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RESEARCH PAPER

An estimation of β_2-adrenoceptor reserve on human bronchial smooth muscle for some sympathomimetic bronchodilators

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Background and purpose: The β_2-Adrenoceptor on human pro-inflammatory cells is exquisitely sensitive to desensitization, whereas β_2-adrenoceptor-mediated relaxation of human airways smooth muscle (HASM) is relatively resistant to this phenomenon. An explanation for this discrepancy is that a large β_2-adrenoceptor 'reserve' exists on HASM cells for sympathomimetic bronchodilators, which protects against desensitization.
Experimental approach: The operational model of agonism was used to estimate the affinity of salbutamol, terbutaline, formoterol and procaterol for the β_2-adrenoceptors in methacholine (MCh)-contracted HASM from which the relationship between fractional receptor occupancy and relaxation was determined. This analysis was performed under conditions of fractional, irreversible, β_2-adrenoceptor inactivation and, for salbutamol and terbutaline only, by the comparative method of Barlow *et al.* The affinity of salbutamol for the β_2-adrenoceptor guinea-pig eosinophils and the receptor/occupancy-response relationship for the suppression of the respiratory burst (an index of pro-inflammatory cell function) was also determined.
Key results: For salbutamol and terbutaline, both pharmacological approaches yielded in HASM discrepant affinity estimates (values differed, maximally, by 0.67 $\log_{10}$ unit). However, affinity values more closely agreed (difference <0.47 $\log_{10}$ unit), when operational analysis was performed on data corrected for 'fade' of the MCh-induced contraction. Plots of fractional β_2-adrenoceptor occupancy versus relaxation indicated a receptor 'reserve' for all agonists tested at all levels of response. In contrast, minimal receptor reserve was detected for the ability of salbutamol to suppress respiratory burst activity in eosinophils.
Conclusions and implications: These data may help explain the relative inability of sympathomimetic bronchodilators to render HASM tolerant to β_2-adrenoceptor-mediated relaxation.
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Keywords: β_2-adrenoceptor reserve; asthma; agonist affinity estimation; airways smooth muscle; bronchodilatation; comparative method; fractional receptor inactivation; DCITC; operational curve fitting

Abbreviations: CGP 20712A, 2-hydroxy-5-[2-[[2-hydroxy-3-[4-[1-methyl-4-(trifluoromethyl)imidazol-2-yl]phenoxy]propyl]amino]ethoxy]benzamide; DCITC, 5(2-(((1'-(4'-isothiocyanatophenylamino)thiocarbonyl)-amino)-2-methylpropyl)amino-2-hydroxypropoxy)-3,4-dihydrocarbostyril; HASM, human airways smooth muscle; HBSS, Hanks' balanced salt solution; ICI 198,615, (1-((2-methoxy-4-(((phenylsulphonyl)amino)carbonyl)phenyl)methyl)-1H-indazol-6-yl)carbamic acid cyclopentyl ester; KHS, Krebs-Henseleit solution; LTB_4, leukotriene B_4; MCh, methacholine

Introduction

Short-acting β_2-adrenoceptor agonists are the most effective bronchodilators currently available and can reduce human airways smooth muscle (HASM) tone irrespective of the causative mediator(s). Indeed, their propensity to evoke rapid

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bronchodilatation accounts for their widespread use in asthma symptoms management. However, a degree of tolerance to this beneficial response may occur with repeated dosing (see Salpeter *et al.*, 2004 for meta-analysis). In addition, the *protective* effects of β_2-adrenoceptor agonists against bronchoconstrictor stimuli may also be compromised (Salpeter *et al.*, 2004), which has been shown to render the airways twice as sensitive to allergen and exercise and to increase the late phase asthmatic response and associated inflammation (Cockcroft *et al.*, 1995; Manolitsas *et al.*, 1995; Gauvreau *et al.*, 1997; Aldridge *et al.*, 2000). In spite of this apparent limitation, regular use of β_2-adrenoceptor agonists rarely produces a clinically problematic impairment of bronchodilatation except in the most severely afflicted and poorly controlled asthmatic subjects in whom these drugs are used on a regular basis as a rescue medication (Barnes, 2007).

The *relative* resistance of HASM to desensitization is unexplained especially given the rapid, and sometimes complete, tolerance that develops to many β_2-adrenoceptor-mediated responses in pro-inflammatory and immune cells (Barnes, 1993). One hypothesis that is often used to explain (at least in part) this discrepancy is that a large β_2-adrenoceptor 'reserve' exists on the smooth muscle cells for clinically used, short-acting, β_2-adrenoceptor agonists that protects against this phenomenon (Lemoine and Kaumann, 1982; Lemoine and Overlack, 1992; Barnes, 1995; Hanania *et al.*, 2002). Despite the popularity of this idea, supporting empirical data are sparse (Chong and Peachell, 1999) and, in fact, evidence contrary to this hypothesis has been published (McGraw *et al.*, 1999).

The term 'receptor reserve' when used without clarification is vague and ambiguous. The proportion of agonist-occupied receptors ([AR]) required to produce a given degree of response is dependent upon the efficacy of the agonist, receptor density and efficiency of receptor–effector coupling. In the same tissue, receptor density and efficiency of receptor–effector coupling are invariant parameters. Accordingly, to achieve an equivalent functional response, the fraction of unoccupied receptors will vary only in an agonist-dependent manner. Thus, theoretically, two agonists that interact with the same receptor to produce the same level of response may occupy a markedly different fraction of the total functional receptor population (Gunst *et al.*, 1987; 1989; Kenakin, 1987). Moreover, a large receptor reserve may exist for an agonist that evokes a response that is 50% of the maximum but not for a response that is equal to 90% of the maximum (Gunst *et al.*, 1987; 1989; Kenakin, 1987). Therefore, in a given tissue the number of receptors that needs to be occupied to produce a given response will depend on the agonist in question *and* the level of response at which the measurement is made.

In 1966 a pharmacological method was described using partial irreversible receptor inactivation (alkylation) to estimate the efficacy, affinity and receptor reserve of an agonist (Furchgott, 1966). Irreversible antagonists are composed of a pharmacophore, which imparts affinity and selectivity for a receptor, and a reactive moiety that leads to the formation of a covalent bond(s) with the receptor, thereby preventing dissociation of the ligand (Baker and Deyrup, 1994). In the studies described herein, this method has been employed to determine the relationship between fractional receptor occupancy and relaxation of HASM for several (salbutamol, terbutaline, procaterol, formoterol) β_2-adrenoceptor agonists used clinically as bronchodilators. Specifically, the aim of the study was to establish if a large receptor density could provide a plausible explanation for the relative resistance of HASM cells to functional desensitization. To this end, a carbostyril-based irreversible β-adrenoceptor antagonist, DCITC [5(2-(((1′-(4′-isothiocyanatophenylamino)thiocarbonyl)-amino)-2-methyl-propyl) amino - 2 - hydroxypropoxy) - 3,4- dihydrocarbostyril; Figure 1; Deyrup *et al.*, 1998], was employed to inactivate a fraction of functional β_2-adrenoceptors on HASM to a level where the upper asymptote of agonist concentration–response (E/[A]) curves was significantly depressed. DCITC was selected for this purpose as it is selective for the β-adrenoceptor subtype and without non-specific actions on muscle tone (Deyrup *et al.*, 1998). Given the *theoretical* limitations of this pharmacological method (see *Discussion* and Leff *et al.*, 1990a; Colquhoun, 1998; Strange, 2008), agonist affinity was also determined by using an alternative approach. Thus, agonist E/[A] curves were constructed under conditions of high induced tone thereby rendering them partial agonists relative to a reference full agonist (Barlow *et al.*, 1967). This, so-called, comparative method was also employed by using eosinophils, which express fewer β_2-adreneceptors than HASM (Yukawa *et al.*, 1990; Johnson, 1998) and, thus, could mediate responses that are more prone to desensitization. It is noteworthy that the comparative method is less subject to the error that is, theoretically, introduced by receptor alkylation and is believed to yield more reliable estimates of agonist affinity (Leff and Harper, 1989; Leff *et al.*, 1990b). In addition, although the method can only be used for partial agonists (Barlow *et al.*, 1967), it provides an internal check to gauge the error in K_A that the method of partial irreversible receptor inactivation is believed to produce (Leff *et al.*, 1990b).

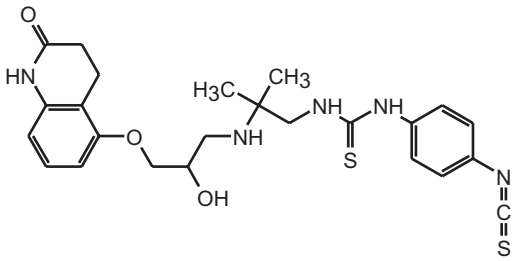

Figure 1 Structure of 5(2-(((1′-(4′-isothiocyanatophenylamino) thiocarbonyl)-amino)-2-methyl-propyl)amino-2-hydroxypropoxy)-3, 4-dihydrocarbostyril (DCITC) (Deyrup *et al.*, 1998).

The results of the present study are consistent with the hypothesis that a large β_2-adrenoceptor density exists on HASM that protects against desensitization and, therefore, a clinically relevant loss of responsiveness to sympathomimetic bronchodilators.

Methods

Acquisition and preparation of HASM for isometric tension recording

Ethical approval for the use of human tissues was granted by the Co-joint Health Research Ethics Board of the University of

Calgary. All experiments were performed by using HASM strips derived from the major bronchi of 16 donors (5 female, 11 male; age range: 15–62 years; median age 34 years). The lungs with major airways intact were obtained from the International Institute for the Advancement of Medicine (Edison, NJ, USA).

Intralobar bronchi were placed in oxygenated Krebs-Henseleit solution (KHS) of the following composition (in mM: NaCl 118, KCl 4.7, $MgSO_4.7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.7, $CaCl_2$ 1.5) supplemented with diclofenac (2 μM), mepyramine (1 μM) and ICI 198,615 [1-((2-methoxy-4-(((phenylsulphonyl)amino)carbonyl)phenyl)methyl)-1H-indazol-6-yl)carbamic acid cyclopentyl ester] (1 μM) to prevent, respectively, effects on smooth muscle tone due to the release of prostanoids, histamine and cysteinyl leukotrienes (Ellis and Undem, 1994). Phentolamine (1 μM) and CGP 20712A (2-hydroxy-5-[2-[[2-hydroxy-3-[4-[1-methyl-4-(trifluoromethyl)imidazol-2-yl]phenoxy]propyl]amino]ethoxy]benzamide) (500 nM) were also added to the KHS to block α- and β_1-adrenoceptor activation respectively. Each bronchus, which typically provided at least eight preparations, was cut longitudinally and stripped free of extrinsic connective tissue. Transverse muscle strips (~2 × 2 × 8 mm) were prepared and mounted vertically, under a final tension of 20 mN, in 5 mL tissue baths containing oxygenated KHS (with the inhibitor/receptor antagonists above-mentioned) at 37°C and were allowed to equilibrate, with frequent washing, for at least 120 min before beginning the experiment. Changes in tension were measured isometrically by using Powerlab MTL0201 force-displacement transducers (AD Instruments, Colorado Springs, CO, USA), recorded on a PowerLab/800 recording unit and analysed using Chart (version 5.1) data acquisition software (ADI instruments).

Preparation of guinea-pig eosinophils

All animal care and procedures complied with the guidelines of the Canadian Council on Animal Care and were approved by the University of Calgary Animal Care Committee. Eosinophils were elicited into the peritoneum of male Dunkin-Hartley guinea-pigs and purified as described previously (Lindsay *et al.*, 1998a,b) Briefly, an eosinophil/macrophage-rich peritoneal exudate was produced by injecting guinea-pigs weekly with human serum (1 mL per animal) for 2–4 weeks. Three to 6 days after the last injection, guinea-pigs were anaesthetized and the peritoneal cavity of each animal lavaged with 50 mL sterile glucose. The lavage fluid was washed in Hanks' balanced salt solution (HBSS), pooled and the eosinophils were separated by centrifugation through discontinuous Percoll density gradients (1.080, 1.085, 1.090 and 1.100 $g{\cdot}mL^{-1}$). Using this procedure, eosinophils were recovered from the 1.085/1.090 $g{\cdot}mL^{-1}$ and 1.090/1.100 $g{\cdot}mL^{-1}$ Percoll interfaces and were >97% pure and >95% viable as assessed by Trypan blue exclusion. Cells were pooled, washed twice in HBSS and resuspended in HEPES (10 mM, pH 7.4) containing 0.1% ($v{\cdot}v^{-1}$) bovine serum albumin in HBSS.

Measurement of respiratory burst

The ability of eosinophils to generate hydrogen peroxide (H_2O_2) was used as an index of respiratory burst activity and was assessed by measuring the horseradish peroxidase-catalysed oxidation of scopoletin (Lindsay *et al.*, 1998a,b). Eosinophils were resuspended at a concentration of 10^8 $cells{\cdot}mL^{-1}$ in buffer A [in mM: HEPES 10 – pH 7.4, NaCl 138, KCl 6, NaH_2PO_4 1, $NaHCO_3$ 5, glucose 5.5 and bovine serum albumin 0.1% ($v{\cdot}v^{-1}$)], and 10 μL was added to 990 μL of the same buffer supplemented with $CaCl_2$ (1 mM), $MgCl_2$ (1 mM), superoxide dismutase (30 U), horseradish peroxidase (1 U) and scopoletin (4 μM) in polystyrene cuvettes. The cell suspension was incubated with vehicle or the β_2-adrenoceptor agonist of interest for 5 min at 37°C and then challenged with leukotriene B_4 (LTB_4; 100 nM, ~$p[A]_{70}$). Generation of H_2O_2 was measured fluorimetrically ($\lambda_{excitation}$ = 350 nm; $\lambda_{emission}$ = 460 nm; slit width = 5 nm) by using a thermostatically controlled recording spectrophotofluorimeter fitted with a stirring mechanism. Changes in fluorescence were monitored continuously for 20 min, and negative first derivative plots of the reduction in fluorescence were constructed to obtain the peak rates of scopoletin extinction. These values were converted to rates of H_2O_2 generation and quantified by interpolation from a standard curve constructed to known concentrations of H_2O_2.

Determination of agonist potency

Monophasic *E*/[A] curves were constructed to the β_2-adrenoceptor agonist of interest and the experimental data fitted, subsequently, to the following form of the Hill equation using Prism software (GraphPad Inc, San Diego, CA, USA) (Motulsky and Christopoulos, 2005):

$$E = \frac{E_{max}}{1 + 10^{(p[A]_{50} - p[A])^n}} \quad (1)$$

where E is the effect, E_{max} is the upper asymptote, which represents the maximum β_2-adrenoceptor agonist-induced response, p[A] is the –log molar concentration of agonist, $p[A]_{50}$ is a location parameter equal to the –log molar concentration of agonist producing $E_{max}/2$, and n is the gradient of the *E*/[A] curve at the $p[A]_{50}$ level.

Estimation of agonist equilibrium dissociation constants by fractional receptor inactivation

The equilibrium dissociation constant (K_A) of salbutamol, terbutaline, procaterol and formoterol in HASM was estimated by operational model fitting (Black and Leff, 1983; Black *et al.*, 1985) using one or two experimental approaches. The first method involved 'irreversibly' inactivating a fraction of the total functional β_2-adrenoceptor population with the alkylating agent, DCITC (Deyrup *et al.*, 1998) according to Furchgott (1966) and was used for all agonists studied. In the second method, which could only be applied to salbutamol and terbutaline, the comparative method of Barlow *et al.* (1967) was employed, where *E*/[A] curves of the agonists of interest and isoprenaline, a reference full agonist, were compared. These experiments were conducted under conditions where the concentration of methacholine (MCh) used to precontract the tissue rendered salbutamol and terbutaline partial agonists ($\alpha < 1$) relative to isoprenaline ($\alpha = 1$).

Preliminary studies established that the comparative method could not be used to estimate the K_A of procaterol or formoterol because they remained full agonists relative to isoprenaline irrespective of the concentration of MCh used to raise tone.

Two-curve design and analysis. This method was used for salbutamol, terbutaline and procaterol and involved constructing two *E*/[A] curves: one before and one after β_2-adrenoceptor alkylation. Each muscle strip was first contracted with MCh at a concentration (1 μM) that elicits ~70% of the maximum attainable response (Roffel *et al.*, 1990). Once the response was stable, a *E*/[A] curve was constructed to the β_2-adrenoceptor agonist of interest. Each tissue was washed until basal tone was re-established and then exposed to DCITC or its vehicle for 30 min at a concentration that reduced the maximum asymptote (see Figure 2 for details). Each tissue was washed frequently with KHS for 30 min and then re-challenged with MCh (1 μM). When the contraction had reached a plateau, a second *E*/[A] curve was constructed to the same β_2-adrenoceptor agonist. Thus, from each donor two *E*/[A] curves were obtained. This was the preferred protocol as it allows two *E*/[A] curves to be compared that are uncomplicated by inter-tissue differences in maximal obtainable response and agonist sensitivity.

E/[A] curves generated for each β_2-adrenoceptor agonist before and after DCITC were fitted simultaneously to the operational model of agonism (Motulsky and Christopoulos, 2005), which describes a theoretical relation between pharmacological effect and agonist concentration (Black and Leff, 1983; Black *et al.*, 1985). Thus,

$$E = \frac{E_m \tau^n [A]^n}{(K_A + [A])^n + \tau^n [A]^n} \quad (2)$$

where E_m is the theoretical maximum response of the tissue, [A] is the agonist concentration, and τ is the operational efficacy of the agonist, which is the ratio of the total functional receptor population to the concentration of AR complexes required to produce half-maximal effect (Leff *et al.*, 1990c). Each pair of *E*/[A] curves was fitted to Equation 2 assuming a common value of E_m, K_A and n (Black and Leff, 1983; Leff *et al.*, 1990c). Only τ, which at sub-maximal responses decreases proportionally with the remaining fraction of non-inactivated receptors, was allowed to vary between individual *E*/[A] curves (Black and Leff, 1983; Leff *et al.*, 1990c). This analysis yielded for each experiment a single estimate of E_m, n and K_A as well as a τ value for agonist before (τ) and after (τ') receptor inactivation. The percentage of functionally active receptors (q) remaining after treatment of cells with DCITC is given by $(\tau'/\tau) \times 100$.

Single-curve design and analysis. Formoterol is a long-acting β_2-adrenoceptor agonist whose relaxant action is relatively resistant to repeated washing (Anderson, 1993; Teschemacher and Lemoine, 1999). Accordingly, an alternative experimental approach was employed to estimate the K_A of this agonist as an analysis by the preferred two-curve design was not possible (Leff *et al.*, 1990c). In this series of studies, one *E*/[A] curve was constructed to formoterol per smooth muscle strip that had been treated with DCITC (120 nM; 30 min) or its vehicle. To minimize error resulting from inter-donor differences in tissue sensitivity, the effect of DCITC on agonist-induced relaxation was determined on contiguous muscle taken from the same airway. Using this method, single estimates of E_m, K_A and n were determined by simultaneously fitting all replicate *E*/[A] curves (before and after alkylation) to Equation 2. As before, individual τ and τ' values were determined from each *E*/[A] curve from which a mean and standard error were then calculated (Leff *et al.*, 1990c).

Estimation of agonist equilibrium dissociation constants by comparison with a full reference agonist

This approach was used for salbutamol and terbutaline only. HASM was contracted by a concentration of MCh (see text for details) that, in preliminary experiments, rendered salbutamol and terbutaline partial agonists relative to isoprenaline. In a single piece of tissue, consecutive *E*/[A] curves were constructed to isoprenaline and then the β_2-adrenoceptor agonist of interest. Each pair of *E*/[A] curves (i.e. the full agonist and partial agonist *E*/[A] curves) were then fitted simultaneously to Equations 1 and 2 respectively (Motulsky and Christopoulos, 2005), which yielded an estimate of K_A and τ for the partial agonist, the E_m and n of the tissue and a $p[A]_{50}$ for each full agonist curve (Leff *et al.*, 1990c). A similar method was used to estimate the K_A of salbutamol for the inhibition of LTB_4-induced H_2O_2 generation in guinea-pig eosinophils. In this experiment, formoterol was used as a reference full agonist as high concentrations of isoprenaline interfered with the oxidation of scopoletin.

Influence of 'fade' of the MCh-induced contraction on operational parameter estimates

In preliminary experiments involving DCITC, considerable 'fade' of the MCh-induced contraction often was seen over the time required to construct complete agonist *E*/[A] curves. In contrast, 'fade', while still detectable, was less of a problem in experiments performed in the absence of DCITC because agonist *E*/[A] curves were easy to define and could be completed more quickly. In contrast, for the comparative method, contractions were induced by higher concentrations of MCh than were used in the receptor inactivation protocol, and these responses were considerably less prone to 'fade'. Thus, in the receptor inactivation studies, an attempt was made to 'correct' for the spontaneous reduction in tone prior to operational curve fitting. Each tissue was first exposed to MCh (1 μM). Once the response had plateaued, the degree of 'fade' was recorded over a period of 120 min. The tissue was then thoroughly washed, allowed to recover and then processed for the determination of agonist affinity by using either the one or two *E*/[A] curve protocol described above. In the subsequent analysis, the degree of 'fade' measured at the beginning of the experiment was subtracted from subsequent time-matched control in the same tissue in which β_2-adrenoceptor-mediated relaxation was investigated. This method is unavoidably imprecise, because it cannot be assumed that the magnitude and rate of 'fade' are invariant between subsequent MCh-induced contractions. Accordingly, the

operational parameter values, in particular efficacy and E_m, 'corrected' in this manner must be considered estimates only (see *Discussion*).

Determination of receptor reserve

Receptor occupancy–effect curves were constructed to each β_2-adrenoceptor agonist by using the K_A determined by receptor inactivation (corrected) and/or the comparative method as indicated. At each concentration of agonist and, therefore, at each level of response, fractional β_2-adrenoceptor occupancy [i.e. the ratio of agonist occupied β_2-adrenoceptors to the total number of available receptors (R_A/R_t)] in control (i.e. DCITC naïve) tissues was determined (see Ruffolo, 1982). Thus,

$$R_A/R_t = [A]/(K_A + [A]) \quad (3)$$

As each E/[A] curve was constructed to a β_2-adrenoceptor agonist in half-log increments of concentration, the point at which maximal relaxation is reached cannot be determined with accuracy. Thus, an estimate of this value was obtained by determining the fraction of agonist-occupied receptors required to elicit 95% of the maximum response (Gunst *et al.*, 1987). To allow comparison between agonists, the fraction of agonist-occupied receptors required to elicit 25%, 50% and 75% of the maximum was also determined.

General statistics

Data points, and values in the text and figure legends, represent the mean ± SEM of 'N' independent determinations. When appropriate, data were analysed statistically by using one-way ANOVA followed, if necessary, by Bonnferoni's multiple comparison test. The null hypothesis was rejected when $P < 0.05$.

Materials

Diclofenac and LTB_4 were purchased from Cayman Chemicals (Ann Arbor, MI, USA). DCITC (Figure 1) was donated by Dr Stephen Baker (University of Florida, USA). Isoprenaline, procaterol, terbutaline, formoterol, salbutamol, phentolamine, mepyramine, ICI 198,615, HBSS, horseradish peroxidase, Percoll, superoxide dismutase, scopoletin, H_2O_2, CGP 20712A and other drugs, salts and analytical reagents were obtained from Sigma-Aldrich (Oakville, ON, Canada).

Results

Estimating the affinity of salbutamol, terbutaline, procaterol and formoterol for the β_2-adrenoceptor by fractional receptor inactivation

On HASM strips pre-contracted with MCh (1 μM), the four β_2-adrenoceptor agonists studied elicited concentration-dependent relaxations that were comparable in magnitude (Table 1; Figure 2). After estimating the 'fade' of the MCh-induced contraction (see *Methods* and text following), the molar rank order of potency was formoterol > procaterol > salbutamol > terbutaline (Table 1; Figure 2).

Pretreatment (30 min) of HASM strips with DCITC (up to 120 nM) had no effect on baseline tone or on the magnitude of contractions induced by MCh (1 μM). However, both the potency and maximal asymptote of the E/[A] curves that described the relaxation of HASM strips by the four agonists studied was significantly reduced (Table 1; Figure 2). The data set in Figure 2 (i.e. the individual control E/[A] curves and the associated individual E/[A] curves obtained in the presence of DCITC) were fitted globally to the operational model of agonism shown in Equation 2 (Black and Leff, 1983; Black *et al.*, 1985), by using either the single- or two-curve method described in *Methods*. This analysis showed that salbutamol, terbutaline, procaterol and formoterol were full agonists in human bronchus with mean K_A values of 3 μM, 15.8 μM, 427 nM and 112 nM respectively. Operational model fitting also showed that salbutamol and terbutaline had lower efficacy ($\tau = 21.7$ and 32 respectively) relative to both procaterol ($\tau = 73$) and formoterol ($\tau = 164$; Table 2).

Estimating the affinity of salbutamol and terbutaline for the β_2-adrenoceptor by comparison with a reference full agonist

On HASM strips pre-contracted with MCh (5 μM), salbutamol evoked concentration-dependent relaxations with a mean $p[A]_{50}$ (M) and E_{max} of 6.42 ± 0.04 and 65.0 ± 3.9% respectively ($N = 10$). Similar results were obtained for terbutaline ($p[A]_{50}$ (M) = 6.13 ± 0.05; E_{max} = 78.2 ± 5.7%, $N = 7$) in HASM strips contracted with a slightly higher concentration of MCh

Table 1 Effect of the alkylating agent, DCITC, on the potency and magnitude of human bronchial smooth muscle relaxation elicited by several β_2-adrenoceptor agonists used clinically as bronchodilators

Agonist	N	*−DCITC*		*+DCITC*[a]		*Rightward shift (mean fold)*
		$p[A]_{50}$ (M)	*E_{max} (%)*	*$p[A]_{50}$ (M)*	*E_{max} (%)*	
Salbutamol	8	6.75 ± 0.05	90.7 ± 2.1	5.66 ± 0.10	37.1 ± 3.7	12.3
Terbutaline	11	6.31 ± 0.10	99.6 ± 2.7	5.01 ± 0.09	44.2 ± 4.6	19.9
Procaterol	7	7.98 ± 0.08	103.9 ± 5.5	6.34 ± 0.07	60.1 ± 4.5	43.6
Formoterol	5	9.01 ± 0.10	105.9 ± 1.2	6.92 ± 0.10	65.5 ± 8.5	123.0

Each agonist E/[A] curve in Figure 2 was fitted to Equation 1 from which parameter estimates of $p[A]_{50}$ and E_{max} were derived. Data are expressed as mean ± SEM of N independent determinations. See text and legend to Figure 2 for further details.
DCITC, 5(2-(((1′-(4′-isothiocyanatophenylamino)thiocarbonyl)-amino)-2-methyl-propyl)amino-2-hydroxypropoxy)-3,4-dihydrocarbostyril.
[a]Tissues were incubated for 30 min with DCITC at 50 nM (salbutamol, terbutaline), 100 nM (procaterol) or 120 nM (formoterol).

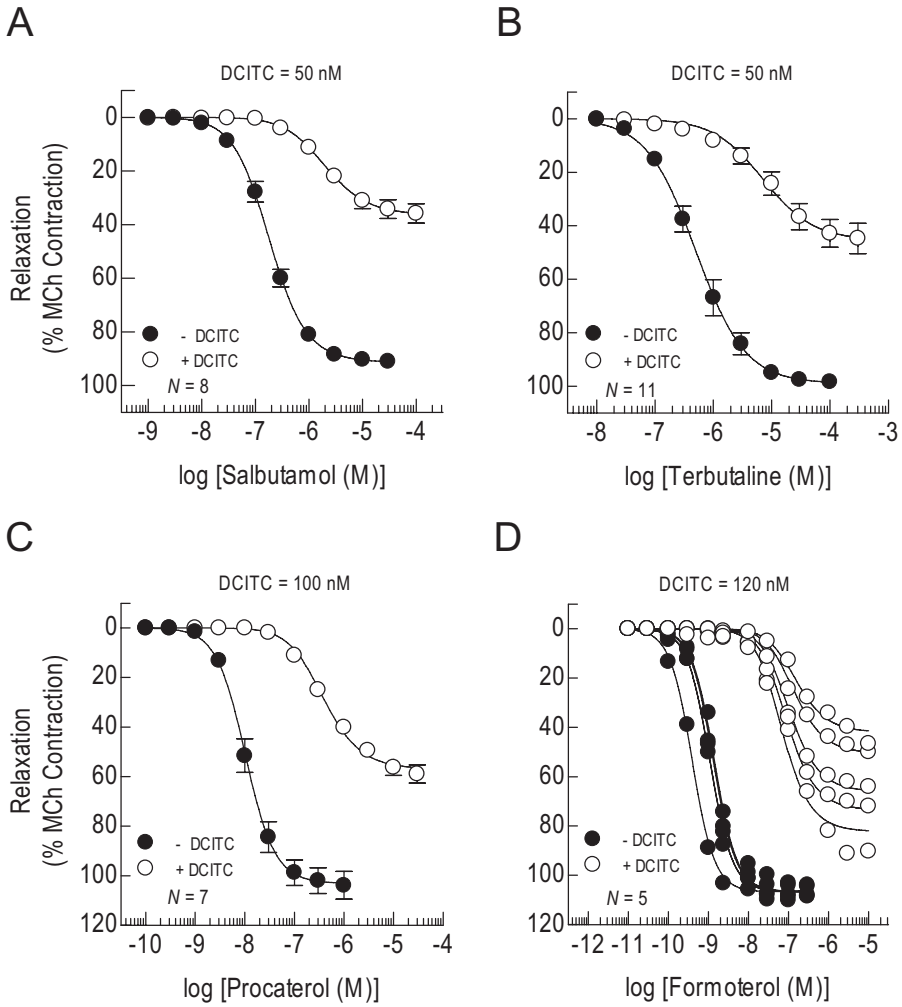

Figure 2 Application of the receptor inactivation method to estimate the K_A of several sympathomimetic bronchodilators for the β_2-adrenoceptor on human airways smooth muscle. Strips of human bronchus were contracted with methacholine (MCh) (1 μM). When responses had plateaued, a cumulative *E*/[A] curve was constructed to salbutamol (A), terbutaline (B) and procaterol (C). Tissues were allowed to re-equilibrate, exposed to the alkylating agent, DCITC [5(2-(((1′-(4′-isothiocyanatophenylamino)thiocarbonyl)-amino)-2-methyl-propyl)amino-2-hydroxypropoxy)-3,4-dihydrocarbostyril], for 30 min at the concentrations indicated in each panel and then washed extensively with Krebs-Henseleit solution over the next 30 min. A second contraction to MCh (1 μM) was evoked and a cumulative *E*/[A] curve to salbutamol, terbutaline and procaterol was re-established. In panel (D), a single cumulative *E*/[A] curve was constructed to formoterol in tissue pretreated with either vehicle or DCITC. In panels (A), (B) and (C), each point represents the mean ± SEM of *N* independent determinations using tissue from different donors. Data from these experiments were analysed by operational curve fitting (see *Methods*) from which estimates of K_A, τ, τ', n, E_m and $p[A]_{50}$ were derived (see Tables 1 and 2).

(7 μM). These concentrations of MCh rendered both salbutamol and terbutaline partial agonists, relative to the reference full agonist isoprenaline (E_{max} > 90% reversal), with an intrinsic activity (α) of 0.68 ± 0.03 (N = 10) and 0.83 ± 0.03 (N = 7) respectively (Figure 3). Simultaneously fitting each pair of salbutamol and isoprenaline *E*/[A] curves that make up Figure 3A to Equations 1 and 2 respectively yielded for salbutamol an affinity estimate that was significantly lower (0.6 $\log_{10}$ unit) than the same parameter determined by receptor alkylation (Table 2). This discrepancy was also seen when terbutaline was analysed in the same way (i.e. the affinity of this agonist was 0.67 $\log_{10}$ unit lower than the same parameter determined by fractional receptor inactivation; Table 2). In addition, the theoretical maximum relaxant response (E_m), expressed as percentage reversal of the MCh-induced contraction, was significantly less after application of the comparative method (97–98%) than was suggested by receptor alkylation (113%; Table 2). In this respect, on HASM strips not exposed to MCh, isoprenaline (1 μM) failed to cause significant relaxation (data not shown). Thus, in these experiments E_m, determined by fractional receptor inactivation, was overestimated.

Effect of 'fade' of the MCh-induced contraction on agonist parameter estimates determined by operational model fitting

Typically, the time required to construct *E*/[A] curves to the four β_2-adrenoceptor agonists studied in MCh (1 μM)-contracted tissues was short (all within 60 min) with the higher efficacy agonists (formoterol, procaterol) exerting their relaxant action more rapidly than agonists (salbutamol, terbutaline) of relatively lower efficacy. Moreover, the magnitude

Table 2 Operational parameter estimates derived by applying the inactivation and comparative methods for β_2-adrenoceptor-mediated relaxation of human bronchial smooth muscle evoked by a panel of agonists used clinically as bronchodilators

Agonist	*Method*	*N*	*Parameter Estimates*					
			pK_A (M)	E_m *(%)*	n	*log*τ	*log*τ′	q*([*τ′/τ*] × 100)*
Salbutamol	Inactivation[a]	8	5.52 ± 0.06	112.5 ± 6.7	1.307 ± 0.14	1.336 ± 0.07	–0.128 ± 0.06	3.4
	Inactivation – corrected[a]	8	5.71 ± 0.09	94.5 ± 2.5*	1.498 ± 0.14	1.067 ± 0.09*	–0.251 ± 0.14	4.7
	Comparative[b]	10	6.12 ± 0.05*,†	96.8 ± 2.6*	1.372 ± 0.06	332 ± 0.04	–	–
Terbutaline	Inactivation[a]	11	4.80 ± 0.11	113.2 ± 2.4	1.121 ± 0.32	1.507 ± 0.52	–0.002 ± 0.05	3.1
	Inativation – corrected[a]	11	5.00 ± 0.08	101.5 ± 3.0*	1.277 ± 0.05	1.307 ± 0.04*	–0.088 ± 0.07	4.0
	Comparative[b]	7	5.47 ± 0.05*,†	97.7 ± 2.5*	1.362 ± 0.04	705 ± 0.04	–	–
Procaterol[c]	Inactivation[a]	7	6.18 ± 0.17	112.9 ± 4.3	1.628 ± 0.09	1.864 ± 0.11	0.060 ± 0.05	1.9
	Inactivation – corrected[a]	7	6.43 ± 0.11	104.6 ± 7.4	1.816 ± 0.14	1.598 ± 0.06*	0.066 ± 0.05	3.1
Formoterol[c]	Inactivation[a]	5	6.95	114.3	1.184	2.214 ± 0.12	0.092 ± 0.10	0.8
	Inactivation – corrected[a]	5	6.87	106.7	1.557	2.049 ± 0.03*	0.109 ± 0.08	1.1

Each pair of agonist *E*/[A] curves shown in Figures 2 and 3 were analysed by operational curve fitting from which parameter estimates of pK_A, logτ, logτ', *n*, E_m and *q* were derived. Data are expressed as mean ± SEM of '*N*' independent determinations. See text and legends to Figures 2 and 3 for further details.
DCITC, 5(2-(((1′-(4′-isothiocyanatophenylamino)thiocarbonyl)-amino)-2-methyl-propyl)amino-2-hydroxypropoxy)-3,4-dihydrocarbostyril; MCh, methacholine.
[a]Tissues were incubated for 30 min with DCITC at 50 nM (salbutamol, terbutaline), 100 nM (procaterol) or 120 nM (formoterol).
[b]Tone was induced by 5 μM and 7 μM MCh for salbutamol and terbutaline experiments respectively.
[c]Comparative method could not be used as procaterol, and formoterol remained full agonists relative to isoprenaline irrespective of the concentration of MCh used to raise tone.
*$P < 0.05$ relative to inactivation method; †$P < 0.05$ relative to inactivation – corrected method.

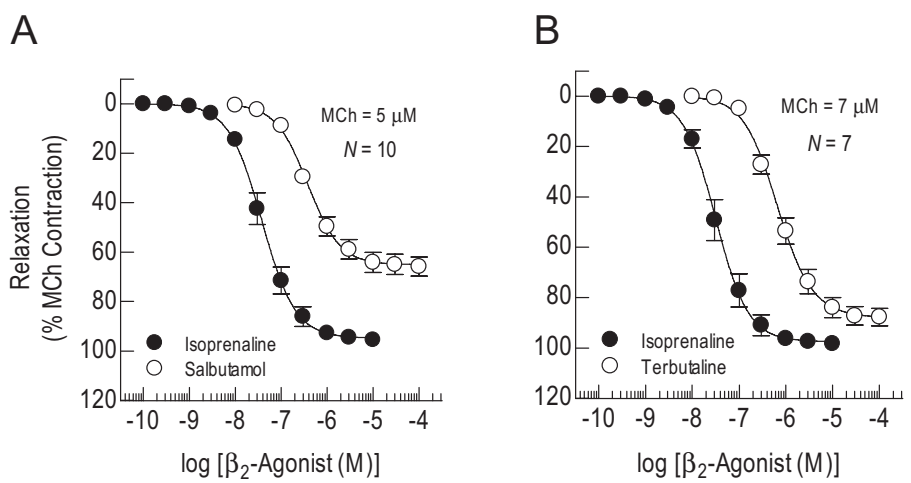

Figure 3 Application of the comparative method to estimate the K_A of salbutamol and terbutaline for the β_2-adrenoceptor on human airways smooth muscle. Strips of human bronchus were contracted with methacholine (MCh) at the concentration indicated in each panel. When responses had plateaued, a cumulative *E*/[A] curve was constructed to isoprenaline, a reference full agonist. Tissues were allowed to re-equilibrate, and a second contraction to the same concentration of MCh was evoked. A cumulative *E*/[A] curve was then constructed to salbutamol (A) and terbutaline (B). Each pair of isoprenaline and test β_2-adrenoceptor agonist *E*/[A] curves was analysed by operational curve fitting (see *Methods*) from which K_A, τ, *n* and E_m were derived (see Table 2 for model parameter estimates). Each point represents the mean ± SEM of *N* independent determinations using tissue from different donors. In these experiments, isoprenaline caused almost complete reversal of induced tone (>93%) with p$[A]_{50}$ (M) values of 7.37 ± 0.09 and 7.40 ± 0.08 in tissues contracted with 5 μM and 7 μM MCh respectively.

of MCh-induced tone over this same time frame was well maintained (mean fade <5%). In contrast, after fractional β_2-adrenoceptor alkylation with DCITC, the kinetics of relaxation induced by salbutamol, terbutaline, procaterol and formoterol were significantly slowed. Indeed, the time required to construct complete *E*/[A] curves was markedly increased (in some preparations to 120 min). These protracted kinetics of relaxation created two potential sources of error in the estimation of τ and E_m. First, inspection of agonist *E*/[A] curves constructed in the presence of DCITC indicated significant 'fade' of the MCh-induced contraction (15–40%) during the prolonged time required to complete agonist *E*/[A] curves, which will result in τ and E_m being overestimated (Dougall *et al.*, 1991). Second, agonist-induced relaxations of DCITC-treated tissues were difficult to define and, therefore, measure precisely. Thus, to gain a more accurate estimate of K_A, τ, *n* and E_m from the fractional receptor inactivation data, the degree of 'fade' of the MCh-induced contraction in the presence of DCITC was approximated (see *Methods*). Agonist *E*/[A] curves were 'corrected' accordingly and fitted globally to the operational model of agonism. As predicted, the analysis of these 'corrected' data yielded significantly lower efficacy and

Table 3 Relationship between $[A]_{50}$ and K_A of salbutamol, terbutaline, procaterol and formoterol before and after fractional, irreversible β_2-adrenoceptor blockade with DCITC

Agonist	*−DCITC*			*+DCITC*		
	K_A *(μM)*	$[A]_{50}$ *(μM)*	$K_A/[A]_{50}$	K_A *(μM)*	$[A]_{50}$ *(μM)*	$K_A/[A]_{50}$
Salbutamol	1.95	0.18	10.8	1.95	2.2	0.89
Terbutaline	10.0	0.49	20.4	10.0	9.8	1.02
Procaterol	0.37	0.01	37.0	0.37	0.46	0.80
Formoterol	0.13	0.001	130	0.13	0.12	1.08

$[A]_{50}$ values are taken from Table 1.
K_A values are derived from the inactivation method and have been 'corrected' for fade of the methacholine-induced contraction (Table 2).
DCITC, 5(2-(((1′-(4′-isothiocyanatophenylamino)thiocarbonyl)-amino)-2-methyl-propyl)amino-2-hydroxypropoxy)-3,4-dihydrocarbostyril.

maximum response estimates while the affinity of salbutamol and terbutaline were slightly increased (Table 2). Indeed, the pK_A of salbutamol and terbutaline now approached the affinity of these agonist determined by the comparative method (values within 0.41 and 0.47 $\log_{10}$ unit respectively), and the corrected E_m values were not significantly different (Table 2).

Effect of DCITC on the relationship between the agonist potency and agonist affinity

In tissues not exposed to DCITC, formoterol, procaterol, terbutaline and salbutamol were 10- to 130-fold more potent in promoting smooth muscle relaxation relative to their respective affinity for the β_2-adrenoceptor (Table 3). After fractional, irreversible β_2-adrenoceptor blockade with DCITC, this difference was eliminated for each agonist (i.e. $K_A/[A]_{50} \sim 1$) indicating the depletion of spare receptors, such that the relationship between receptor occupancy and response was linear.

Relationship between β_2-adrenoceptor occupancy and relaxation

Using the K_A values of salbutamol, terbutaline, procaterol and formoterol estimated by fractional receptor inactivation (corrected for 'fade'), β_2-adrenoceptor occupancy expressed as a function of relaxation was, in each case, described by a curvilinear relationship that deviated significantly from the line of identity (where response is directly proportional to occupancy; Figure 4). Thus, a β_2-adrenoceptor 'reserve' for these four agonists was present on human bronchus. Indeed, reference to Table 2 shows that although DCITC inactivated >95% of the functional receptor population ($q < 5\%$) a significant relaxant response was still evoked. Although the proportion of 'spare' receptors declined incrementally with increasing agonist concentration, a receptor reserve was evident at all measured levels of response (Figure 4; Table 4). However, the relationship between receptor reserve and relaxation was not uniform across the four agonists examined. Indeed, the proportion of receptors required to evoke a fixed level of response increased with agonists of decreasing efficacy with the following rank order: formoterol > procaterol > terbutaline > salbutamol (Figure 4; Table 4).

The β_2-adrenoceptor occupancy–response relationship was also curvilinear for salbutamol and terbutaline using the K_A values estimated by the comparative method. However, given that these experiments were performed under higher degrees of MCh-induced tone (salbutamol, 5 μM; terbutaline, 7 μM) the receptor reserve at all levels of response was, predictably, less than that determined by receptor inactivation due to increased functional antagonism (i.e. the potency of salbutamol and terbutaline were lower thereby reducing the $K_A/[A]_{50}$ ratio; Figure 4A,B; Table 4).

Estimating the affinity of salbutamol for the β_2-adrenoceptor on eosinophils by comparison with a reference full agonist

On guinea-pig eosinophils stimulated with LTB_4 (100 nM; $p[A]_{70}$), salbutamol evoked a concentration-dependent inhibition of the rate of H_2O_2 generation with a mean $p[A]_{50}$ (M) and E_{max} of 6.27 ± 0.09 and 34.8 ± 14.2% respectively ($N = 5$). Under these experimental conditions salbutamol behaved as a partial agonist ($\alpha = 0.56 \pm 0.08$; $N = 5$), relative to formoterol (E_{max} ~63% reversal). Simultaneously fitting each pair of salbutamol and formoterol $E/[A]$ curves that make up Figure 5A to Equations 1 and 2 respectively yielded for salbutamol a pK_A, $\log\tau$, n and E_m of 6.024 ± 0.05, 0.097 ± 0.02, 1.187 ± 0.05 and 62.4 ± 6.8% respectively. Reference to Table 2 shows that the affinity of salbutamol for the β_2-adrenoceptor calculated by using the comparative method was the same in eosinophils (924 nM) and HASM (759 nM) and differed by only 2.1-fold from the K_A determined by receptor alkylation (3 μM in tissues corrected for fade of the MCh-induced contraction). It should however be noted that the affinity of salbutamol in eosinophils could be slightly underestimated, as the reference agonist (formoterol), could be behaving as a full agonist in this system.

Relationship between β_2-adrenoceptor occupancy and inhibition of H_2O_2 generation

Using a K_A of salbutamol of 946 nM, β_2-adrenoceptor occupancy was expressed as a function of the inhibition of H_2O_2 generation (Figure 5B). Given that the K_A and $[A]_{50}$ of salbutamol were very similar (ratio = 1.76), this relationship deviated only slightly from the line of identity. Thus, on this functional response evoked by the $p[A]_{70}$ of LTB_4, the β_2-adrenoceptor 'reserve' for salbutamol was very modest at all levels of response (Figure 5B).

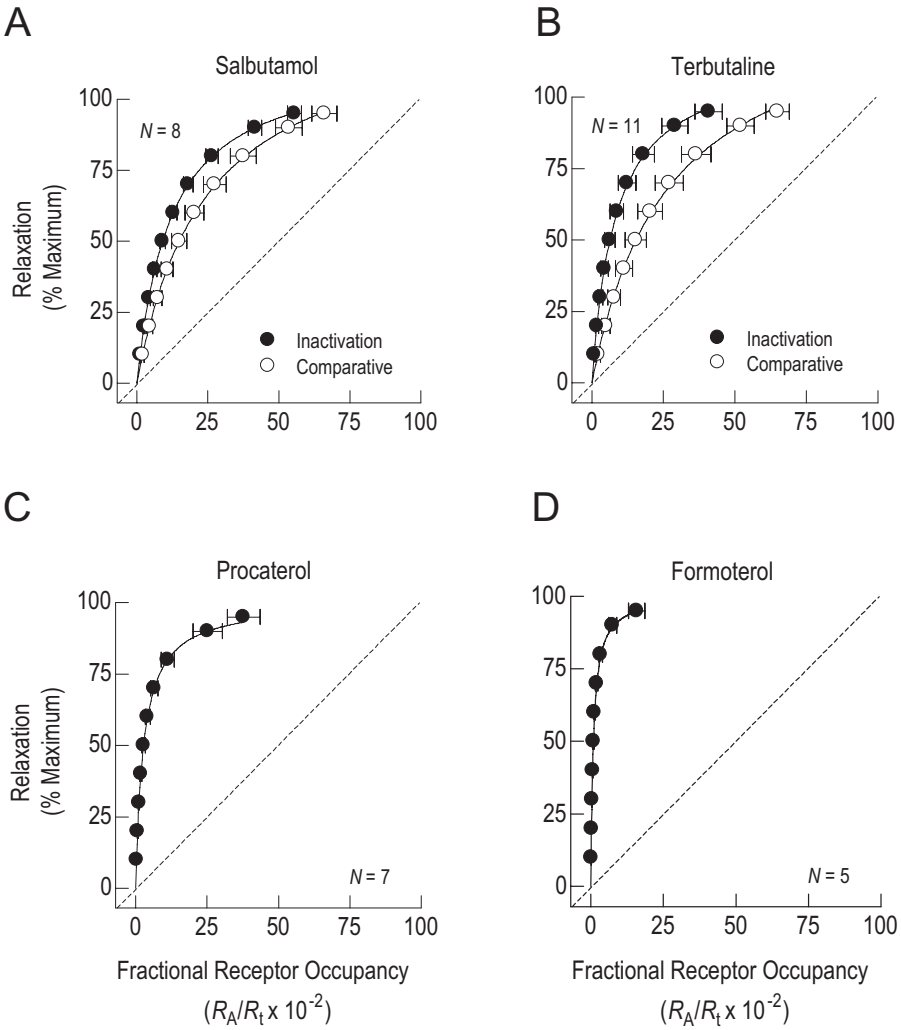

Figure 4 Relationship between β_2-adrenoceptor occupancy and relaxation of human airways smooth muscle. The K_A of salbutamol (A), terbutaline (B), procaterol (C) and formoterol (D) derived by either the inactivation (corrected for 'fade') and/or comparative method was used to calculate the relationship between fractional receptor occupancy (R_A/R_t) and relaxant responses shown in Figure 2 according to Equation 3. The dashes in each panel indicate the line of identity where response is directly proportional to receptor occupancy.

Table 4 Relationship between β_2-adrenoceptor occupancy and relaxation of human bronchial smooth muscle for a panel of agonists used clinically as bronchodilators

Agonist	*Method*	*N*		*Response level (% Max)*			
				25%	*50%*	*75%*	*95%*
Salbutamol	Inactivation	8	Occupancy (%)	3.3 ± 0.4	9.0 ± 1.0	21.7 ± 1.9	55.4 ± 2.5
			Receptor excess (fold)	30	11	4.6	1.8
	Comparative	10	Occupancy (%)[b]	5.8 ± 1.2	14.9 ± 2.7	31.9 ± 4.3	66.1 ± 4.1
			Receptor excess (fold)	17	6.7	3.3	1.5
Terbutaline	Inactivation	11	Occupancy (%)	2.3 ± 0.7	6.3 ± 1.8	14.8 ± 3.4	40.8 ± 4.8
			Receptor excess (fold)	44	16	6.8	2.5
	Comparative	7	Occupancy (%)	6.3 ± 1.8	11.3 ± 3.0	31.3 ± 5.1	64.9 ± 4.1
			Receptor excess (fold)	16	8.8	3.2	1.5
Procaterol	Inactivation	7	Occupancy (%)	0.9 ± 0.18	2.8 ± 0.6	8.4 ± 1.7	37.8 ± 5.7
			Receptor excess (fold)	111	36	12	2.6
Formoterol	Inactivation	5	Occupancy (%)	0.3 ± 0.04	0.9 ± 0.1	2.5 ± 0.4	15.8 ± 2.8
			Receptor excess (fold)	333	111	40	6.3

The K_A of each β_2-adrenoceptor agonist derived by either the inactivation (corrected for 'fade') and/or comparative method was used to calculate the relationship between fractional receptor occupancy (R_A/R_t) and relaxation according to Equation 3 using the control *E*/[A] curves shown in Figure 2. Data are expressed as mean ± SEM of '*N*' independent determinations. See text and legend to Figure 4 for further details.

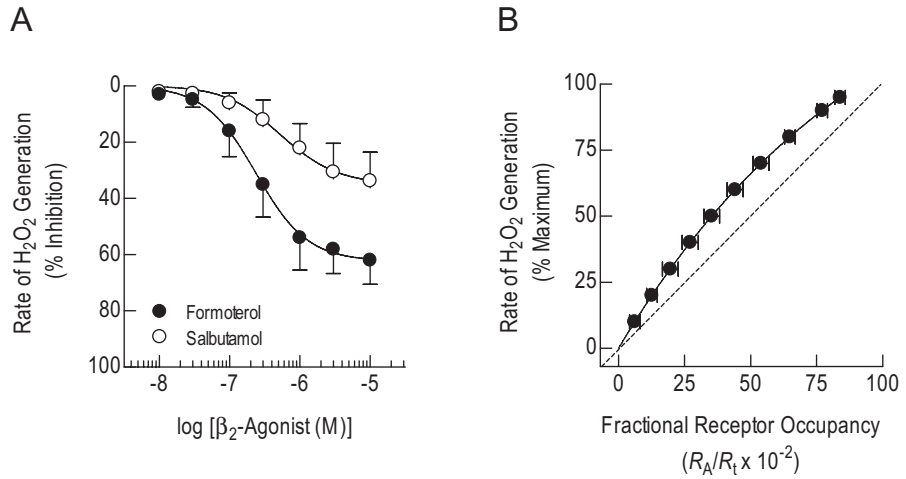

Figure 5 Application of the comparative method to estimate the K_A of salbutamol for the β_2-adrenoceptor on guinea-pig eosinophils and the relationship between receptor occupancy and inhibition of H_2O_2 generation. In panel (A) eosinophils were pretreated (5 min) with salbutamol, formoterol (both 10 nM to 10 μM) or their respective vehicles and then exposed to leukotriene B_4 (100 nM; $p[A]_{70}$). Formoterol and salbutamol *E*/[A] curves were then constructed for the rate of inhibition of H_2O_2 production, which were analysed, subsequently, by operational curve fitting (see text for model parameter estimates). The K_A of salbutamol (946 nM) determined by this method was then used to calculate the relationship between fractional receptor occupancy (R_A/R_t) and responses according to Equation 3. The dashes in panel (B) indicate the line of identity where response is directly proportional to receptor occupancy. Each point represents the mean ± SEM of five independent determinations.

Discussion

The present studies demonstrate that human bronchial smooth muscle *in vitro* expressed a large β_2-adrenoceptor reserve for salbutamol-, terbutaline-, procaterol- and formoterol-induced relaxation. In contrast, β_2-adrenoceptor density was not in excess on eosinophils at any level of response for the ability of salbutamol to inhibit the generation of H_2O_2, an index of respiratory burst activity. Collectively, these two sets of data support the hypothesis that a high density of β_2-adrenoceptors on HASM cells in subjects with asthma may protect against desensitization. However, it is important to remember that the relationship between fractional receptor occupancy and response will vary if that agonist-induced response is conducted in tissues that are susceptible to functional antagonism. In the case of airway smooth muscle, the potency (and efficacy) of sympathomimetic bronchodilators is inversely related to the concentration of spasmogen used to raise tone (Buckner and Saini, 1975; Torphy *et al.*, 1983). This, in turn, will be reflected by a reduction in the $K_A/[A]_{50}$ ratio and, so, receptor reserve. The data in Figure 4 illustrate this point. Thus, the receptor reserve is less at all levels of response for salbutamol- and terbutaline-induced relaxation when the comparative method was applied to determine agonist affinity because higher concentrations of MCh were used to raise tone than those used in the receptor alkylation studies. This is potentially important as the effect of functional antagonism on the potency and efficacy of β_2-adrenoceptor agonists will not be equal across different tissues (e.g. airway smooth muscle vs. eosinophils). Nevertheless, two pieces of evidence, when considered together, support the idea that relaxation of HASM may be more resistant to β_2-adrenoceptor desensitization than is the suppression of immune and pro-inflammatory cell function. First, HASM expresses 30 000 to 40 000 β_2-adrenoceptors per cell, whereas the neutrophil, eosinophil (guinea-pig and human), alveolar macrophage and airway epithelial cell have considerably fewer (4000 to 8000/cell) and T-lymphocytes less still (~750/cell) (Johnson, 2002). Second, the potency of β_2-adrenoceptor agonists, such as salbutamol, for the attenuation of a variety of pro-inflammatory responses in different cell types often is weak and of low efficacy such that the $K_A/[A]_{50}$ ratios approach unity (this study and Sekut *et al.*, 1995; Ezeamuzie and Al-Hage, 1998; Seldon *et al.*, 1998; Perkins *et al.*, 2007). It is worth noting that formoterol inhibited respiratory burst in eosinophils, maximally, by 63% and had an $[A]_{50}$ of 240 nM. This relatively low potency (*cf.* HASM) might indicate that it is not a full agonist in this system. In this event, the pK_A of salbutamol, determined by the comparative method, will have been under-estimated and the β_2-adrenoceptor occupancy-response relationship shown in Figure 5B will be more curvilinear due to the increase in the $K_A/[A]_{50}$ ratio. However, on many pro-inflammatory and immune cell functional responses, incomplete inhibition is often seen even with very high efficacy β_2-adrenoceptor agonists such as isoprenaline (e.g. Berends *et al.*, 1997; Drury *et al.*, 1998; Gibson-Berry *et al.*, 1993). Thus, the 63% inhibition of eosinophil respiratory burst reported herein with formoterol might, indeed, reflect the maximum that can be achieved even with an agonist of higher efficacy.

Theoretical objections to estimating agonist affinity by pharmacological methods

To determine the relationship between fractional receptor occupancy and relaxation it was first necessary to estimate the affinity of salbutamol, terbutaline, procaterol and formoterol for the β_2-adrenoceptor. To this end, where possible, two pharmacological approaches were adopted: the method of Furchgott (1966), which involves fractional, irreversible,

β_2-adrenoceptor inactivation with an alkylating agent, and the comparative method of Barlow *et al*. (1967), in which *E*/[A] curves of agonists of interest and a reference full agonist are compared. However, estimating the affinity of an agonist for a G-protein-coupled receptor using pharmacological means is *theoretically* invalid due, in its most simplest form, to the operation of ternary complex mechanisms (Leff and Harper, 1989; Leff *et al*., 1990a; Colquhoun, 1998; Strange, 2008). This is because pharmacological approaches do not accurately measure the formation of an agonist receptor complex (AR in Equation 4). In fact, computer simulations indicate that both methods are likely to overestimate affinity. This is especially true for full agonists; nevertheless, errors may also be produced for partial agonists although these are predicted to be smaller (Leff and Harper, 1989; Leff *et al*., 1990a; Colquhoun, 1998).

$$A + R \underset{}{\overset{K_A}{\rightleftharpoons}} AR + T \underset{}{\overset{K_{AR}}{\rightleftharpoons}} ART \rightarrow \text{Response} \qquad (4)$$

$$K_A = \frac{[A][R]}{[AR]} \qquad (5)$$

The origin of this error lies in traditional receptor theory that makes the assumption that affinity and efficacy are independent parameters. However, this cannot be the case when agonism depends on the formation of an active ternary complex (Equation 4). In this situation agonist, A, interacts with receptor, R, to form a binary AR unit. AR then forms an active ternary complex by binding to a transducer, T – Gs in the present study – which promotes, directly or indirectly, a response. K_A is the agonist dissociation constant for the formation of AR and its reciprocal defines affinity. K_{AR} is the dissociation constant for the formation of an active ART complex. This constant is agonist-dependent, and its reciprocal defines efficacy (Leff and Harper, 1989).

The problem with applying traditional methods to estimate K_A is that efficacy and affinity are not independent parameters. Thus, the formation of ART cannot be achieved without reducing or even depleting the concentration of AR. Accordingly, the equilibrium between A and R in Equation 4 is displaced to the right such that the affinity measured by the inactivation and comparative methods overestimates the true K_A. Stated differently, as [AR] declines (Equation 5) due to the formation of ART, K_A is increased. An additional problem is that the error in K_A will be directly related to the efficacy of the agonist as K_{AR} is a measure of how effectively AR and T interact to form an active state. Thus, theoretically, both the inactivation and comparative methods do not measure K_A but a function of multiple equilibrium constants that include K_A and K_{AR}.

Extensive simulations (Leff *et al*., 1990a) have established that the overestimation in K_A will be greatest when full agonists are analysed by the inactivation method when compared with partial agonists determined by either method. Leff *et al*. (1990b) have used this predicted error in affinity estimation as a test to determine whether, in fact, this occurs experimentally. Thus, if the theoretical predictions are correct, application of the two methods to a partial agonist should provide values of K_A that do *not* coincide. Using this simple test, it was found that the K_A of salbutamol estimated by the inactivation and comparative method were very similar (difference <2.5-fold; Table 2). Similar results were obtained when terbutaline was used as agonist. Thus, in this experimental system, there was no indication that the receptor inactivation method introduced the error in affinity predicted by theory.

Results similar to these have also been reported for the binding of agonists to other G-protein-coupled receptors including muscarinic M_2 (Leff *et al*., 1990b), muscarinic M_3 (Waud, 1969) and the prostacyclin receptor (Ayer *et al*., 2008) indicating that under certain circumstances, pharmacological methods can provide reliable estimates of affinity despite the operation of mechanisms where pharmacological approaches theoretically measure a function of multiple equilibria (Leff *et al*., 1990a; Strange, 2008). It was not possible to apply the same experimental test to procaterol and formoterol as neither of these agonists could be made partial relative to isoprenaline. However, as there was a substantial receptor reserve for salbutamol and terbutaline, which have relatively lower efficacies (Table 2), logic dictates that an excess of β_2-adrenoceptors will also exist on HASM for procaterol and formoterol at all levels of response.

Several explanations may account for why the overestimation of K_A predicted by theory is not seen experimentally (see Strange, 2008 and references therein). One possibility is that receptor density exceeds the number of G-proteins such that the formation of ART in Equation 4 does not significantly deplete [AR]. Another plausible explanation is that only relatively few ternary ART complexes are formed and that the functional response is more dependent on the magnitude of downstream signal amplification. Again, such a mechanism would not markedly deplete [AR]. Finally, there are data to suggest that ART ternary complexes are unstable in the presence of GTP, which is present in cells at high concentrations. In this scenario, the dissociation of AR from T would result in the concentration of AR being largely preserved.

Influence of pharmacological 'fade' on operational parameter estimates

A significant practical issue in the enumeration of parameter estimates by operational model fitting was the influence of 'fade' of the MCh-induced contraction. This was particularly problematic in tissues subjected to partial β_2-adrenoceptor inactivation with DCITC because of the time required to construct complete agonist *E*/[A] curves (typically 90–120 min) and the difficulty in defining responses. This problem in determining operational parameters has been documented previously in an investigation of salmeterol-induced relaxation of guinea-pig trachea (Dougall *et al*., 1991). In that study, the time required to construct *E*/[A] curves took so long to complete (>5h) that the magnitude of 'fade' would have resulted in efficacy and E_m being overestimated. Our data with salbutamol and terbutaline are consistent with this prediction (Table 2). Indeed, correcting for the contribution of fade normalized values of E_m and reduced the difference in efficacy estimates by half. However, lack of coincidence of τ indicates that the correction imposed was imprecise (see *Methods* for explanation) and did not accurately account for the spontaneous reduction in induced tone. Agonist affinity

estimates also varied depending on whether they were derived by the 'corrected' inactivation or comparative methods. However, as shown in Table 2, the discrepancies were very modest (twofold to threefold for salbutamol and terbutaline). Moreover, as discussed above, theory predicts that the application of the inactivation method will overestimate agonist affinity unless the concentration of β_2-adrenoceptors greatly exceeds the concentration of Gs (Leff and Harper, 1989). In the present study, K_A values derived by the comparative method were, in fact, higher (albeit modestly) than those derived by receptor inactivation. Thus, these findings question again (see Leff *et al.*, 1990b) the theoretical objections that have been raised about the pharmacological estimation of agonist affinity (see Leff *et al.*, 1990a for detailed discussion).

In conclusion, this paper provides empirical evidence that a panel of β_2-adrenoceptor agonists used clinically as rescue medications in the treatment of asthma, can evoke complete relaxation of human bronchial smooth muscle by occupying only a fraction of the total receptor population. Thus, these results support the long-held belief that a high density of β_2-adrenoceptors on HASM cells may, in many subjects with asthma, protect against desensitization and, therefore, a clinically relevant loss of responsiveness to sympathomimetic bronchodilators. Moreover, the excess of functional receptors for the four agonists studied may be even greater than these data suggest as β_2-adrenoceptor density in both normal and asthmatic subjects progressively increases with decreasing airway diameter (Spina *et al.*, 1989; Hoffman *et al.*, 1997). Finally, this study also confirms the results of others (Waud, 1969; Leff *et al.*, 1990b; Ayer *et al.*, 2008) that pharmacological methods can provide reasonably reliable estimates of agonist affinity despite the limitations predicted by theory.

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Conflict of interest

The author states no conflict of interest.

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