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RESEARCH PAPER

Formoterol and salmeterol induce a similar degree of β_2-adrenoceptor tolerance in human small airways but via different mechanisms

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BACKGROUND AND PURPOSE

Steroids prevent and reverse salbutamol-induced β_2-adrenoceptor tolerance in human small airways. This study examines the effects of the long-acting β_2 agonists (LABAs) formoterol and salmeterol, and the ability of budesonide to prevent desensitization.

EXPERIMENTAL APPROACH

Long-acting β_2 agonists in the presence and absence of budesonide were incubated with human precision-cut lung slices containing small airways. Tolerance was deduced from measurements of reduced bronchodilator responses to isoprenaline and correlated with β_2-adrenoceptor trafficking using a virally transduced, fluorescent-tagged receptor. The ability of the LABAs to protect airways against muscarinic-induced contraction was also assessed.

KEY RESULTS

Following a 12 h incubation, both formoterol and salmeterol attenuated isoprenaline-induced bronchodilatation to a similar degree and these effects were not reversible by washing. Pre-incubation with budesonide prevented the desensitization induced by formoterol, but not that induced by salmeterol. Formoterol also protected the airways from carbachol-induced bronchoconstriction to a greater extent than salmeterol. In the epithelial cells of small airways, incubation with formoterol promoted receptor internalization but this did not appear to occur following incubation with salmeterol. Budesonide inhibited the formoterol-induced reduction in plasma membrane β_2-adrenoceptor fluorescence.

CONCLUSIONS AND IMPLICATIONS

Although both formoterol and salmeterol attenuate isoprenaline-induced bronchodilatation, they appear to induce β_2-adrenoceptor tolerance via different mechanisms; formoterol, but not salmeterol, enhances receptor internalization. Budesonide protection against β_2-adrenoceptor tolerance was correlated with the retention of receptor fluorescence on the plasma membrane, thereby suggesting a mechanism by which steroids alter β_2-adrenoceptor function.

Abbreviations

CCh, carbachol; COPD, chronic obstructive pulmonary disease; LABA, long-acting β_2-agonist; PCLS, precision-cut lung slice; SABA, short-acting β_2-agonist; YFP, yellow fluorescent protein

Introduction

Receptor desensitization is a naturally occurring homeostatic process to prevent the over excitation of the receptor. Repeated administration of β_2-adrenoceptor agonists can promote tolerance to these drugs thereby decreasing therapeutic efficacy and hence increasing hospitalizations from asthmatic and chronic obstructive pulmonary disease (COPD) exacerbations. In general, desensitization of G-protein couple-receptors is dependent on the duration and/or frequency of repeated application of agonist. Short or infrequent exposures of the agonist to the receptor can cause the uncoupling of the receptor from adenylyl cyclase; increased duration of exposure can internalize receptors, and further increases in agonist use can cause receptor degradation. Each mechanism decreases the efficacy of the agonist; however, they can all be reversed in a time-dependent manner (Johnson, 2006). Steroids have been demonstrated to prevent the desensitization of the β_2-AR, and therefore can reduce the number of exacerbations and improve asthma control (Pauwels *et al.*, 1997; Hancox and Taylor, 2001; O'Byrne *et al.*, 2001).

Formoterol and salmeterol are both long-acting β_2-agonists (LABAs) and differ from short-acting β_2-agonists (SABAs), such as salbutamol, by having a longer duration of action (8–12 h), compared with SABAs (4 h). The chemical structures of LABAs possess greater lipophilicity that may promote a longer duration of action by more readily penetrating the cell membrane and hence have greater access to internal sites of the β_2-receptor. Formoterol differs from salmeterol in that its onset of action is a few minutes compared with salmeterol, which can take an hour to reach its maximal efficacy. The reason for this is unknown, but the longer binding hydrophobic aryloxyalkyl tail of salmeterol while being beneficial in anchoring itself to the receptor, thus giving it the longer duration of action property, may interfere with the binding rate. When β_2-agonists are administered to patients to relieve the symptoms of airflow obstruction, they are prescribed to deliver similar levels of efficacy; therefore, it is important to consider β_2-agonists at equi-effective concentrations when making comparisons of receptor desensitization in *in vitro* systems.

In our previous study (Cooper and Panettieri, 2008) we demonstrated β_2-adrenoceptor tolerance to the SABA, salbutamol, and the ability of the steroid, dexamethasone to prevent and reverse the desensitization of the β_2-adrenoceptor. It was concluded that the SABA induced a receptor tolerance upstream of adenylyl cyclase, as relaxation to forskolin was unaffected. Dexamethasone was not shown to increase cell surface receptor number, suggesting other uncharacterized mechanisms were at play.

In the present study, using precision-cut lung slices (PCLS) derived from normal healthy subjects, we demonstrated that chronic formoterol and salmeterol exposure profoundly decreases the efficacy of β_2-agonists to attenuate carbachol (CCh)-induced luminal narrowing. Budesonide prevented the tolerance to β_2-agonists. Finally, we showed differential effects of LABAs on internalization of a virally transduced β_2-adrenoceptors in airway epithelial cells. Identifying the precise molecular mechanisms by which β_2-adrenoceptor tolerance occurs and how steroids reverse this tolerance may offer new therapeutic targets to improve the efficacy of bronchodilators in asthma and COPD.

Methods

Reagents

Carbachol, isoprenaline, formoterol fumerate, salmeterol xinofoate, budesonide, low melting point agarose (IX-A), Ham's F-12 medium (supplemented with 2 mM glutamine, 100 $U \cdot mL^{-1}$ penicillin, 100 $\mu g \cdot mL^{-1}$ streptomycin, 1.0 $mg \cdot mL^{-1}$ primocin (Amaxa, Walkersville, MD, USA), 15 mM HEPES; pH 7.6) All reagents were obtained from Sigma (St. Louis, MO, USA), unless otherwise stated.

PCLS preparation and airway function

Precision-cut slices from healthy whole lungs (obtained from National Disease Research Interchange) were prepared as previously described (Cooper and Panettieri, 2008; Cooper *et al.*, 2009). Following their preparation, the lung slices were placed in a 12 well plate in 1.0 mL Ham's F-12 medium and were held in place using a platinum weight with nylon attachments and viewed under a microscope (Mag.; 40x). A baseline image was taken (0% contraction) followed by the addition of the lowest concentration of CCh to begin the concentration–response (10^{-8}–10^{-4} M). Images were collected 10 min after each dose. Once the airway had reached about 80–90% full contraction or would not contract further, the β_2-adrenoceptor activity was examined. A concentration–response to isoprenaline (10^{-9}–10^{-4} M) was conducted in the presence of the final concentration of CCh with images taken 5 min after each dose until no further relaxation occurred. Previous in-house experiments have determined that these time points are sufficient to allow maximal effect at each concentration of isoprenaline. Airway lumen area was measured using a macro written within Image Pro-Plus (version 6.0) software (Media Cybernetics) and given in units of μm^2. A log EC_{50} and E_{max} value for each airway was derived from a concentration–response curve. The minimum lumen area following contraction was normalized to 0% relaxation, the original baseline normalized to 100% relaxation, and the effects of the β_2-agonist were measured with respect to these parameters. Two airways from each donor were used per condition; *n* values indicate number of donors. Data expressed as mean ± SEM. Statistical differences were shown by utilizing a non-paired *t*-test.

Adenovirus production

To generate a replication incompetent adenovirus expressing the human β_2-adrenoceptor under control of the CMV IE promoter, a cDNA encoding a human β_2-adrenoceptor with a carboxyl terminal yellow fluorescent protein tag (β_2AR-YFP) was generated using standard techniques. The residue at position 16 was arginine. Transfer into adenovirus serotype 5 was performed by WelGen Inc. (Worcester, MA, USA). Briefly, plasmid DNA was digested with HindIII and NotI to release the insert and it was ligated into pLEPCMV predigested with the same enzymes. The resulting plasmid was digested with Pi-PspI and ligated to a linearized pREP plasmid that contained the remaining adenovirus type 2 genome (Wang *et al.*,

2000). The ligation product was packaged into a cosmid using Epicentre's lambda phage packaging kit and the packaging products used to infect *Escherichia coli* cells. After incubation overnight, positive clones were selected and cosmid DNA purified. Purified cosmid DNA (2 μg) was digested with I-Ceu I and transfected into HEK293 cells with Lipofectamine 2000 according to the manufacturer's instructions. The HEK293 cells were grown at 37°C with 5% CO_2. Adenovirus plaques were seen 8 days after transfection and a low titre of virus [approximately 10^9 virus particles (vp) mL^{-1}] was used for functional testing in HEK293 cells. For lung slice infection, low-titre virus was further amplified to 10^{12} vp mL^{-1} on 2 sequential cesium chloride gradients and dialysed to reduce the salt concentration. For infection, 4–8 slices were pooled in a 35 mm dish, rinsed 3 times in 2 mL $Ca^{2+}Mg^{2+}$-free PBS, and incubated in a small volume (50–100 μL) of 0.2% Pluronic F-127 detergent (Calbiochem, San Diego, CA, USA) containing 10^8 vpμl^{-1} in the same buffer at 37°C for 1–3 h. After high titre infection, 3 mL fresh culture medium was added and the incubation continued with media changes at 4 day intervals. Fluorescence was sustained for at least 12 days post infection.

Measurement of β_2-AR trafficking

β_2-Adrenoceptor trafficking was monitored by time-lapse microscopy using a Zeiss LSM 410 confocal microscope equipped with a 40 × 1.2 numerical aperture water immersion objective. Slices were assembled in a coverslip perfusion chamber (Bioptechs Butler, PA, USA) along with a square of nylon mesh to gently compress the slice against the coverslip. Confocal stacks (typically 24 slices at 1.5 μm) were collected at hourly intervals for as long as 48 h with perfusion at rate of 0.1 mL·min^{-1}. For solution changes, the perfusion rate was increased to 0.5 mL·min^{-1} for 6 min. Excitation was with the 488 nm line from an Argon Krypton laser (0.2% of maximum output) and the 500–585 nm emission collected using 585 DCXR and 500 DCXR dichromatic mirrors. To maximize sensitivity, there was no emission filter. Sampling in the *x*–*y* plane was optimized at 0.16 microns × 0.16 microns per pixel. The *z*-axis was undersampled to minimize phototoxicity. Projections were rendered using a custom macro written in Image J (Rasband, 1997–2009, http://rsb.info.nih.gov/ij/). Ciliary motility was confirmed at the beginning and the end of each experiment to control for slice viability.

Results

β-Adrenoceptor agonists induce bronchodilatation of human small airways

To determine the concentrations of formoterol and salmeterol that are equi-effective with the concentration of salbutamol we previously used (0.1 μM) to produce β-adrenoceptor desensitization in human small airways (Cooper and Panettieri, 2008), concentration–response curves to the LABAs were carried out. Following the contraction of human small airways to CCh, the airways were relaxed with salbutamol, formoterol or salmeterol. Airways were allowed to reach a plateau before the following increased concentration was added to the slices. EC_{50} values for the β-agonists were 0.20 μM, 1.3 nM and 8.6 nM for salbutamol, formoterol and salmeterol respectively. Interestingly, equi-effective concentrations of formoterol and salmeterol to 0.1 μM salbutamol were determined to be 0.3 nM for both compounds (Figure 1).

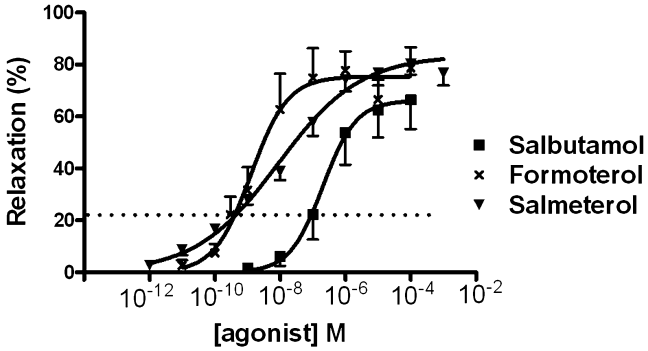

Figure 1

Concentration–response curves of human small airways to salbutamol, formoterol and salmeterol showing airway relaxation of a contractions induced by 30 μM carbachol. The dotted line indicates equi-effective concentrations to 0.1 μM salbutamol. Each data point is expressed as mean ± SEM. Each group contains three airways from each of the three donors (nine total airways).

Budesonide prevents formoterol-, but not salmeterol-induced desensitization of human small airways

To determine whether formoterol and salmeterol differentially decreased the β_2-adrenoceptor response to the β_2-agonist isoprenaline, human lung slices containing a small airway were incubated with either formoterol or salmeterol (0.3 nM) for 12 h. The airways were contracted to CCh in the absence of the LABA, and then treated with isoprenaline in the continued presence of CCh. The prolonged incubation with the LABAs, when removed, had little effect on the CCh-induced bronchoconstriction as there was no change in CCh log EC_{50} values or maximum contractions (data not shown). However, the airways did demonstrate evidence of β_2-adrenoceptor tolerance following the 12 h incubation with either formoterol or salmeterol: the maximum relaxation to isoprenaline was decreased from 72.9 ± 3.8% to 30.8 ± 7.4% ($P < 0.001$) and 44.5 ± 9.8% ($P = 0.01$) respectively; and sensitivity of the airways to isoprenaline decreased: EC_{50} values increased from 0.09 ± 0.07 μM to 0.64 ± 0.28 μM ($P < 0.001$) and to 0.20 ± 0.22 μM ($P = 0.02$) respectively (Figure 2); the differences between the effects of salmeterol and formoterol were not statistically significant. In separate experiments, budesonide (10 nM) was incubated for 1 h before the addition of either formoterol or salmeterol (thus the incubation time was 13 h in total). Budesonide alone only modestly increased the maximum relaxation response to isoprenaline (30.0%, $P = 0.17$). By contrast, pre-incubation with budesonide significantly increased the maximum relaxation to isoprenaline and prevented β_2-adrenoceptor desensitization by formoterol to 65.3% ($P = 0.04$) of that noted in the

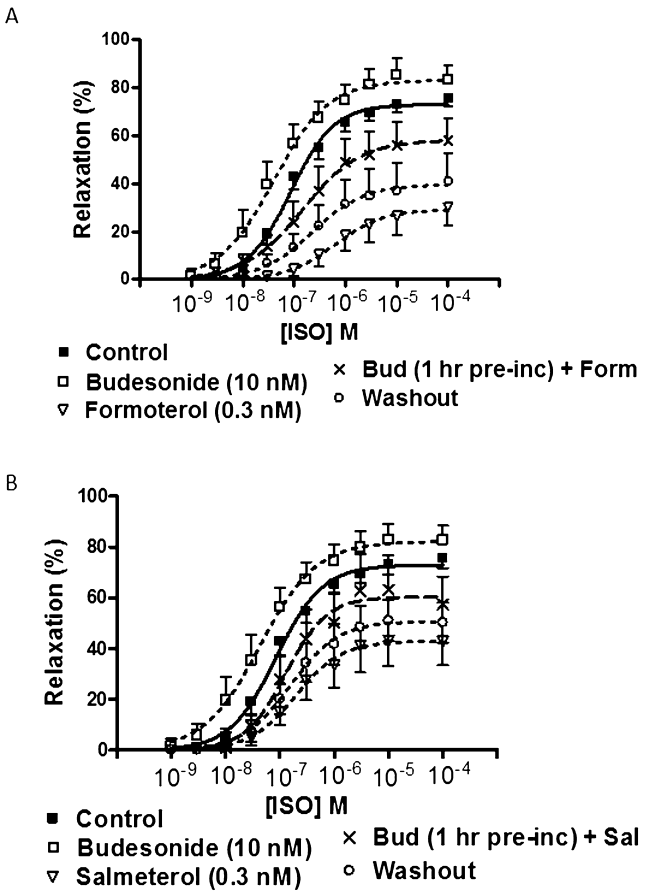

Figure 2

Chronic exposure to salmeterol and formoterol attenuates β_2-adrenoceptor-mediated relaxation. Concentration–response curves to isoprenaline (10^{-9}–10^{-4} M in the presence of 30 μM CCh) after a 12 h incubation of (A) formoterol or (B) salmeterol (0.3 nM) with lung slices containing human small airways. Budesonide (10 nM) was added to slices 1 h before the LABA and remained in contact with the airway for the entire LABA incubation period. In parallel studies, the LABA was vigorously washed out and fresh media was added to the slice for 6 h. Each data point is expressed as mean ± SEM. Each group contains two airways from each of four donors (eight airways in total).

absence of budesonide. The pre-incubation with budesonide before the addition of salmeterol also increased the maximum relaxation to isoprenaline and prevented the desensitization by 55.6% but this was not significantly different from salmeterol alone. In parallel studies, the β_2-agonists were vigorously washed from the tissue following their 12 h incubation and relaxed with isoprenaline. The wash-out protocol consisted of three media and well changes followed by a 6 h incubation in LABA-free media. Despite this aggressive washing, some of the effects of formoterol or salmeterol on β_2-adrenoceptor tolerance were retained (Figure 2). Additionally, budesonide treatment for 6 h following a 12 h incubation with either formoterol or salmeterol did not reverse this prolonged β_2-adrenoceptor tolerance (data not shown).

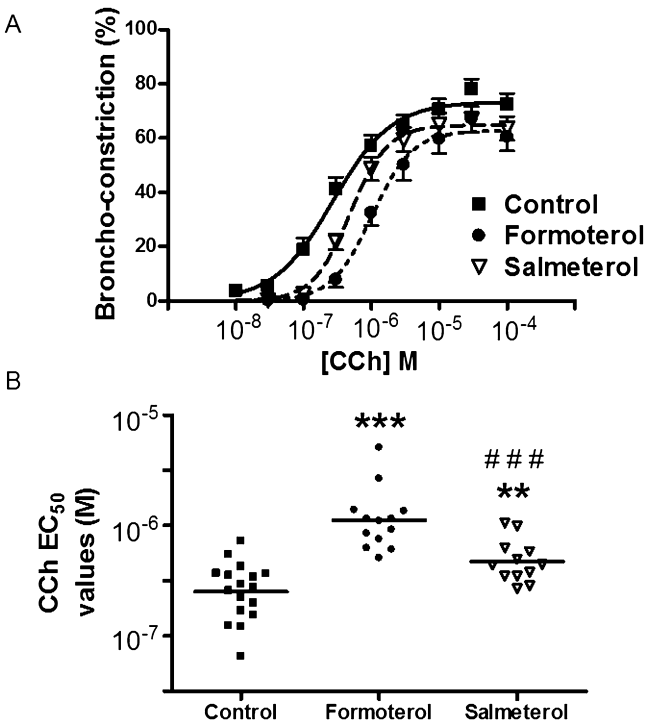

Figure 3

Formoterol decreases the sensitivity of the airways to carbachol-induced bronchoconstriction to a greater extent than salmeterol. (A) Concentration–response curves to carbachol (10^{-8}–10^{-4} M) in the absence and presence of formoterol or salmeterol (0.3 nM). (B) EC_{50} values for carbachol were calculated from airways after a 2 h incubation with formoterol or salmeterol (0.3 nM). The LABAs remained in contact with the airway during the carbachol concentration–response curves. $^{**}P < 0.01$; $^{***}P < 0.001$ versus control group, or $^{\#\#\#}P < 0.001$ versus formoterol group. Each data point is expressed as mean ± SEM. Each group contained at least four airways from each of the three donors (at total of at least 12 airways).

Formoterol decreases the sensitivity to CCh-induced bronchoconstriction more than salmeterol

To determine whether formoterol or salmeterol has an inhibitory effect on CCh-induced bronchoconstriction, airways were incubated with either β_2-agonist for 2 h at equi-effective concentrations (0.3 nM) and remained in the presence of the tissue during a full concentration–response curve to CCh. The presence of formoterol or salmeterol reduced CCh-induced bronchoconstriction, as shown by rightward shifts in the concentration–response curves (Figure 3A), and significantly increased the CCh EC_{50} values (Figure 3B). Formoterol was more effective in preventing CCh-induced bronchoconstriction than salmeterol ($P < 0.001$).

Formoterol and salmeterol promote distinct patterns of β_2-adrenoceptor trafficking in lung slices

To determine whether β_2-adrenoceptor tolerance was related to changes in β_2-adrenoceptor distribution, the effects of

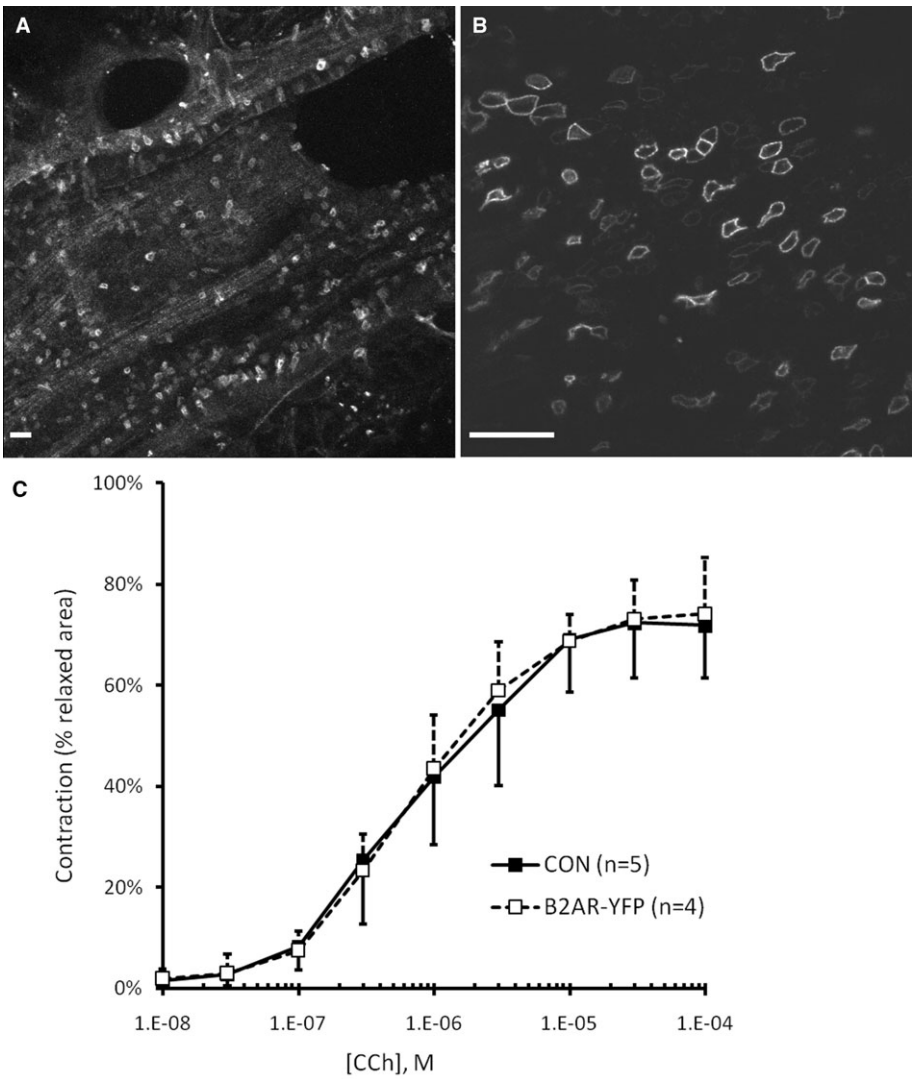

Figure 4

Expression of Ad5-CMV-human β_2-adrenoceptor-YFP in human lung slices. (A) Low magnification view of receptor expression in the airway epithelial cells of a bisected ~0.5 mm diameter bronchiole. The image is a projection of a 200 μm stack of confocal images collected at five micron intervals. See Figure S1 for an animation of this effect. (B) A higher magnification *en face* view of the of the airway epithelium demonstrating that expression in ~30% of the cells is restricted to the plasma membrane. (C) Mean concentration–response curves to carbachol in mock- and β_2-adrenoceptor-YFP-infected airways. Each data point is expressed as mean ± SEM and each group contained four or five airways from a single donor.

β_2-agonists on the localization of β_2-adrenoceptors in human airways were determined. An adenoviral vector was used to transduce human airways with β_2-adrenoceptors tagged with yellow fluorescent protein (β_2-adrenoceptor-YFP) and β_2-adrenoceptor-YFP localization was monitored by time-lapse microscopy. Expression of the fusion protein was readily observed on the plasma membranes of airway epithelial cells (Figure 4A, B), but was infrequent in airway smooth muscle cells. The expression of β_2-adrenoceptor-YFP in airway epithelial cells had no effect on the concentration–response curve for CCh-induced bronchoconstriction (Figure 4C). Therefore, the β_2-adrenoceptor trafficking in airway epithelial cells was used as a surrogate marker for trafficking in airway smooth muscle cells.

Time-lapse studies provided a direct comparison of the steady-state distribution of β_2-adrenoceptor-YFP cells before and at hourly intervals during treatment under conditions of continuous perfusion. In pilot experiments, changes in the steady-state distribution of receptors induced by 0.1 μM salbutamol were modest, so effects of higher equi-effective doses were studied. Treatment with 1 μM isoprenaline (Figure 5A) or 1 μM salbutamol (Figure 5B) induced punctuate intracellular fluorescence and a reduced, but not completely, plasma membrane fluorescence. Occasionally, a similar pattern of fluorescence was noted in long spindle-shaped cells reminiscent of airway smooth muscle cells (Figure 5C) that exhibited similar internalization responses to isoprenaline (Figure 5D). Essentially identical observations were also made in rat

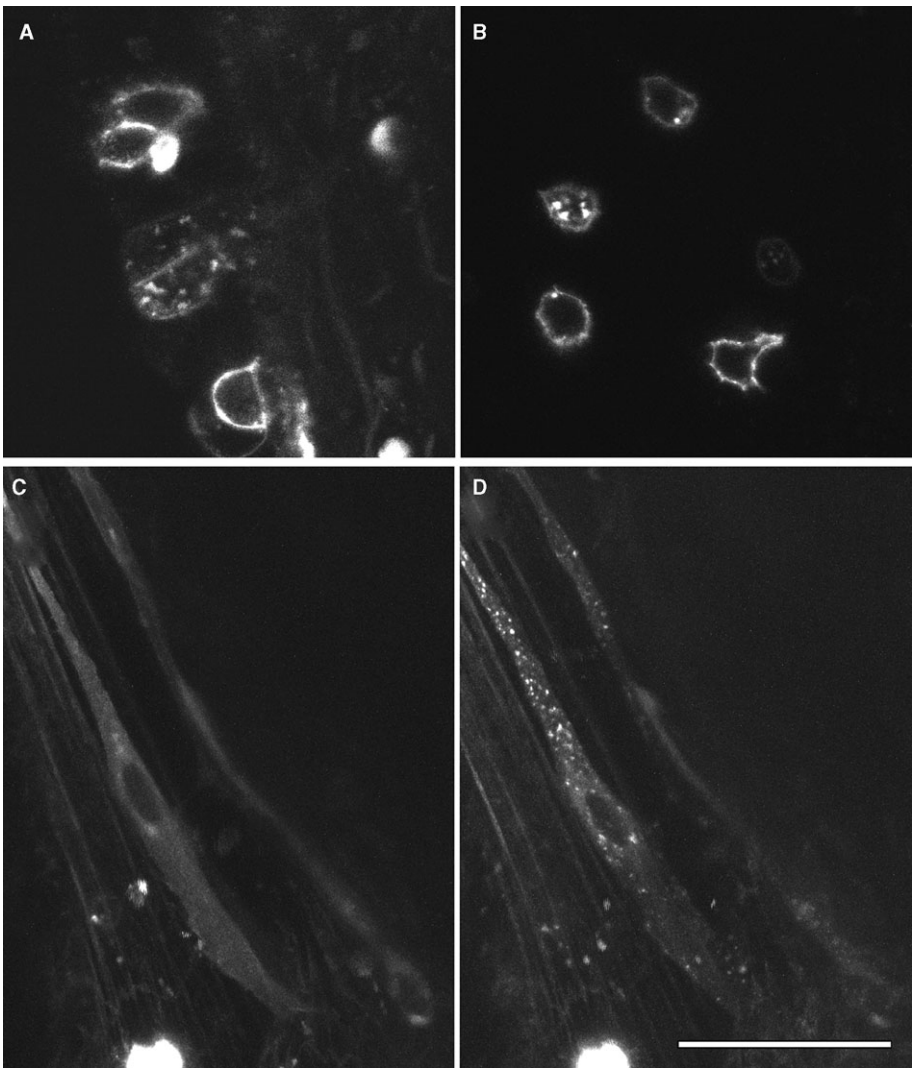

Figure 5

β-Agonist-induced trafficking of human β_2-adrenoceptor-YFP in airway epithelial and smooth muscle cells. (A) Side view of airway epithelial cells demonstrating receptor internalization after treatment for 7 h with 1.0 μM isoprenaline. (B) *En face* view of airway epithelial cells after treatment for 8 h with 1.0 μM salbutamol. (A and B) Single confocal slices extracted from three-dimensional stacks over a 24 h time course. The complete time course can be viewed in Figures S2 and S3. (C) β_2-Adrenoceptor-YFP infected cells with smooth muscle morphology in a human cultured lung slice. (D) The same cell after treatment for 1 h with isoprenaline showing accumulation of particulate intracellular fluorescence. The scale bars are 40 μm.

airways where smooth muscle transduction is much more efficient than in human airways (data not shown). Because β_2-adrenoceptor-YFP trafficking in airway epithelial cells appears similar to that in airway smooth cells, the effects of treatment with 5 nM formoterol and 50 nM salmeterol were also studied. Compared with untreated cells (Figure 6A), formoterol (12 h) promoted receptor internalization with a coincident reduction in plasma membrane fluorescence (Figure 6B). The internalized fluorescence, however, was in smaller aggregates than those noted for either isoprenaline or salbutamol. Subsequent treatment with isoprenaline (8 h) induced larger intracellular aggregates (Figure 6C) as compared with those following treatment with only isoprenaline (Figure 5A) or salbutamol (Figure 5B). By contrast, treatment with salmeterol (12 h) failed to promote a significant accumulation of intracellular aggregates or a reduction in fluorescence on or near the plasma membrane (Figure 6E). Further, after salmeterol treatment, there was little trafficking to large perinuclear aggregates following treatment with isoprenaline (Figure 6F). Treatment with higher concentrations of formoterol (50 and 500 nM) or salmeterol (250 and 1000 nM) resulted in greater reductions in plasma membrane fluorescence and corresponding increases in intracellular fluorescence for both LABAs (Figure 6G, H), but the qualitative nature of the trafficking responses remained distinct.

We also evaluated the effect of 10 nM budesonide on β_2-adrenoceptor trafficking using the YFP-tagged receptor. β_2-adrenoceptor-YFP receptor localization in airways was evaluated before and after treatment with formoterol or salmeterol for 18 h in the absence or presence of budesonide,

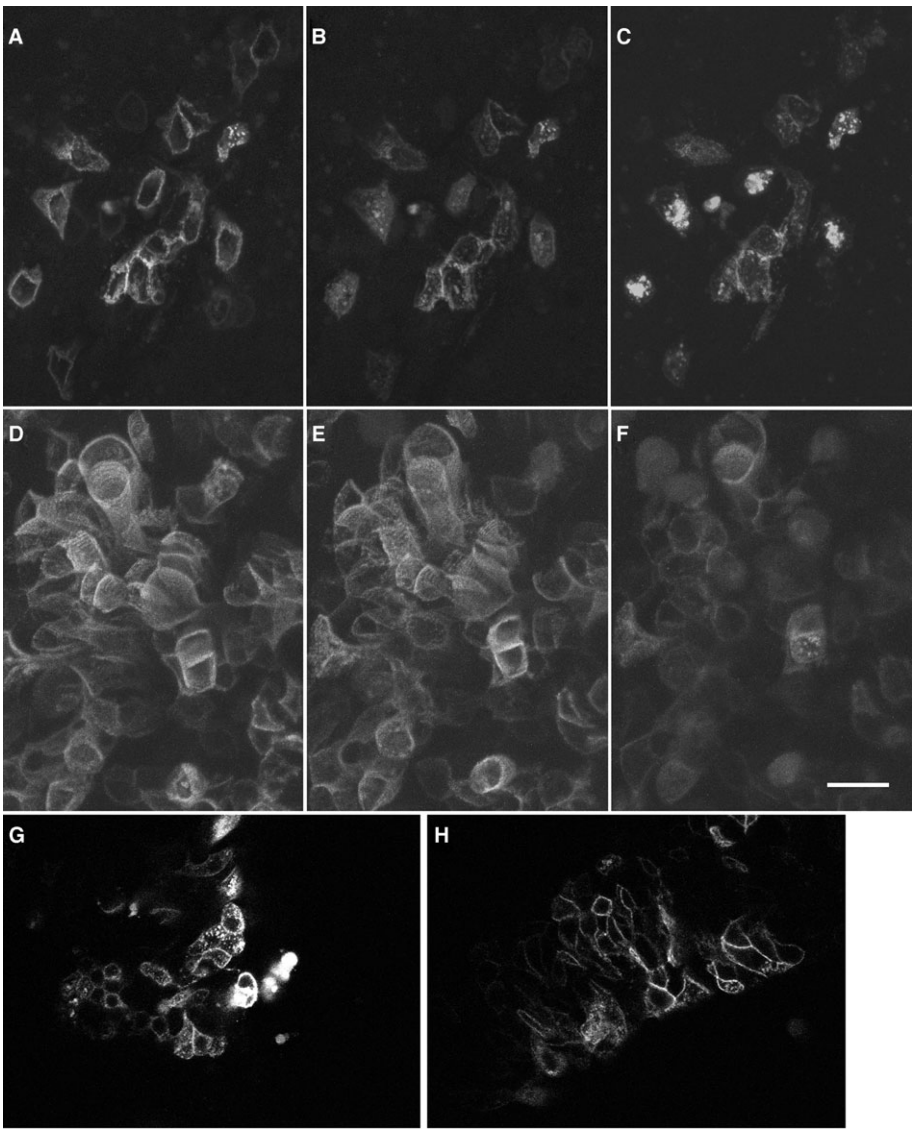

Figure 6

Induction of trafficking of human β_2-adrenoceptor-YFP by formoterol and salmeterol. (A) *En face* view of living human airway epithelial cells in a lung slice with receptor expression largely restricted to the plasma membrane. (B) The same field after treatment for 12 h with 5 nM formoterol showing internalization of receptors coincident with a reduction in plasma membrane expression. (C) The same field after an 8 h treatment with 1.0 μM isoprenaline following formoterol exposure (3D time-lapse can be viewed in Figure S4). (D) *En face* view of living human airway epithelial cells immediately before salmeterol treatment. (E) The same field after treatment for 12 h with 50 nM salmeterol. (F) The same field after 8 h treatment with 1.0 μM isoprenaline following salmeterol exposure. Each image is a maximum intensity projection of a confocal stack. The scale bar is 20 μm (3D time-lapse can be viewed in Figure S5). (G, H) Single confocal slices from fixed human airways after treatment with 50 nM formoterol (G) or 250 nM salmeterol (H) for 12 h.

added 1 h before the β-agonist (Figure 7). Consistent with the ability of budesonide to suppress functional tolerance, budesonide prevented the reduction in plasma membrane fluorescence that was noted following formoterol treatment.

Discussion and conclusions

Although LABAs differentially modulate β_2-adrenoceptor activation as full or partial agonists *in vitro*, the molecular mechanisms that mediate these effects remain elusive. Despite unique attributes, the efficacies of various LABAs remain comparable in the treatment of patients with asthma and COPD. We postulate that the comparable efficacies of LABAs are primarily due to the use of equi-effective concentrations at stimulating bronchodilatation. Using equi-effective concentrations and human PCLS, we have characterized the attributes of the LABAs most commonly used worldwide, formoterol and salmeterol, on the induction and steroid reversal of β_2-adrenoceptor tolerance, on the prevention of CCh-induced bronchoconstriction (bronchoprotection) and on β_2-adrenoceptor trafficking.

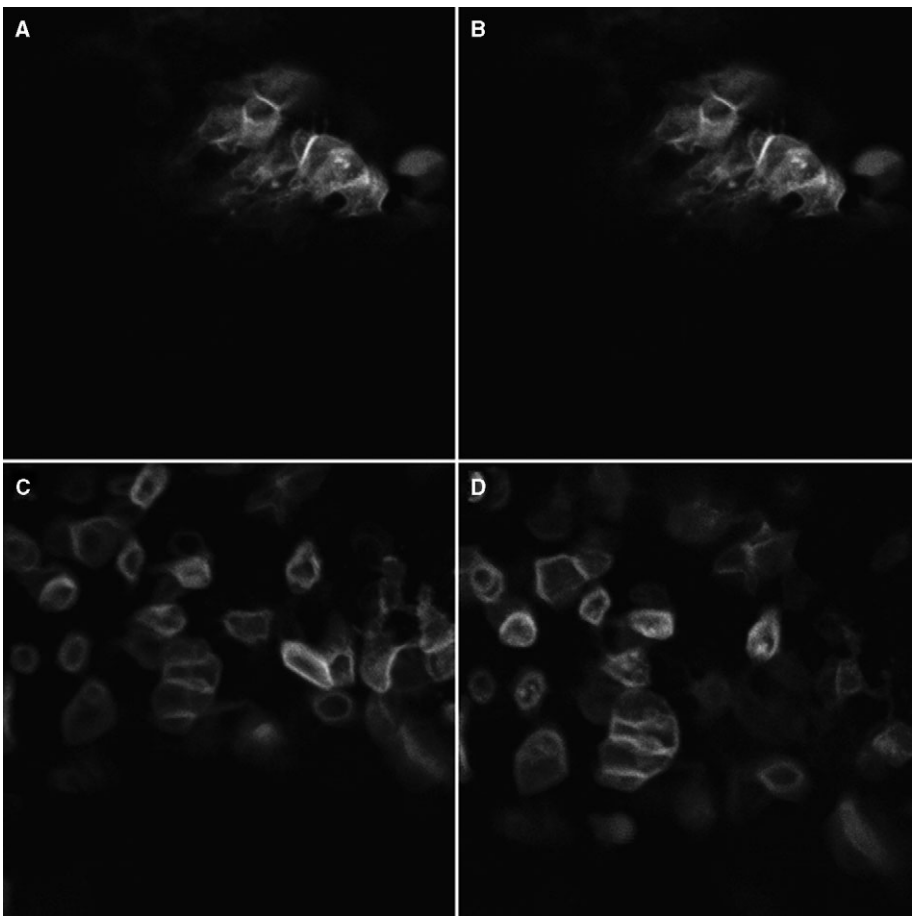

Figure 7

Budesonide suppresses the reductions in plasma membrane fluorescence induced by formoterol and salmeterol. (A, B) Confocal fluorescence images of β_2-adrenoceptor-YFP expression in airway epithelial cells in airways before (A) and after (B) they had been incubated with 10 nM budesonide and 5 nM formoterol for 18 h. (C, D) Images collected from airways before (C) and after (D) they had been incubated with 10 nM budesonide and 50 nM salmeterol.

In asthma and COPD, β_2-agonists are a cornerstone in the management of airflow obstruction and are prescribed to deliver similar levels of efficacy. In order to study β_2AR tolerance, we characterized equi-effective concentrations of formoterol and salmeterol and compared those with the effects of salbutamol, a SABA. Previously, we showed that the prolonged treatment with SABA increased tolerance to isoprenaline, an effect that was prevented and reversed by co-incubation with dexamethasone. In order to directly compare equi-effective concentrations of LABAs with the 0.1 μM salbutamol that was previously demonstrated to induce tolerance, increasing concentrations of salmeterol or formoterol were studied and cumulative concentration–response curves determined. Formoterol with a fast onset of action required less than 10 min before a plateau occurred; salmeterol had a slower onset of action and required up to 40 min to reach a plateau of bronchodilatation (data not shown). Using this experimental paradigm, we determined that 0.3 nM salmeterol and 0.3 nM formoterol are equi-effective to 0.1 μM salbutamol at reversing CCh-induced bronchoconstriction in human small airways. In contrast, others have shown that formoterol is more potent than salmeterol in human airways (Naline *et al.*, 1994; Molimard *et al.*, 1998; Sturton *et al.*, 2008). Our data show that salmeterol has an E_{max} value similar to that of formoterol; this is comparable with clinical studies that demonstrated similar mean peak bronchodilatation induced by these two compounds, albeit at different concentrations (Cazzola *et al.*, 1995), but conflicts with results obtained *in vitro* (Sturton *et al.*, 2008).

To determine whether equi-effective concentrations of the β_2-agonists induced β_2-adrenoceptor tolerance similarly, the effects of prolonged treatment with formoterol, salmeterol and salbutamol were examined on isoprenaline-induced bronchodilatation. Just as observed with prolonged (12 h) salbutamol exposure (Cooper and Panettieri, 2008), formoterol and salmeterol elicited a profound rightward shift in the isoprenaline concentration–response curve and decreased the maximum relaxation to isoprenaline while having little effect on the muscarinic-induced bronchoconstriction, obtained in the absence of the LABA. There was no significant difference between the abilities of formoterol and salmeterol (Figure 2) to decrease β_2-adrenoceptor function (i.e. induce tolerance). In previous studies, partial agonists appeared to have less

effect on β_2-adrenoceptor tolerance than full agonists (January *et al.*, 1998); these studies suggested that salmeterol acts as a partial agonist. Unlike the aforementioned studies that used concentrations of β_2-agonists based on receptor occupancy (January *et al.*, 1998; Scola *et al.*, 2004), we studied equi-effective concentrations and showed that salmeterol similar to formoterol acts as a full agonist and had the same effect on β_2-adrenoceptor tolerance. Recently, others have also defined salmeterol as a full agonist and demonstrated that salmeterol induces β_2AR tolerance to comparable levels as formoterol and indacaterol (Scola *et al.*, 2009). Duringer *et al.* using equi-effective concentrations of formoterol and salmeterol, also showed that they produced a similar maximum relaxation response and that a 4 h pre-incubation with salmeterol decreased airway sensitivity to isoprenaline (Duringer *et al.*, 2009). Collectively, these data indicate that controversy still exists as to whether LABAs are partial or full agonists. However, our results provide compelling evidence that at equi-effective doses, salmeterol and formoterol induce β_2-adrenoceptor tolerance to similar levels.

Substantial concerns surround the use of LABAs alone in the management of asthma. Interestingly, LABA monotherapy in COPD appears to improve clinical outcomes (Cazzola *et al.*, 1995). One clinical concern regarding LABA monotherapy focuses on β_2-adrenoceptor tolerance. As a platform to study reversal of β_2-adrenoceptor tolerance, PCLS were either aggressively lavaged to remove LABA or treated with steroids to prevent β_2-adrenoceptor tolerance. However, the tolerance induced by both salmeterol and formoterol was not eliminated by aggressive washing. The precise mechanisms that prevent lavage from restoring β_2-adrenoceptor sensitivity remain unclear but are unlikely to depend on ligand-cell surface receptor binding. Accordingly, β_2-adrenoceptor tolerance may be due to receptor internalization pathways within the cell that are not reversible by lavage as has been recently demonstrated for other G-protein couple-receptors (Calebiro *et al.*, 2009; Ferrandon *et al.*, 2009). Our studies are in concert with the results from clinical trials, where salmeterol and formoterol have been found to have similar efficacies and durations of action (van Noord *et al.*, 1996).

In parallel studies, we incubated the PCLSs with budesonide for 1 h before exposure to the β_2-agonist for 12 h. Similar to our previous studies with dexamethasone (Cooper and Panettieri, 2008), budesonide alone had little effect on CCh-induced bronchoconstriction. Budesonide pretreatment, however, prevented the desensitization induced by formoterol and re-established the maximum relaxation to isoprenaline to 65.3%. Curiously, budesonide had a greater effect on formoterol-induced rather than salmeterol-induced tolerance. Interestingly, budesonide has recently been shown to prevent the decrease in the bronchodilatation response to formoterol induced by pro-inflammatory cytokines in murine trachea, but to have no effect on the impaired response to salmeterol (Adner *et al.*, 2010). Our data also suggest that formoterol induces β_2-adrenoceptor tolerance to a greater extent than salmeterol, although this difference was not statistically significant. The 10 nM concentration of budesonide was chosen for our studies because this concentration of steroid inhibits TNFα-induced CCR1 in human PCLS (data not shown) and serves as a positive control for steroids that modulate PCLS function. We have also shown that it produces similar effects to fluticasone propionate on chemokine expression (Banerjee *et al.*, 2008). Further, in clinical studies, the budesonide concentration in airway and lung tissue (measured in central and peripheral lung), following a 1000 μg inhalation, approximates to 10 nM tissue concentration (van den Brink *et al.*, 2008).

The ability of β-agonists to prevent CCh-induced bronchoconstriction correlates with their clinical efficacy at preventing allergen- or exercise-induced bronchoconstriction. To assess the differential effects of LABAs on bronchoprotection, slices were pretreated with β_2-agonists and CCh-induced bronchoconstriction was then measured. Both formoterol and salmeterol inhibited CCh-induced bronchoconstriction, significantly shifting the concentration–response curves to the right. Surprisingly, although the concentrations of formoterol and salmeterol used to incubate the slices were equi-effective at inducing bronchodilatation before CCh-induced bronchoconstriction took place, formoterol caused a greater rightward shift of the dose–response curve than salmeterol. The precise molecular mechanisms mediating these effects remain unclear. A prolonged pretreatment time for the LABAs before CCh treatment was used to compensate for the disparity between the onset of action between salmeterol and formoterol. Our data indicate that the mechanism by which these LABA induce bronchoprotection is different from that involved in reversing bronchoconstriction. Although the exact mechanisms of salmeterol-induced relaxation remain unknown, low concentrations of formoterol induce airway relaxation by decreasing airway smooth muscle Ca^{2+} sensitivity. At higher concentrations, formoterol additionally slows or inhibits Ca^{2+} oscillations within smooth muscle cells to relax the airways (Ressmeyer *et al.*, 2010). Whether these mechanisms explain the differential effects of salmeterol and formoterol on bronchoprotection requires further study.

To determine the potential contribution of β_2-adrenoceptor trafficking to tolerance, a fluorescent protein-tagged receptor (Kallal *et al.*, 1998) was expressed in airways using an adenoviral vector and the steady-state expression monitored by time-lapse microscopy. Current reagents are inadequate to identify endogenous β_2-adrenoceptor using immune-cytochemical approaches; hence viral transduction of a tagged receptor was used. In human small airways, expression of β_2-adrenoceptor-YFP was largely restricted to epithelial cells. As both human airway epithelial cells and alveolar macrophages undergo β_2-adrenoceptor desensitization and down-regulation in response to inhaled β_2-agonist (Turki *et al.*, 1995), we characterized β_2-adrenoceptor expression in airway epithelial cells and assumed that similar responses occur in airway smooth muscle. In supporting of this assumption, long spindle-shaped fluorescent cells were occasionally observed that exhibited β_2-adrenoceptor-YFP trafficking patterns similar to those in epithelial cells. Further, in rat airways, where airway smooth muscle transduction is much more efficient than in human airways, similar trafficking patterns were also observed (R.C. Kurten, unpubl. obs.).

The time-lapse microscopy experiments revealed differences in the redistribution of receptors from the plasma membrane to the cell interior in response to the short- versus LABA used. Both salbutamol and isoprenaline stimulated

receptor internalization into small vesicles that increased in size with time. These responses are consistent with those described in cell lines and probably correspond to trafficking to early and recycling endosomes with eventual trafficking to lysosomes for degradation (von Zastrow and Kobilka, 1992; Kallal *et al.*, 1998; Moore *et al.*, 1999; 2004). By contrast, formoterol treatment promoted receptor internalization into small vesicles only. Moreover, the loss in plasma membrane fluorescence after formoterol treatment was reduced compared with that induced by salbutamol or isoprenaline, perhaps indicating an enhancement in receptor recycling (Morrison *et al.*, 1996; Cao *et al.*, 1999; Millman *et al.*, 2004; Moore *et al.*, 2004; Hanyaloglu *et al.*, 2005; Hanyaloglu and von Zastrow, 2007; Parent *et al.*, 2009). Further, on subsequent treatment with isoprenaline in the absence of formoterol, β_2-adrenoceptors in formoterol-pretreated cells moved into large punctuate vesicles similar to those observed after treatment with either isoprenaline or salbutamol alone.

β_2-Adrenoceptor trafficking in response to salmeterol was qualitatively distinct from that induced by formoterol, isoprenaline or salbutamol. First, the loss in plasma membrane fluorescence and the appearance of small intracellular vesicles were much reduced after salmeterol treatment as compared with formoterol treatment. The use of higher doses of formoterol and salmeterol did not alter these distinctions. Similar results have been noted in cultured cell lines (Kallal *et al.*, 1998) and attributed to a reduction in high-affinity β-arrestin binding (Moore *et al.*, 2007) required for β_2-adrenoceptor endocytosis (Ferguson *et al.*, 1996; Goodman *et al.*, 1996). Although these results are mechanistically consistent with the somewhat reduced tolerance to isoprenaline bronchodilatation induced by salmeterol compared with formoterol in our assays (Figure 2), these differences were not statistically significant. However, EC_{70} doses were used for the trafficking assays whereas EC_{20} doses were used in the bronchodilatation assays. However, treatment with isoprenaline (in the absence of salmeterol) after prolonged salmeterol treatment failed to promote the accumulation of β_2-adrenoceptors in large perinuclear vesicles noted after formoterol treatment followed by isoprenaline. These data suggest that salmeterol treatment has little impact on the steady-state distribution of β_2-adrenoceptors and that this effect persists on restimulation with isoprenaline. Pretreatment with budesonide prevented the reductions in plasma membrane fluorescence promoted by formoterol and this could suppress the development of tolerance. β_2-adrenoceptor expression from the adenoviral vector is driven by a constitutive CMV promoter, so the effect of budesonide cannot be at the level of β_2-adrenoceptor gene transcription. Post-transcriptional mechanisms that may account for the observed effects of budesonide include stabilization of the β_2-adrenoceptor mRNA (Danner *et al.*, 1998) or effects on endosomal trafficking.

The changes in receptor distribution that we observed in human airway cells are not absolute. That is, although the intensity of plasma membrane fluorescence declines with β_2-agonist treatment, the signal does not disappear completely. Consistent with our observations, the bronchodilatation and bronchoprotection induced by the β_2-agonists were also reduced over time, but were not completely abolished. Interestingly, the retention of β_2-adrenoceptor-YFP fluorescence at or near the cell surface following salmeterol treatment does not reduce tolerance as compared with treatment with formoterol or salbutamol, where β_2-adrenoceptor were internalized into small and large vesicles respectively. Desensitization and tolerance, in part, are thus independent of the reductions in β_2-adrenoceptor density on the cell surface that would be expected to occur following the initiation of a slow recycling and/or enhanced lysosomal targeting pathway. Although endosomes are frequently considered conduits for the degradation and recycling of surface receptors, endosomes also provide a signalling platform for diverse families of receptors (Murphy *et al.*, 2009). Our previous studies indicate that tolerance to β_2-agonists occurs before the activation of adenylyl cyclase, as forskolin rapidly and effectively dilates desensitized airways (Cooper and Panettieri, 2008). An intriguing possibility is that the differential trafficking of β_2-adrenoceptors in response to β_2-agonists characterizes the nature of the response including the time to onset and the duration of action. Indeed, experiments using FRET reporters to monitor intracellular cAMP following activation of parathyroid receptor 1 (Ferrandon *et al.*, 2009) and the thyroid-stimulating hormone receptor (Calebiro *et al.*, 2009) show that hormonal signalling to adenylyl cyclase is not necessarily restricted to the plasma membrane, but also occurs on endosomes. Similarly, losses in spatially restricted signalling by β_2-adrenoceptors correlate with disease pathogenesis in the heart (Nikolaev *et al.*, 2010).

In summary, our studies show that formoterol and salmeterol act comparably as bronchodilators in human small airways. Both bronchodilators at equi-effective doses induced a similar degree of β_2-adrenoceptor tolerance that, in the case of formoterol was significantly reduced by budesonide. Formoterol was more potent than salmeterol at inducing bronchoprotection but both produced similar maximal effects at preventing CCh-induced bronchoconstriction. This observation indicates a limitation of the present study in that the selection of the parameter with which to characterize equi-effective doses is arbitrary. One could select bronchoprotection against specific doses of CCh or bronchodilatation measured after a different exposure interval. We selected bronchodilatation after 10–20 min to best reflect the clinical problem of β-agonist tolerance. The differences in receptor distribution following β_2-agonist treatment were not directly related to differences in the magnitude of β_2-adrenoceptor tolerance. While this could be because the β_2-agonist doses used to study receptor trafficking (EC_{70}) were higher than those used to study contractility (EC_{20}), the doses used were consistently equi-effective with respect to bronchodilatation. Recognizing the limitations imposed by using airway epithelial cell β_2-adrenoceptor trafficking as an indicator of airway smooth muscle β_2-adrenoceptor trafficking, we suggest that distinct β_2-adrenoceptor trafficking patterns are probably more relevant to shaping tissue responses to β_2-agonists than they are in terminating the responses. Future studies are needed to confirm this for human airway smooth muscle cells. We have described an approach by which mechanisms responsible for shaping β_2-agonist responses can be defined within the native tissue structure in physiologically responsive human airways. Further analysis of β_2-adrenoceptor trafficking and signalling using this platform should provide insights into the mechanisms that determine the lung

responses to β_2-agonists and provide new therapeutic targets or strategies to prevent or reverse tolerance to β_2-agonists.

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Conflicts of interest

DN and ID work for AstraZeneca UK; the other authors do not have any conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Animated reconstruction (–20 to +20 degree views) for Figure 4A.
Figure S2 3D time-lapse for Figure 5A (~24 h 1 uM isoprenaline).
Figure S3 3D time-lapse for Figure 5B (~24 h 1 uM albuterol).
Figure S4 3D time-lapse for Figure 6A–C (~24 h 5 nM formoterol followed by 1 uM isoprenaline).
Figure S5 3D time-lapse for Figure 6D–F (~24 h 50 nM salmeterol followed by 1 uM isoprenaline).

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