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Influence of agonist intrinsic activity on the desensitisation of β_2-adrenoceptor-mediated responses in mast cells

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1 The aim of the present study was to determine whether the intrinsic activity of an agonist influences the extent of desensitisation of β_2-adrenoceptor-mediated responses in human lung mast cells.

2 The effects of a wide range of β-adrenoceptor agonists (10^{-10}–10^{-5} M) on the IgE-mediated release of histamine from mast cells were determined. The intrinsic activity of agonists was established by comparing the maximal inhibitory response (E_{max}) of an agonist relative to the maximal response obtained with the full agonist, isoprenaline. The intrinsic activity order for the inhibition of histamine release was isoprenaline (1.0) > formoterol (0.94) > fenoterol (0.89) > terbutaline (0.84) > salbutamol (0.69) > clenbuterol (0.65) > salmeterol (0.30) > dobutamine (0.20).

3 There was a significant ($P<0.05$) positive correlation ($r=0.81$) between the extent to which β-adrenoceptor agonists inhibited histamine release and the degree to which the agonists caused elevations in cAMP in mast cells.

4 Further studies investigated the effects of long-term (24 h) incubation of mast cells with β-adrenoceptor agonists on the subsequent ability of isoprenaline to inhibit histamine release. At concentrations of agonists selected to occupy a large percentage (88%) of β_2-adrenoceptors, there was a significant ($P<0.05$) correlation ($r=0.73$) between the relative intrinsic activity of agonists as inhibitors of histamine release and the extent of functional desensitisation induced by the agonists. At lower receptor occupancies, however, there was no correlation between the relative intrinsic activity of agonists and the extent of agonist-induced desensitisation.

5 These data indicate that, under experimental conditions where high receptor occupancies prevail, agonist intrinsic activity influences the extent of desensitisation of β_2-adrenoceptor-mediated responses in mast cells.

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

Introduction

Bronchodilator β_2-adrenoceptor agonists are important drugs used in the treatment of asthma (Waldeck, 2002). The primary action of these drugs is to relax airway smooth muscle although additional effects could include the stabilisation of inflammatory cell activity (Barnes, 1999). In this context, effects on lung mast cells could be important. β_2-Adrenoceptor agonists have been shown to inhibit the stimulated generation of a wide variety of mediators from lung mast cells, mediators that have been shown to provoke bronchoconstriction, and to promote inflammation (Butchers *et al*., 1980; 1991; Church & Hiroi, 1987; Lau *et al*., 1994; Nials *et al*., 1994).

One problem that may cause disadvantage to the continued use of β_2-adrenoceptor agonists is the development of tolerance (Svedmyr, 1990). To what extent tolerance to β_2-adrenoceptor agonists develops in the therapeutic context is hard to gauge. Nevertheless, in the experimental context, a large number of clinical studies has shown that tolerance to β_2-adrenoceptor agonists can be induced quite readily, suggesting that the process should be considered as a potential drawback to the use of bronchodilators (O'Connor *et al*., 1992; Cockcroft *et al*., 1993; Yates *et al*., 1997; Van der Woude *et al*., 2001). Furthermore, it has been demonstrated, both *in vivo* and *in vitro*, that tolerance to the mast cell-stabilising properties of β_2-adrenoceptor agonists develops more readily than tolerance to the smooth muscle relaxant effects of these drugs (Van der Heijden *et al*., 1984; O'Connor *et al*., 1992; Cockcroft *et al*., 1993; Chong & Peachell, 1999).

At the molecular level, tolerance may reflect receptor desensitisation. Receptor desensitisation is a complex multi-step process that can involve uncoupling, internalisation and degradation of receptors (Su *et al*., 1980; Lefkowitz, 1998; Ferguson, 2001). In the context of β_2-adrenoceptors, phosphorylations of the receptor catalysed by cAMP-dependent protein kinase (PKA) and G-protein-coupled receptor kinase (GRK) are thought to be important in mediating the components of desensitisation (Kohout & Lefkowitz, 2003).

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We have previously reported that long-term exposure of mast cells to β-adrenoceptor agonists leads to a reduction in the subsequent effectiveness of β-adrenoceptor agonists to inhibit mediator release from mast cells (Chong *et al*., 1995; 2000; Drury *et al*., 1998; Chong & Peachell, 1999). Our studies also suggest that the extent of functional desensitisation induced by different β-adrenoceptor agonists can be quite variable (Chong *et al*., 1997; 2003; Scola *et al*., 2004). Studies by others in transfected systems have shown a correlation between agonist efficacy and the extent of receptor desensitisation (January *et al*., 1997; 1998; Benovic *et al*., 1998; Clark *et al*., 1999). The aim of the present study was to determine whether this principle carries any currency in a primary cell system and, more specifically, to establish whether the intrinsic activity of β_2-adrenoceptor agonists influences the extent of functional desensitisation to β_2-adrenoceptor-mediated responses in human lung 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 and glucose 5.6. Tyrode's-BSA was Tyrode's buffer which additionally contained: $CaCl_2 \cdot 2H_2O$ 0.5 mM, $MgCl_2 \cdot 6H_2O$ 1 mM, BSA 1 mg ml^{-1} and 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 mM, $MgCl_2 \cdot 6H_2O$ 1 mM, glucose 5.6 mM and 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 and EDTA 2. The pH of Tris buffer was titrated to 7.4.

Preparation of compounds

Stock solutions (10 mM) of (−)-isoprenaline bitartrate were prepared in 0.05% sodium metabisulphite (dissolved in 0.9% NaCl). Salbutamol hemisulphate, terbutaline hemisulphate, dobutamine hydrochloride and clenbuterol hydrochloride were prepared as stock solutions (10 mM) in distilled water. Salmeterol base, formoterol fumarate and fenoterol hydrobromide 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 to 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 g^{-1} of tissue) containing collagenase Ia (350 U ml^{-1} of Tyrode's-BSA) and agitated by using a water-driven magnetic stirrer immersed in a water bath set at 37°C. The supernatant 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 ($120 \times g$, room temperature, 8 min), the supernatant discarded and the pellets reconstituted in Tyrode's-BSA (100 ml). The pellet was washed further two times. The dispersion procedure generated $0.2–1 \times 10^6$ mast cells g^{-1} 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 floatation 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 ($400 \times g$, 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 inhibitor 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 ml^{-1} 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. Moreover, long-term incubation (24 h) of mast cells with the vehicle, DMSO, which was used to prepare some of the drugs

(salmeterol, formoterol, fenoterol), had no effect on the isoprenaline inhibition of histamine release (Scola *et al*., 2004).

Assays for cAMP

Total cell cAMP levels were monitored according to methods that have been described 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 (51±4% 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 ($\geqslant 3\times10^6$ cells per condition) after treatment (24 h) with buffer or a given β-adrenoceptor agonist. After these incubations, the cells were washed three times in PBS buffer. Membranes were then prepared by homogenising in ice-cold Tris buffer using an Ultra Turrax homogeniser for 20 s, followed by four strokes ($\times4$) of a Teflon homogeniser. The homogenate was centrifuged ($500\times g$, 40 min) and 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, was 74±2% 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, fenoterol, terbutaline, dobutamine, clenbuterol, salbutamol (all Sigma, Poole, U.K.); [^{125}I] CYP (New England Nuclear, Stevenage, U.K.); cAMP EIA kits (Amersham, Little Chalfont, U.K.). Salmeterol (GSK, Stevenage, U.K.) and formoterol (Yamanouchi, Ibaraki, Japan) were both 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, repeated-measures ANOVA was performed.

Results

Effects of β*-adrenoceptor agonists on mast cell responses*

The effect of the β-adrenoceptor agonist isoprenaline (10^{-10}–10^{-5} M) on the IgE-mediated release of histamine from mast cells was investigated. Isoprenaline inhibited the release of histamine in a concentration-dependent fashion with maximal inhibition of ~50% (Figure 1a). The effects of seven additional β-adrenoceptor agonists on histamine release from mast cells were also studied (Figure 1b). Relative to the full agonist isoprenaline, these agonists showed variable potencies and intrinsic activities (Table 1). Only formoterol appeared clearly to be a full agonist. The relative intrinsic activity order for the inhibition of histamine release was isoprenaline ⩾ formoterol > fenoterol > terbutaline > salbutamol > clenbuterol > salmeterol > dobutamine.

As the human lung mast cell is known to express a homogeneous population of β_2-adrenoceptors (Chong *et al*., 2002) and as β_2-adrenoceptors are known to be coupled to adenylyl cyclase, the effects of β-adrenoceptor agonists (10^{-5} M) on mast cell cAMP levels were determined (Figure 2). All agonists tested, with the exception of salmeterol, induced significant ($P<0.05$) elevations in mast cell cAMP. There was a close correlation between the ability of an agonist to elevate cAMP and to inhibit histamine release (Figure 3)

Effects of β*-adrenoceptor agonists on desensitisation*

We have previously reported that long-term (24 h) incubation of mast cells with a β-adrenoceptor agonist leads to a reduction in the subsequent ability of a β-adrenoceptor agonist to inhibit mediator release from mast cells (Chong *et al*., 1995; 2000; Drury *et al*., 1998; Chong & Peachell, 1999). This is illustrated in Figure 4 where long-term (24 h) treatment with the full agonist isoprenaline (10^{-6} M), the partial agonist salbutamol (10^{-6} M) or the weak partial agonist salmeterol (10^{-6} M) leads to a reduction in the effectiveness of isoprenaline (10^{-10}–10^{-5} M) to inhibit IgE-mediated histamine release.

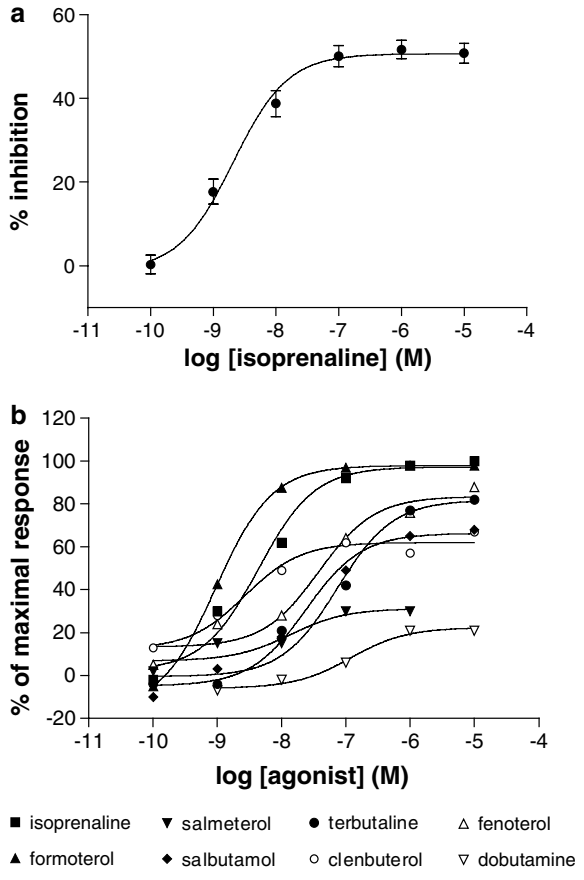

Figure 1 Effect of β-adrenoceptor agonists on histamine release from mast cells. Cells were incubated without or with a β-adrenoceptor agonist for 10 min before challenge with anti-IgE (1 : 300) for 25 min for histamine release. (a) The inhibitory effects of isoprenaline are expressed as the % inhibition of control histamine release, which was 30±3%, and values are means±s.e.m., $n=64$. (b) A wide range of β-adrenoceptor agonists was also studied. All agonists were not studied in the same experiments but in the following sets: isoprenaline, salmeterol, formoterol ($n=15$); isoprenaline, salbutamol, terbutaline ($n=23$); isoprenaline, clenbuterol ($n=18$) with dobutamine also studied in six of these experiments; isoprenaline, fenoterol ($n=8$). Values have been normalised relative to the E_{max} value for isoprenaline for each experiment. Values are means (error bars have been omitted for clarity).

Table 1 Potency (pD_2) and intrinsic activity values for the inhibition of histamine release for a series of β-adrenoceptor agonists

Agonist	n	*pD_2*	*Intrinsic activity*
Isoprenaline	64	8.5±0.1	1
Formoterol	15	9.0±0.2	0.94±0.06
Salmeterol	15	7.8[a]	0.3[a]
Salbutamol	23	7.6±0.2	0.69±0.06
Terbutaline	23	7.2±0.2	0.84±0.05
Clenbuterol	18	8.7±0.2	0.65±0.09
Dobutamine	6	6.9[a]	0.20[a]
Fenoterol	8	7.4±0.3	0.89±0.06

Values were determined from the concentration–response data in Figure 1 and further experimental details can be found in the legend to that figure. Intrinsic activities were determined by the following formula: E_{max} for the inhibition of histamine release by a β-adrenoceptor agonist $\div E_{max}$ for the inhibition of histamine release by isoprenaline. Values are means±s.e.m. except those indicated by [a], where the modest responses of mast cells to salmeterol and dobutamine, in some individual experiments, made it difficult to generate reliable parameter values. Hence, the mean concentration–responses for salmeterol and dobutamine were used to determine potency and intrinsic activity values.

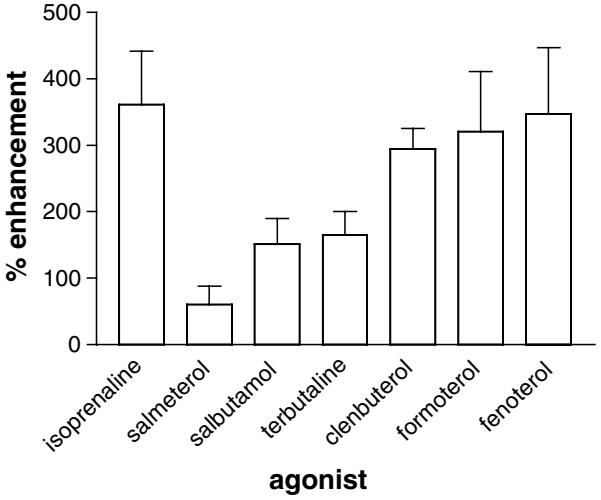

Figure 2 Elevations in cAMP induced by β-adrenoceptor agonists. Mast cells (91±6% purity) were incubated without or with an agonist (10^{-5} M) for 10 min and total cell cAMP levels were determined. Values are expressed as the percent enhancement in cAMP levels over basal levels. All agonists caused statistically significant ($P<0.05$ at least) increases in cAMP except salmeterol. Values are means±s.e.m. from four experiments.

Surprisingly, the weakest agonist, salmeterol, caused the most extensive levels of functional desensitisation and the full agonist, isoprenaline, the least.

In a further series of experiments, we investigated the effects of a wide range of β-adrenoceptor agonists on the functional desensitisation. Mast cells were incubated (24 h) with an agonist (10^{-6} M) and the subsequent ability of isoprenaline (10^{-6} M) to inhibit histamine release was determined. The extent of functional desensitisation induced by the agonists was highly variable (Figure 5). In these same experiments, the extent to which the concentration (10^{-6} M) of agonist used for desensitisation inhibited histamine release was also determined such that relative intrinsic activities could be established (Figure 5). There was no correlation between the relative intrinsic activity of an agonist and the extent of functional desensitisation induced by the agonist (Figure 5).

To determine whether the extent of functional desensitisation observed with β-adrenoceptor agonists reflects the degree of β_2-adrenoceptor downregulation, the effect of long-term treatment of mast cells with selected β-adrenoceptor agonists on receptor density was investigated (Figure 6). Mast cell-enriched preparations were incubated (24 h) with or without an agonist (10^{-6} M), after which the cells were washed extensively and then disrupted in order to prepare membranes. The density of β-adrenoceptors, following treatments, was determined using the radioligand, [^{125}I]CYP. The data demonstrate that only formoterol caused significant ($P<0.05$) levels of receptor downregulation (~50%), whereas isoprenaline, salbutamol, terbutaline and salmeterol all caused similar levels of downregulation (25–35%) that were not significant

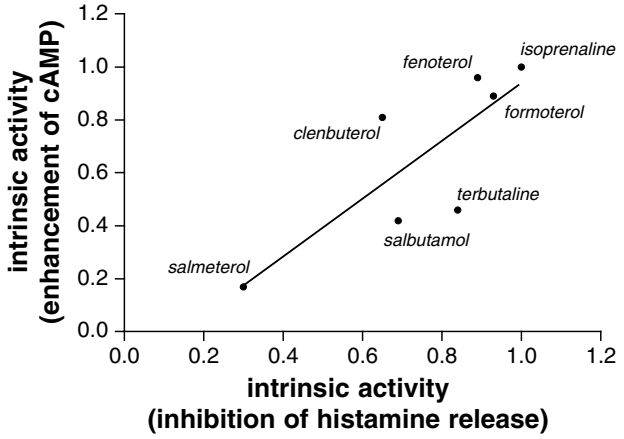

Figure 3 Correlation between elevations in cAMP and inhibitory effects of β-adrenoceptor agonists in mast cells. Intrinsic activities for the inhibition of histamine release can be found in Table 1 and the intrinsic activities for the elevations in cAMP have been calculated from the data shown in Figure 2. There was a statistically significant ($P<0.05$) correlation ($r=0.81$) between agonist-induced elevations in cAMP and inhibitory effects of agonists.

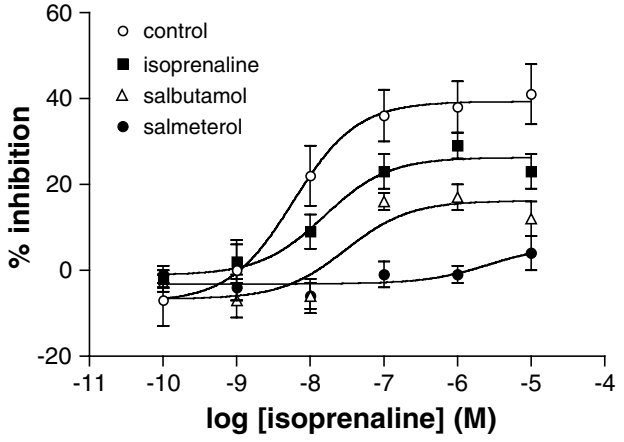

Figure 4 Desensitisation of β_2-adrenoceptor-mediated responses in mast cells. Cells were incubated (24 h) without (control) or with isoprenaline, salmeterol or salbutamol (all at 10^{-6} M) after which the cells were washed extensively. Cells were then incubated without or with increasing concentrations of isoprenaline for 10 min before challenge with anti-IgE (1 : 300) for 25 min for histamine release. Values are expressed as the % inhibition of unblocked histamine releases which ranged from 34 ± 2 to 38 ± 3% after 24 h treatments with buffer or agonists. Values are means ± s.e.m., $n=8$.

($P>0.05$). Therefore, the degree of receptor downregulation induced by these agonists does not parallel the extent of functional desensitisation.

Influence of receptor occupancy on desensitisation

Although our previous experiments (Figures 4 and 5) do not support the idea that the intrinsic activity of an agonist influences the extent of functional desensitisation, these experiments employed equivalent concentrations (10^{-6} M) of agonists to induce desensitisation. Since the affinities of these agonists for the β_2-adrenoceptor vary, agonist-dependent

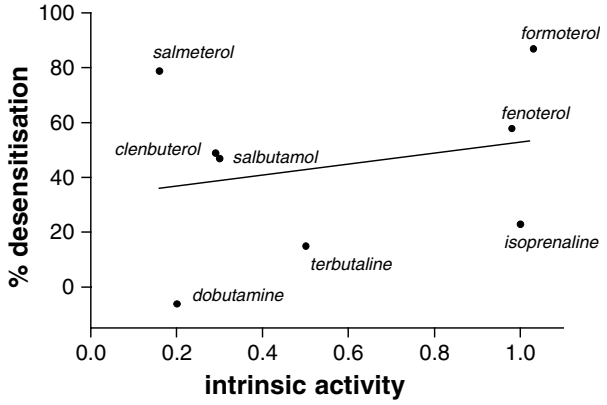

Figure 5 Correlation between intrinsic activity and desensitisation. Mast cells were incubated for 24 h with or without a β-adrenoceptor agonist (all at 10^{-6} M) and then washed extensively. Cells were then incubated without or with isoprenaline (10^{-6} M) for 10 min before challenge with anti-IgE (1 : 300) for 25 min. 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. In the same experiments, the extent to which β-adrenoceptor agonists (10^{-6} M) inhibited histamine release was also determined and intrinsic activities were determined by the following formula: [inhibition of histamine release by a β-adrenoceptor agonist at 1 μM ÷ inhibition of histamine release by 1 μM isoprenaline]. There was no correlation ($r=0.24$, $P>0.05$) between intrinsic activity and desensitisation. The correlation was generated from data from 11 experiments.

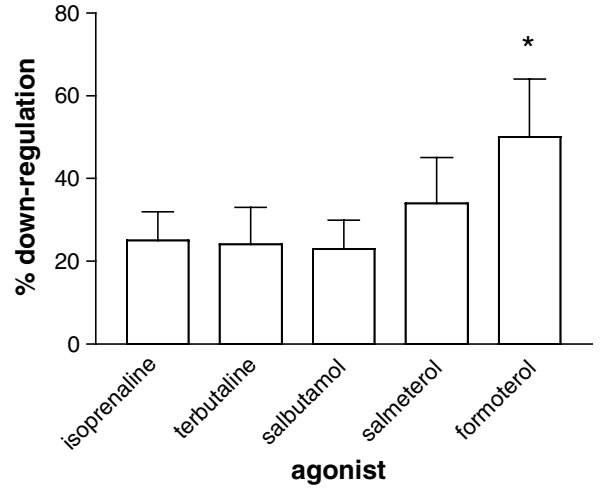

Figure 6 Effects of desensitising treatments on β_2-adrenoceptor density. Mast cell-enriched preparations (51 ± 4% purity) were incubated (24 h) without (control) or with a β-adrenoceptor agonist (10^{-6} M), the cells washed three times and then membranes prepared. Receptor density (B_{max}) was determined employing the radioligand [^{125}I]CYP. Values are expressed as the % downregulation of β_2-adrenoceptor density relative to the control B_{max} which was 20 ± 5 fmol mg^{-1} protein. The affinity (K_D) of [^{125}I]CYP was 0.08 ± 0.02 nM and long-term drug treatments with agonists did not alter this value to a statistically significant degree ($P>0.05$). Treatment with formoterol caused a significant ($P<0.05$) reduction in receptor density, whereas the effects of other β-adrenoceptor agonists were not statistically significant ($P>0.05$). Values are means ± s.e.m. from nine (isoprenaline), five (salbutamol, terbutaline) and four experiments (salmeterol, formoterol). Asterisk denotes a significant ($P<0.05$) effect.

Table 2 Affinities of β-adrenoceptor agonists for the human β_2-adrenoceptor and concentrations of agonists required for 88% occupancy

Agonist	K_D (μM)	*[Agonist] required for 88% occupancy* (μM)
Isoprenaline	0.197	1.5
Salbutamol	1.510	10
Salmeterol	0.007	0.05
Formoterol	0.015	0.10
Fenoterol	0.136	1.0
Dobutamine	1.462	10
Clenbuterol	0.028	0.20
Terbutaline	4.012	30

The K_D values cited for the β-adrenoceptor agonists were obtained from the literature (e.g., January *et al*., 1997; Kikkawa *et al*., 1997; Green *et al*., 2001) and are mean values of between three to 11 determinations for each agonist, except for clenbuterol where only one value was found. These K_D values were then used to calculate the concentrations of agonists required to occupy 88% of the receptors by using a modification ($[agonist]^{88\%} = 7.33 \times K_D$) of the equation, occupancy = [agonist] ÷ ([agonist] + K_D).

Table 3 Desensitisation induced by concentrations of agonists calculated to occupy 88% of β_2-adrenoceptors

Agonist	*Intrinsic activity*	*% Desensitisation of isoprenaline*	*% Desensitisation of formoterol*
Isoprenaline	1	54±12	62±10
Salbutamol	0.60±0.07	68±10	95±10
Fenoterol	1.00±0.05	94±8	124±13
Clenbuterol	0.42±0.15	47±8	33±13
Dobutamine	0.07±0.06	28±9	−9±15
Salmeterol	0.30±0.10	66±9	47±9
Formoterol	1.05±0.1	95±7	98±11
Terbutaline	0.86±0.12	63±9	88±17

Mast cells were incubated for 24 h without or with a β-adrenoceptor agonist at concentrations calculated to occupy 88% of the receptors (see Table 2). The cells were then washed extensively and incubated with either isoprenaline (10^{-6} M) or formoterol (10^{-6} M) for 10 min before challenge with anti-IgE for 25 min for histamine release. The % desensitisation was calculated by the following formula: [1−(inhibition of histamine release by 1 μM isoprenaline or formoterol following desensitising treatment ÷ inhibition of histamine release by 1 μM isoprenaline or formoterol)] × 100. In the absence of any desensitising treatment, isoprenaline (10^{-6} M) and formoterol (10^{-6} M) inhibited histamine release by 44±7 and 36±7%, respectively. In the same experiments, the extent to which β-adrenoceptor agonists (at concentrations calculated to occupy 88% of the receptors) inhibited histamine release was also determined and intrinsic activities were determined by the following formula: [inhibition of histamine release by a β-adrenoceptor agonist ÷ inhibition of histamine release by isoprenaline]. Values are means±s.e.m., $n = 7$.

differences in receptor occupancy would be expected at an equivalent concentration of agonists. In order to determine whether receptor occupancy influences the functional desensitisation, K_D values were obtained from the literature and concentrations of agonists that would occupy 88% of the available receptors determined (Table 2). Mast cells were then incubated overnight (24 h) with agonists, at concentrations calculated to occupy 88% of the receptors, and the subsequent ability of both isoprenaline (10^{-6} M) and formoterol (10^{-6} M) to inhibit IgE-mediated histamine release determined. In order to establish relative intrinsic activities, the extent to which these agonists (at concentrations calculated to occupy 88% of the receptors) inhibited histamine release was also determined in these same experiments (Table 3). The data indicate that significant ($P<0.05$) correlations exist between the relative intrinsic activity of agonists and the extent of functional desensitisation induced when desensitising concentrations of agonists are tailored to occupy equivalent and a large proportion (88%) of the receptors (Figure 7).

In further studies, we sought to establish the effect of varying receptor occupancies on the functional desensitisation. Mast cells were incubated overnight (24 h) with increasing concentrations (at least four doses within the concentration range, 10^{-10}–10^{-5} M) of a given β-adrenoceptor agonist and then the subsequent effectiveness of isoprenaline (10^{-10}–10^{-5} M) to inhibit IgE-mediated histamine release determined. The situation for the agonist salbutamol, as a representative case, is shown in Figure 8a, demonstrating a progressive reduction in the inhibitory effects of isoprenaline with increasing desensitising concentration of salbutamol. Knowing the affinity (Table 2) of salbutamol at the β_2-adrenoceptor, these data were reworked to evaluate the influence of receptor occupancy on the extent of functional desensitisation (Figure 8b). The data show that the relationship between receptor occupancy and functional desensitisation is hyperbolic such that quite a pronounced degree of functional desensitisation (36%) can be obtained at low (6%) receptor occupancy.

The relationship between receptor occupancy and desensitisation was evaluated for additional β-adrenoceptor agonists and, for the majority of agonists, hyperbolic relationships hold (Figure 9). Interestingly, for the full agonist isoprenaline (and for clenbuterol to some extent), the relationship between receptor occupancy and desensitisation was linear rather than hyperbolic.

Discussion

Receptor desensitisation is an agonist-driven and time-dependent process that can involve uncoupling, sequestration and downregulation of receptors (Lefkowitz, 1998; Ferguson, 2001). The β_2-adrenoceptor has been the best-studied receptor with respect to unravelling mechanisms that regulate desensitisation. In this regard, phosphorylations of the receptor induced by PKA and GRK appear to be critical (Kohout & Lefkowitz, 2003; Tran *et al*., 2004). Consensus opinion suggests that lower concentrations of agonist initiate PKA-driven phosphorylations of the β_2-adrenoceptor and, because of the promiscuity of PKA for alternative G-protein-coupled receptors, 'heterologous' desensitisation results. At higher concentrations of agonist, GRK-driven phosphorylations of the β_2-adrenoceptor occur and, as the receptor needs to be in an agonist-occupied state for the receptor to serve as a substrate for GRK, this results in 'homologous' desensitisation.

A number of studies has investigated the influence of agonist efficacy on desensitisation primarily in reconstituted or transfected cell systems (Pittman *et al*., 1984; January *et al*., 1997; 1998; Benovic *et al*., 1998; Borgland *et al*., 2003). In this context, components of desensitisation, whether these be kinase-driven phosphorylations, uncoupling, sequestration or

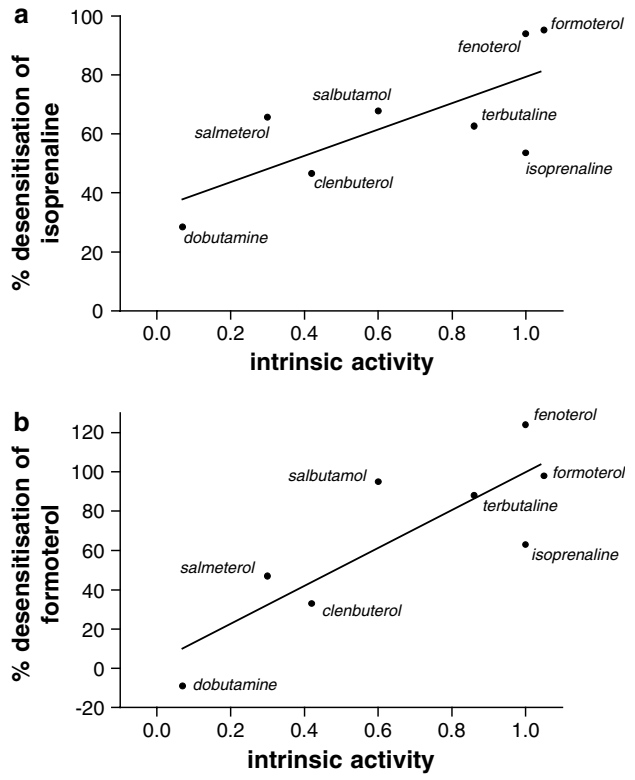

Figure 7 Correlation between intrinsic activity and desensitisation at high (88% for all agonists) receptor occupancy. Specific experimental details and data can be found in Table 3. There were significant positive correlations between the intrinsic activity of agonists and desensitisation induced by agonists of the (a) isoprenaline ($r=0.73$; $P<0.05$) and (b) formoterol ($r=0.84$; $P<0.01$) inhibitory responses.

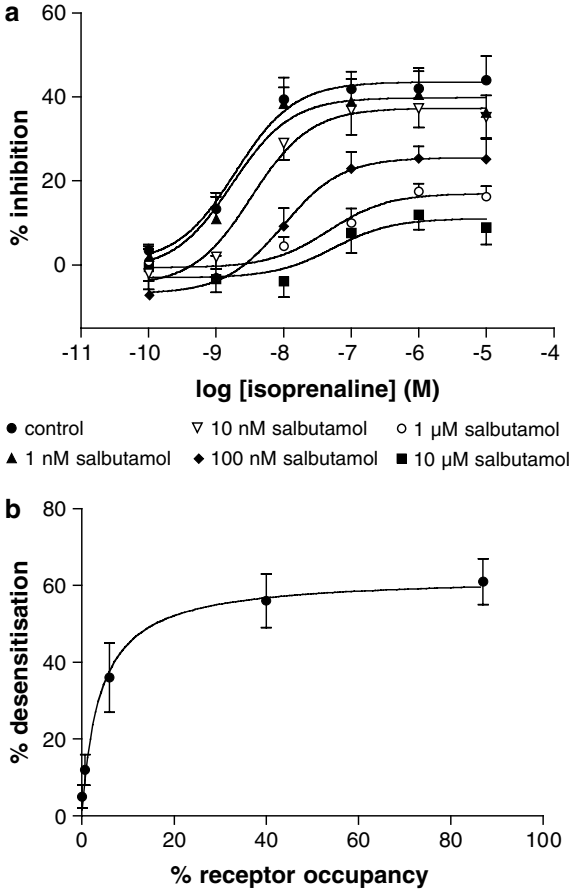

Figure 8 Concentration dependence of the functional desensitisation. (a) Mast cells were incubated without or with increasing concentrations of salbutamol (10^{-9}–10^{-5} M) for 24 h and then washed. The cells were then incubated with isoprenaline (10^{-10}–10^{-5} M) for 10 min before challenge with anti-IgE (1 : 300) for histamine release. Values are expressed as the percent inhibition of the control histamine releases which ranged from 35 ± 3 to 39 ± 3%. Incubations of mast cells with salbutamol ($\geqslant10^{-7}$ M) caused significant ($P<0.05$ at least) reductions in the potency (pD_2) and maximal inhibitory effects of isoprenaline. (b) The data were reworked by considering the inhibitory effects of isoprenaline at 1 μM without or with salbutamol-desensitising treatments. The % desensitisation was calculated by the following formula: [1-(inhibition of histamine release by 1 μM isoprenaline following desensitising treatment $\div$ inhibition of histamine release by 1 μM isoprenaline)] $\times$ 100. Receptor occupancy of salbutamol was calculated using the following equation: [agonist] $\div$ ([agonist] + K_D). Values are means $\pm$ s.e.m. from six experiments.

downregulation of receptors, have been studied. Overall, these studies demonstrate an influence of agonist efficacy on selected components of the desensitisation process (Clark *et al*., 1999). The aim of the present study was to determine whether agonist intrinsic activity influences desensitisation in a primary human cell system.

In initial experiments, we sought to investigate the effects of a range of agonists on IgE-mediated histamine release from mast cells and rank these compounds according to an intrinsic activity order. The drugs used were primarily selected on the basis of previous experience, clinical relevance and suitability based on the studies of others. The activities of different agonists were found to vary substantially and the rank order of intrinsic activity for inhibition of histamine release was isoprenaline $\geqslant$ formoterol $\geqslant$ fenoterol > terbutaline > salbutamol > clenbuterol > salmeterol > dobutamine. Furthermore, there was a good correlation between the extent to which agonists inhibited histamine release and the degree to which agonists elevated mast cell cAMP. This finding suggests that the mechanism by which β-adrenoceptor agonists inhibit mast cell function is driven by cAMP.

Once the relative intrinsic activities of these agonists were established, our aim was then to evaluate the effects of these agonists on desensitisation. Initially, the effects of long-term (24 h) incubation of mast cells with equivalent concentrations (10^{-6} M) of isoprenaline, salbutamol or salmeterol on the subsequent inhibitory effect of isoprenaline were investigated. An expectation might have been that salmeterol, with the lowest intrinsic activity, would induce the least amount of functional desensitisation and that isoprenaline, as a full agonist, the most. However, the weak partial agonist, salmeterol, induced greater levels of functional desensitisation than the full agonist, isoprenaline. Further studies, employing a wider range of β-adrenoceptor agonists used at equivalent (10^{-6} M) concentrations to induce desensitisation, indicated that there was no correlation between the intrinsic activity of the agonists and the extent of functional desensitisation induced.

Although these studies investigating the effects of a range of β-adrenoceptor agonists, at equivalent desensitising concen-

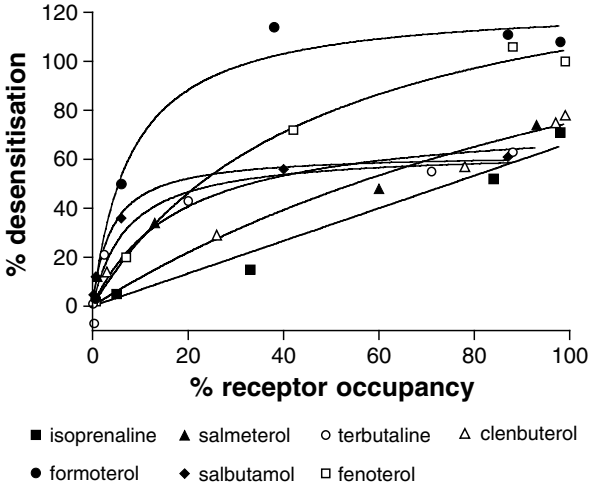

Figure 9 Relationship between receptor occupancy and functional desensitisation. The % desensitisation was calculated by the following formula: [1−(inhibition of histamine release by 1 μM isoprenaline following desensitising treatment ÷ inhibition of histamine release by 1 μM isoprenaline)] × 100. Receptor occupancy was calculated using the following equation: [agonist] ÷ ([agonist] + K_D). K_D values can be found in Table 2. Values are means (error bars omitted for clarity) from 10 (isoprenaline, salmeterol, formoterol), eight (clenbuterol, fenoterol) and six (salbutamol, terbutaline) experiments.

trations, argue against an influence of agonist intrinsic activity on the extent of functional desensitisation, these experiments do not account for differences in the extent of receptor occupancy of the agonists. As the affinities (see Table 2 for agonist K_D values) of the agonists for the β_2-adrenoceptor differ, a wide difference in receptor occupancies would be expected at equivalent concentrations. To address this disparity, desensitising concentrations of the agonists were employed, calculated to occupy an equivalent and high proportion (88%) of the receptors. Under these constrained conditions, there was a good correlation between agonist intrinsic activity and the extent of functional desensitisation induced. It has been suggested that, at high receptor occupancies, agonist-induced changes in the conformational state of the receptor, the extent of which can be influenced by agonist efficacy, drive GRK activity (Williams *et al*., 2000; Kohout & Lefkowitz, 2003). Whether a similar process might hold for the human mast cell is not known.

In further studies, we evaluated the extent of functional desensitisation induced by lower concentrations of agonists corresponding to lower receptor occupancies. The data show a hyperbolic relationship between desensitisation and receptor occupancy such that even weaker partial agonists, such as salmeterol and salbutamol, cause quite prominent levels of functional desensitisation at low receptor occupancies. The reason for this is not immediately apparent although it has been suggested that the reason why partial agonists might mediate larger than expected responses is due to intracellular amplification of the β_2-adrenoceptor-driven cAMP signal (Scott *et al*., 1999). This concept of amplification of the cAMP signalling pathway might also hold in the context of phosphorylations of the β_2-adrenoceptor by PKA and, consequently, receptor desensitisation (Williams *et al*., 2000).

Although amplification of the cAMP-PKA pathway might explain the higher than expected desensitising capability of partial agonists, a few concerns do exist with such an explanation. In a proposed scheme wherein PKA mediates phosphorylation of the β_2-adrenoceptor, PKA might be expected to mediate 'heterologous' desensitisation by phosphorylating other GPCRs. However, our previous studies show that, whereas long-term incubations with a variety of β-adrenoceptor agonists can totally ablate the subsequent response of a β-adrenoceptor agonist, these treatments have hardly any effect on the actions of an alternative receptor-mediated activator of adenylyl cyclase, prostaglandin E_2, indicating that the desensitisation is 'homologous' (Chong *et al*., 2003; Scola *et al*., 2004). Moreover, it is interesting to note that certain other agonists, most notably the full agonist isoprenaline and the partial agonist clenbuterol, display a linear and not a hyperbolic relationship between receptor occupancy and desensitisation. These findings are difficult to reconcile given the fact that isoprenaline, as a full agonist, very ably induces increases in mast cell cAMP levels and so would be expected to activate PKA very effectively. Another important consideration is whether, indeed, PKA-driven desensitisation is an important process after long-term (24 h) incubations. Moreover, the possibility that different agonists might induce desensitisation by different mechanisms cannot be excluded.

An interesting aspect of the present study is the finding that partial and full agonists induce similar levels of β_2-adrenoceptor downregulation in mast cells after a 24 h incubation. Similar findings have been reported for transfected cell systems, with the striking difference that in the present primary cell system the levels of downregulation were modest yet in transfected cells the downregulation was extensive (Williams *et al*., 2000). Furthermore, the levels of receptor downregulation obtained in the present study do not equate with the extent of functional desensitisation observed. Thus, long-term treatment of mast cells with salmeterol, salbutamol or isoprenaline caused about 90, 60 and 30% reductions in the subsequent functional response of mast cells to isoprenaline yet the same treatments of mast cells with all three agonists caused about the same (~25%) reductions in β_2-adrenoceptor density that were not statistically significant ($P > 0.05$). These findings imply that a component of receptor desensitisation other than downregulation, perhaps uncoupling or sequestration, is responsible for the functional desensitisation of β_2-adrenoceptor-mediated responses in mast cells.

From the clinical perspective, the present study may have some relevance. It is possible that mast cells, especially those that reside close to epithelial surfaces, are exposed to high concentrations of inhaled β-adrenoceptor agonists such that high β_2-adrenoceptor occupancies may prevail and quite extensive levels of functional desensitisation may result. Clearly, a subsequent inability of β-adrenoceptor agonists to stabilise mast cell responses effectively would compromise the therapeutic effectiveness of bronchodilators. Perhaps an even more important aspect of the present study is the finding that exposure of mast cells to relatively ineffective inhibitory concentrations of partial agonists, equating with quite low receptor occupancies, can nevertheless substantially impair the subsequent ability of β-adrenoceptor agonists to stabilise mast cells. This could be quite important clinically, for example, in situations where salmeterol is being administered regularly to

control symptoms, but where a fast-acting β_2-adrenoceptor agonist, such as salbutamol, might be required for immediate relief. An inability of salbutamol to stabilise mast cell responses effectively, in this type of situation, would result in impaired therapeutic effectiveness.

In summary, the present study has shown that the intrinsic activity of agonists influences the extent of desensitisation of β_2-adrenoceptor-mediated responses in mast cells under conditions where high receptor occupancies prevail. However, at lower receptor occupancies, this relationship breaks down as, generally, partial agonists induce greater levels of desensitisation than might be anticipated. Additionally, complete ablation of the functional response can occur without significant reductions in β_2-adrenoceptor density. These findings may have some clinical bearing, highlighting the fact that exposure of mast cells to relatively ineffective inhibitory concentrations of β-adrenoceptor agonists can, nevertheless, substantially impair the subsequent ability of agonists to stabilise mast cells.

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