of mast cells with formoterol, salmeterol and salbutamol (all at 10^{-6} M) on the subsequent ability of isoprenaline (10^{-10}–10^{-6} M) to inhibit histamine release (Figure 4). Long-term treatment of mast cells with salmeterol and formoterol substantially compromised ($P<0.001$) the subsequent inhibition of histamine release by isoprenaline. Long-term treatment with salbutamol caused a less profound but statistically significant ($P<0.05$) reduction in the maximal inhibitory effects of isoprenaline (control E_{max}, 39±6%; E_{max} after salbutamol treatment, 20±3%). Long-term treatments

Table 1 E_{max} and pD_2 values for the inhibition of histamine release by isoprenaline, formoterol and salmeterol

	E_{max} (%)	pD_2
Isoprenaline	40±3	8.2±0.3
Formoterol	37±3	9.2±0.3
Salmeterol	13	7.8

Values have been calculated from data in Figure 1 and further details can be found in the legend to that figure. Due to the large variability in the response of mast cells to salmeterol, E_{max} and pD_2 values for salmeterol have been calculated using the mean concentration–response curve (10^{-10}–10^{-6} M) shown in Figure 1. For formoterol and isoprenaline, values are means±s.e.m.

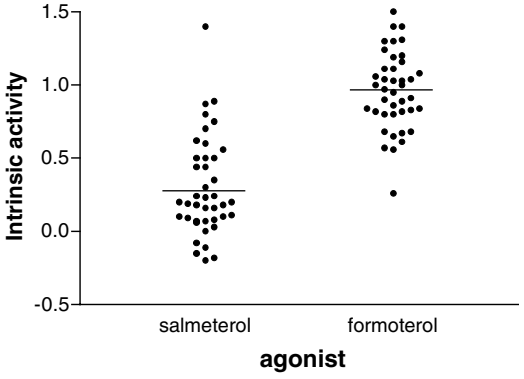

Figure 2 Intrinsic activities of salmeterol and formoterol. Intrinsic activities have been determined relative to the full agonist isoprenaline, using the following equation: (inhibition obtained with 1 μM of a long-acting agonist/inhibition obtained with 1 μM isoprenaline). Each of the points represents a separate mast cell preparation and the horizontal line represents the mean intrinsic activities and these were 0.30±0.05 and 0.96±0.04 for salmeterol and formoterol, respectively. Data have been taken from 41 experiments.

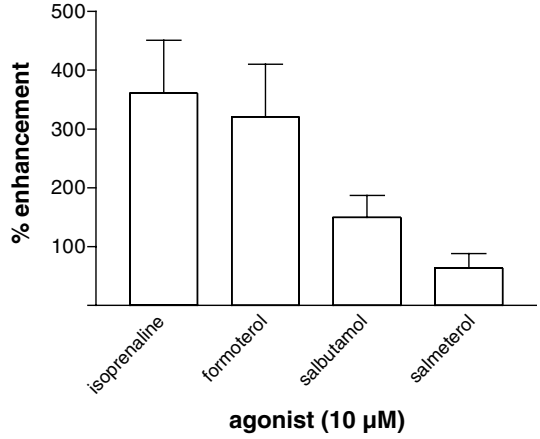

Figure 3 Elevations in cAMP induced by β-adrenoceptor agonists. Mast cells (91±6% purity) were incubated without or with an agonist (10^{-5} M) for 10 min and total cell cAMP levels were determined. Values are expressed as the % enhancement in cAMP levels over basal levels. All agonists caused statistically significant ($P<0.05$ at least) increases in cAMP except salmeterol. Values are means±s.e.m. from four experiments.

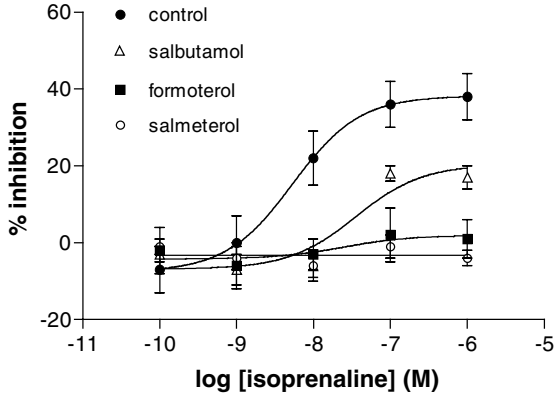

Figure 4 Functional desensitisation of β-adrenoceptor agonist-mediated responses in mast cells. Mast cells were incubated (24 h) without (control) or with either salbutamol (10^{-6} M), formoterol (10^{-6} M) or salmeterol (10^{-6} M) and then washed extensively. Cells were then incubated without or with isoprenaline for 10 min before challenge with anti-IgE (1 : 300) for histamine release. Values are expressed as the % inhibition of the control histamine releases which were 36±6, 34±6, 34±2 and 38±3% following 24 h treatments with buffer, formoterol, salmeterol and salbutamol, respectively. All treatments with β-adrenoceptor agonists caused statistically significant ($P<0.05$ at least) reductions of the isoprenaline inhibition of histamine release. Values are means±s.e.m. from seven experiments.

with formoterol, salmeterol and salbutamol also caused reductions in the subsequent inhibitory actions of formoterol and salbutamol (Figure 5). The functional desensitisation induced by both formoterol and salmeterol was concentration-dependent with formoterol causing greater levels of desensitisation than salmeterol at lower concentrations (Figure 6).

In order to determine whether the aliphatic tail of salmeterol possesses any desensitising capability, mast cells were incubated (24 h) with or without the tail (10^{-6} M), the cells washed extensively and then incubated (10 min) with isoprenaline (10^{-10}–10^{-5} M) before challenge with anti-IgE ($n=8$). There was no difference in the maximal inhibitory effect of isoprenaline following the treatment (control E_{max}, 35±4%; E_{max} after treatment, 32±5%; $P>0.05$) although there was a modest rightward shift in the isoprenaline concentration–response curve (control pD_2, 8.0±0.3; pD_2 after treatment, 7.7±0.3; $P>0.05$). In further studies to determine whether the aliphatic tail might facilitate desensitisation, mast cells were incubated (24 h) with either salmeterol, salbutamol, the aliphatic tail or the aliphatic tail plus salbutamol (all at 10^{-6} M), and the subsequent inhibitory effects of isoprenaline (10^{-6} M) determined (Figure 7). The presence of the aliphatic tail increased the desensitising capability of salbutamol but the increase was not significantly different ($P>0.05$) from desensitisation obtained with salbutamol alone.

In order to establish whether long-term treatments affected the responses to alternative cAMP-elevating agents, mast cells were incubated (24 h) with either formoterol, salmeterol or isoprenaline (all at 10^{-6} M) and then the effects of prostaglandin E_2 (PGE_2), a receptor-mediated activator of adenylate cyclase, forskolin, a direct activator of adenylate cyclase, theophylline, an inhibitor of phosphodiesterases, and the β-adrenoceptor agonists, formoterol and salbutamol, were evaluated (Table 2). Long-term exposure of mast cells to salmeterol, formoterol or isoprenaline had no influence on the inhibitory effects of forskolin and PGE_2. However, long-term treatment of mast cells with salmeterol and formoterol (but not isoprenaline) reduced ($P<0.05$) the effectiveness of theophylline as an inhibitor of histamine release. Overall, long-term treatment of mast cells with salmeterol or formoterol reduced the inhibitory effects of β-adrenoceptor agonists to a greater extent than long-term treatment with isoprenaline.

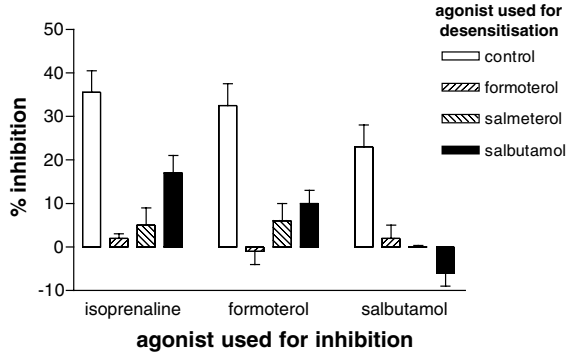

Figure 5 Cross desensitisation experiments. Mast cells were incubated (24 h) without or with either formoterol, salmeterol or salbutamol (all at 10^{-6} M) and then washed extensively. Cells were then incubated without or with either isoprenaline, formoterol or salbutamol (all at 10^{-6} M) for 10 min before challenge with anti-IgE (1 : 300) for histamine release. Values are expressed as the % inhibition of the control histamine releases which were 36±3, 31±3, 35±4 and 33±3% following 24 h treatments with buffer, formoterol, salmeterol or salbutamol, respectively. All treatments with β-adrenoceptor agonists caused statistically significant ($P<0.05$ at least) reductions of the isoprenaline, formoterol or salbutamol inhibition of histamine release. Values are means±s.e.m. from nine experiments.

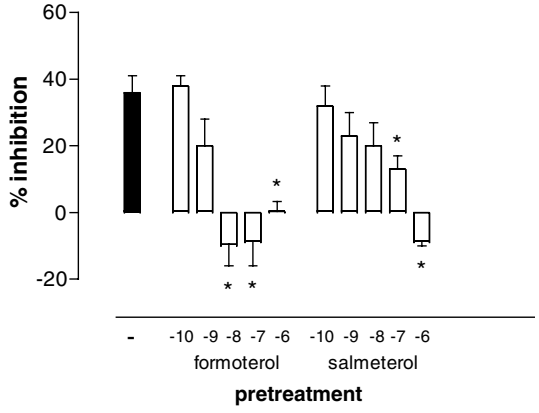

Figure 6 Concentration dependence of the desensitisation. Mast cells were incubated without or with increasing concentrations of salmeterol (10^{-10}–10^{-6} M) or formoterol (10^{-10}–10^{-6} M) for 24 h and then washed. The cells were then incubated with isoprenaline (10^{-6} M) for 10 min before challenge with anti-IgE for histamine release. Columns represent the isoprenaline inhibition of histamine release following treatments. Values are expressed as the % inhibition of the control histamine releases which ranged from 27±5 to 31±6%. Compared to the control isoprenaline inhibition, statistically significant ($P<0.01$) reductions in the inhibition, following treatments with drugs, are indicated by an asterisk. Values are means±s.e.m. from six experiments.

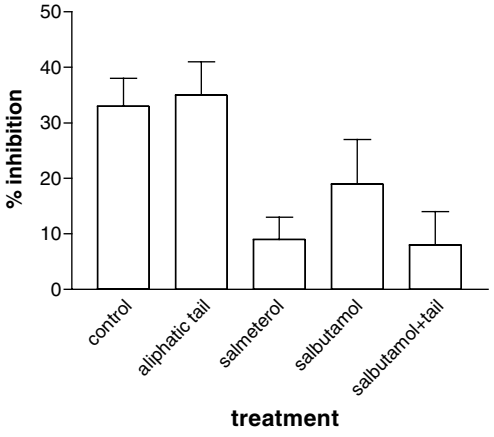

Figure 7 Influence of the aliphatic tail of salmeterol on desensitisation. Mast cells were incubated (24 h) without or with either the aliphatic tail (10^{-6} M), salmeterol (10^{-6} M), salbutamol (10^{-6} M) or salbutamol (10^{-6} M) plus the aliphatic tail (10^{-6} M). After this time, the cells were washed extensively and then incubated (10 min) with isoprenaline (10^{-6} M) before challenge with anti-IgE (1 : 300) for histamine release. Data show the isoprenaline inhibition of histamine release following treatments. Values are expressed as the % inhibition of the control histamine releases which ranged from 29±4 to 36±1%. Compared to the control isoprenaline inhibition, all treatments caused statistically significant ($P<0.05$ at least) reductions in the inhibition except treatment with the aliphatic tail alone. Values are means±s.e.m. from five experiments.

Salmeterol acting as an antagonist

As salmeterol is a partial agonist in this system, the possibility exists that the effects attributed to desensitisation reflect salmeterol acting as an antagonist. Indeed, preincubation of mast cells with salmeterol (10^{-6} M) before incubation with isoprenaline (10^{-10}–10^{-5} M) caused an approximately 100-fold shift in the concentration–response curve for the inhibition of histamine release by isoprenaline (Figure 8). Further experiments indicated that pretreatment of mast cells with the aliphatic tail did not antagonise the isoprenaline (10^{-10}–10^{-5} M) inhibition of histamine release (data not shown).

In order to determine whether the inhibitory effects of salmeterol can be removed by washing, mast cells were incubated (20 min) with or without salmeterol (10^{-6} M) and then the cells were either washed extensively (mimicking washings performed in the desensitisation protocol) or not washed. For comparative purposes, the effects of washing on the inhibitory actions of formoterol (10^{-6} M), isoprenaline (10^{-6} M) and PGE_2 (5×10^{-6} M) were also determined (Table 3). The data show that extensive washing reduces the inhibitory effects of all the agonists tested to a significant ($P<0.05$) degree.

Table 2 Effects of desensitising treatments on cAMP-elevating compounds

	% Inhibition			
	Desensitising treatment			
	Control	*Isoprenaline*	*Formoterol*	*Salmeterol*
Formoterol (1 μM)	45±4	24±7*	2±2*	9±5*
Salbutamol (1 μM)	31±5	15±5	4±5*	1±3*
PGE_2 (5 μM)	43±5	45±6	43±5	44±5
Forskolin (5 μM)	43±6	35±6	37±4	36±7
Theophylline (1 mM)	65±4	55±4	53±5*	50±7*

Cells were incubated (24 h) without (control) or with isoprenaline, formoterol or salmeterol (all at 10^{-6} M) and then washed extensively. The cells were then incubated with a cAMP-elevating compound for 10 min before challenge with anti-IgE (1 : 300) for a further 20 min for histamine release. Values are expressed as the % inhibition of the control histamine releases and these ranged from 29±3 to 34±1%. Values are means±s.e.m. from six experiments. Asterisks denote statistically different ($P<0.05$ at least) levels of inhibition, compared to control inhibition, following desensitising treatments.

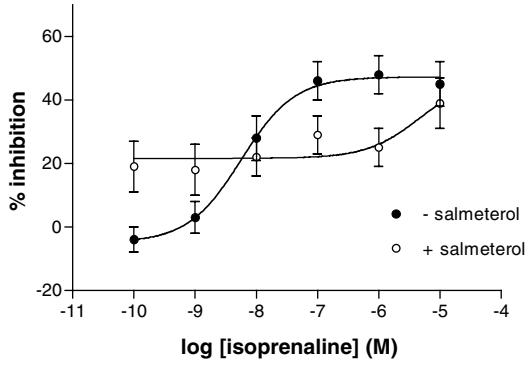

Figure 8 Salmeterol antagonism of isoprenaline inhibition. Mast cells were incubated without or with salmeterol (10^{-6} M) followed by incubation with isoprenaline for 10 min before challenge with anti-IgE (1 : 300) for histamine release. Results are expressed as the % inhibition of the control histamine release in the absence of any agonists and this was 21±4%. Salmeterol (10^{-6} M) alone inhibited the control histamine release by 20±6% and this is the reason why the concentration–response curve, at lower concentrations of isoprenaline in the presence of salmeterol, starts at a higher value than the curve for isoprenaline in the absence of salmeterol. Values are means±s.e.m. from five experiments.

Table 3 Effect of extensive washing on the inhibitory activity of agonists

	% Inhibition			
	Salmeterol	*Formoterol*	*Isoprenaline*	*PGE_2*
Unwashed	34±6	47±9	40±10	36±6
Washed	19±5	26±7	3±5	6±3

Cells were incubated with either salmeterol (10^{-6} M), formoterol (10^{-6} M), isoprenaline (10^{-6} M) or PGE_2 (5×10^{-6} M) for 20 min and the cells were then either washed or not before challenge for 25 min with anti-IgE (1 : 300) for histamine release. Results are expressed as the % inhibition of the control histamine releases and these were 29±2 and 23±3% for unwashed and washed sets. Values are means±s.e.m. from four to five experiments. Washing reduced the inhibitory effects of all agonists to a statistically significant ($P<0.05$ at least) extent.

Radioligand binding studies

In further experiments, the effects of β-adrenoceptor agonists on β_2-adrenoceptor density in cell membranes were determined. Long-term (24 h) exposure of mast cell-enriched preparations to isoprenaline, salmeterol and formoterol (all at 10^{-6} M) caused 13±5 ($P>0.05$), 35±13 ($P>0.05$) and 49±17% ($P<0.05$) reductions, respectively, in β_2-adrenoceptor density (Table 4).

Discussion

Salmeterol and formoterol have both been introduced as long-acting bronchodilators for the therapeutic management of asthma. Both drugs act primarily to relax airway smooth muscle and thereby provide symptomatic relief in asthma (Barnes, 1999). Additional effects could include the stabilisation of pulmonary inflammatory cells and, in this context, inhibition of mast cell responses could be important (Barnes, 1999). Certainly, a number of *in vitro* studies show that β_2-adrenoceptor agonists are effective inhibitors of mediator release from lung mast cells (Butchers *et al*., 1980; 1991; Church & Hiroi, 1987; Lau *et al*., 1994; Nials *et al*., 1994). In keeping with these findings, formoterol was a very effective inhibitor of the stimulated release of histamine from mast cells being about 10-fold more potent than isoprenaline and acting as a full agonist. By contrast, salmeterol was about two-thirds less efficacious as an inhibitor of histamine release and showed highly variable inhibitory activity from preparation to preparation. The variability in activity observed for salmeterol may relate to differences in receptor reserve among mast cell preparations (Drury *et al*., 1998). The finding that formoterol

Table 4 Effects of desensitising treatments on β_2-adrenoceptor density

	Desensitising treatment			
	Control	*Isoprenaline*	*Salmeterol*	*Formoterol*
B_{max} (fmol mg^{-1} protein)	13±6	11±5	9±4	5±1
K_D (nM)	0.06±0.04	0.04±0.01	0.07±0.04	0.03±0.01

Mast cell-enriched preparations (48±7% purity) were incubated (24 h) without (control) or with a β-adrenoceptor agonist (10^{-6} M), the cells washed three times and then membranes prepared. Receptor density (B_{max}) was determined employing [^{125}I]CYP and the K_D values are for this ligand. There was no difference in the K_D values following the different treatments. Treatment with formoterol caused a significant ($P<0.05$) reduction in receptor density, whereas the effects of isoprenaline and salmeterol were not statistically significant ($P>0.05$). Values are means±s.e.m. from four experiments. The mast cell purities were 33, 44, 47 and 67%.

is both more potent and efficacious than salmeterol has also been observed by others in mast cell systems (Lau *et al*., 1994; Nials *et al*., 1994) and in alternative *in vitro* systems (Nials *et al*., 1993; Kallstrom *et al*., 1994). These data suggest that, in the clinical context, formoterol is likely to prevent mast cell degranulation to a greater extent than salmeterol.

As bronchodilators are thought to interact with β_2-adrenoceptors to activate adenylate cyclase, the effects of salmeterol and formoterol on the generation of total cell cAMP were determined. Formoterol increased cAMP in mast cells to a similar extent to that seen with isoprenaline whereas salmeterol caused very modest increases in cAMP that were not statistically significant. Salbutamol, which is related to salmeterol except that it does not have the aliphatic tail, caused increases in cAMP that were about 50% of that seen with isoprenaline and inhibited histamine release to about 60–70% of that obtained with isoprenaline. Therefore, there appears to be a good correlation between the increases in cAMP induced by these agonists and the effects of these compounds on histamine release.

These preliminary data suggest that formoterol and salmeterol affect the responses of mast cells to substantially different degrees. In experiments to address the comparative effects of these two agonists on functional desensitisation in mast cells, an expectation might be, based on the relative efficacies of these compounds as inhibitors of histamine release and activators of adenylate cyclase, that formoterol would promote far greater levels of desensitisation than salmeterol. However, incubation (24 h) of mast cells with either salmeterol or formoterol substantially attenuated the inhibitory effects of isoprenaline and alternative β-adrenoceptor agonists. Indeed, agonists (isoprenaline and salbutamol) with higher intrinsic activities than salmeterol were less effective at causing the functional desensitisation. This outcome was, perhaps, surprising given that studies in transfected cells demonstrate a correlation between the relative efficacy of agonists and the extent of receptor desensitisation (January *et al*., 1997; 1998).

That salmeterol, despite showing weak efficacy, caused substantial levels of functional desensitisation prompted us to determine whether the aliphatic tail might influence the responses to mast cells. That the tail may have some effect on mast cells is illustrated by the fact that, at high concentrations (10^{-5} M), it inhibited histamine release to a similar extent to that seen with salmeterol (10^{-5} M) although these effects of both salmeterol and the tail are probably not β_2-adrenoceptor-mediated (Bergendal *et al*., 1996; Chong *et al*., 1998). Further studies demonstrated that long-term (24 h) incubation of mast cells with the aliphatic tail (10^{-6} M) had no significant effects on the inhibition by isoprenaline of histamine release. Long-term incubation of mast cells with salbutamol plus the aliphatic tail did appear to increase the extent of desensitisation over that seen with salbutamol alone but not to a statistically significant degree. Thus, it is uncertain whether the aliphatic tail makes a direct contribution to the overall desensitising capability of salmeterol.

As salmeterol is a partial agonist in this system, the possibility exists that the effects attributed to desensitisation reflect salmeterol acting as an antagonist. Indeed, salmeterol has been shown to act as an antagonist to higher efficacy agonists in a number of tissue and cell systems (Dougall *et al*., 1991; Kallstrom *et al*., 1994) and similar effects were observed in the present system. In attempts to address whether salmeterol acts as an antagonist or induces desensitisation following long-term treatments with salmeterol, experiments were performed attempting to wash out salmeterol. A short incubation (20 min) of mast cells with salmeterol followed by thorough washing of the cells reversed the salmeterol inhibition of histamine release but not completely. It is difficult to assess, therefore, to what extent salmeterol persists after a longer (24 h) incubation followed by washing. Perhaps the best evidence to suggest that salmeterol is washed away effectively after longer (24 h) incubations comes from binding studies in which the K_D of the radioligand [^{125}I]CYP was no different in membranes generated from control or salmeterol-treated cells. It is probable, therefore, that the attenuation of the isoprenaline inhibition of histamine release following long-term incubation (24 h) of mast cells with salmeterol, followed by extensive washing, reflects desensitisation by salmeterol of β_2-adrenoceptor-mediated effects rather than antagonism.

Long-term treatment of mast cells with salmeterol or formoterol did not affect the responses of forskolin, a direct activator of adenylate cyclase, or PGE_2, a receptor-mediated activator of adenylate cyclase. The inhibitory effects of theophylline were attenuated to a modest degree by treatments with salmeterol or formoterol. Overall, these data suggest that the mechanism by which long-term treatments with salmeterol and formoterol attenuate the inhibition by β-adrenoceptor agonists in mast cells involves effects at the β_2-adrenoceptor itself. It is probable that the functional desensitisation observed in the present system involves receptor-related processes such as uncoupling, sequestration or downregulation of the β_2-adrenoceptor (Lefkowitz, 1998). However, it is interesting to note that, in our studies, the extent of functional desensitisation observed with formoterol and salmeterol does not correlate with the degree of β_2-adrenoceptor downregulation induced by these agonists, perhaps, indicating that processes leading to uncoupling and/or sequestration of the receptor are more important.

An interesting aspect of the present study is that an apparently weak partial agonist can cause extensive levels of functional desensitisation. At equivalent concentrations (1 μM), salmeterol, a weak inhibitor of histamine release and a poor activator of adenylate cyclase, caused greater levels of functional desensitisation than the full agonist isoprenaline (see Table 2). Although this comparison between isoprenaline and salmeterol, at equivalent concentrations, does not account for greater occupancy of β_2-adrenoceptors by salmeterol (K_D, 6.7 nM) than isoprenaline (K_D, 197 nM), even if this factor is taken into consideration (see Figure 9), salmeterol still appears to cause greater levels of functional desensitisation than isoprenaline. This finding would suggest that agonist efficacy is not necessarily the only factor that influences functional desensitisation.

How relevant these studies are in the clinical context is difficult to address. If, indeed, the system employed in the present study is representative of mast cell responses *in vivo*, then formoterol is likely to be a more effective stabiliser of mast cell degranulation than salmeterol. Another consideration is the suggestion that inhalation of bronchodilators can lead to very high (micromolar) topical concentrations of β_2-adrenoceptor agonists in the airways (Anderson *et al*., 1994) although other studies suggest that the concentrations attained are lower (Lipworth & Clark, 1997). Should concentrations of inhaled β_2-adrenoceptor agonists reach micromolar levels *in vivo* then, based on data from the present study, both salmeterol and formoterol are likely to induce extensive tolerance to the mast cell stabilising properties of β_2-adrenoceptor agonists. Another potentially important consideration is that should salmeterol persist in the airways then, as well as causing desensitisation, it may act as an antagonist to higher efficacy agonists, such as salbutamol, that are used on an as-required basis.

In summary, the present study has shown that long-term treatment with salmeterol or formoterol causes extensive levels of functional desensitisation to β_2-adrenoceptor-mediated responses in mast cells. These data may have wider clinical significance in the context of asthma treatment as compromised mast cell inhibition could result following long-term exposure of mast cells to long-acting bronchodilators.

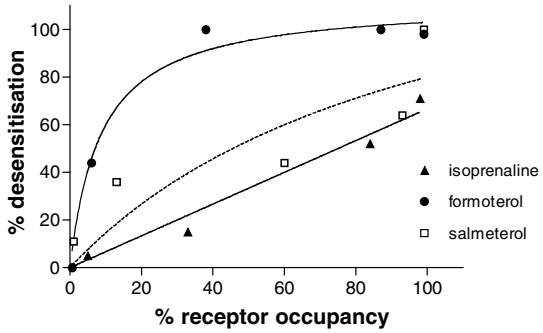

Figure 9 Relationship between receptor occupancy and functional desensitisation. The % desensitisation was calculated by the following formula: (1−(inhibition of histamine release by 1 μM isoprenaline following desensitising treatment/inhibition of histamine release by 1 μM isoprenaline)) × 100. Receptor occupancy was calculated using the following equation: [agonist]/([agonist] + K_D). The K_D values used were 197, 6.7 and 15.3 nM for isoprenaline, salmeterol and formoterol, respectively. These values were taken from the literature and are means of ten (isoprenaline), four (salmeterol) and three (formoterol) determinations for the human β_2-adrenoceptor. The curves for formoterol and salmeterol (hatched line) have been generated by reworking the data in Figure 6. The data for the isoprenaline relationship have been taken from separate unpublished experiments ($n = 10$).

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