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RESEARCH PAPER

β-Blockers have differential effects on the murine asthma phenotype

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BACKGROUND AND PURPOSE

Our previous studies have shown the β_2-adrenoceptor and its endogenous ligand, adrenaline, are required for development of the asthma phenotype in murine asthma models. Chronic administration of some, but not other, β-blockers attenuated the asthma phenotype and led us to hypothesize that biased signalling was the basis of their differential effects, experimentally and clinically.

EXPERIMENTAL APPROACH

We used mice with no detectable systemic adrenaline ($PNMT^{-/-}$) and wild-type (WT) mice to study the effects of four β-blockers, alprenolol, carvedilol, propranolol and nadolol, in an ovalbumin sensitization and challenge (Ova S/C) murine model of asthma. The parameters measured were inflammatory cell infiltration, mucous metaplasia and airway hyperresponsiveness. To interpret the pharmacological action of these ligands quantitatively, we conducted computer simulations of three-state models of receptor activation.

KEY RESULTS

Ova S/C $PNMT^{-/-}$ mice do not develop an asthma phenotype. Here, we showed that administration of alprenolol, carvedilol or propranolol in the absence of interference from adrenaline using Ova S/C $PNMT^{-/-}$ mice resulted in the development of an asthma phenotype, whereas nadolol had no effect. Ova S/C WT mice did develop an asthma phenotype, and administration of alprenolol, propranolol and carvedilol had no effect on the asthma phenotype. However, nadolol prevented development of the asthma phenotype in Ova S/C WT mice. Computer simulations of these four ligands were consistent with the isolated three-state receptor model.

CONCLUSION AND IMPLICATIONS

β-Blockers have different effects on the murine asthma phenotype that correlate with reported differences in activation or inhibition of downstream β_2-adrenoceptor signalling pathways.

Abbreviations

AHR, airway hyperresponsiveness; BALF, broncho-alveolar lavage fluid; E_{max}, maximal effective concentration; i.n., intra-nasal; MEK, MAPK kinase; NHBE, normal human bronchial epithelial cells; Ova S/C, ovalbumin sensitization and challenge; PAFS, periodic acid fluorescent Schiff's; PC_{100}, provocative concentration of methacholine that results in a doubling of baseline airway resistance; WT, wild-type

Tables of Links

TARGETS	
GPCRs[a]	**Enzymes**[b]
β_2-adrenoceptor	Adenylyl cyclase (AC)
	ERK1
	ERK2
	MEK

LIGANDS
Adrenaline (epinephrine)
Alprenolol
Carvedilol
Propranolol

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.,* 2013a,b).

Introduction

Asthma is a chronic inflammatory disorder of the airways that affects 300 million people worldwide (Foundation, 2014). A mainstay of asthma therapy is the use of β_2-adrenoceptor agonists. β_2-Adrenoceptor agonists are the most potent bronchodilators ever discovered and the drugs of choice for treating the bronchospasms associated with an acute asthma attack. However, chronic administration of β_2-adrenoceptor agonists has been associated with a loss of asthma control and a small, but significant, increase in asthma-related deaths (Theron *et al.*, 2013).

The β_2-adrenoceptor has been shown to be required for development of the asthma phenotype in murine models (Nguyen *et al.*, 2009). We have also previously shown that chronic administration of certain β-blockers with inverse agonist properties like nadolol or ICI-118,551 or genetic deletion of the β_2-adrenoceptor significantly attenuates the asthma phenotype in murine asthma models (Callaerts-Vegh *et al.*, 2004; Nguyen *et al.*, 2008, 2009). A subsequent study to investigate the role of constitutive signalling as driving the asthma phenotype, and inverse agonism as the key property for β-blockers, showed that genetically altered mice lacking any detectable circulating adrenaline would not develop the asthma phenotype (Thanawala *et al.*, 2013). We also recently confirmed that adrenaline was required for normal human bronchial epithelial (NHBE) cells to increase mucin production in response to IL-13 and that this effect was mediated by the β_2-adrenoceptor (Al-Sawalha *et al.*, 2015). Taken together, these results showed that ligand activation of the β_2-adrenoceptor was required for development of the asthma phenotype in mice and for mucin production in primary cultures of human cells and thus ruled out inverse agonism as the key property of β-blockers that attenuated the asthma phenotype (Thanawala *et al.*, 2013).

Also, the results of two clinical trials using the β_2-adrenoceptor inverse agonists, nadolol and propranolol, led to different outcomes, further ruling out inverse agonism as the key drug property. Pilot studies treating mild asthmatics with chronic nadolol resulted in decreased airway hyperresponsiveness (AHR) to methacholine (increased the provocative concentrations of methacholine that produce a 20% decrease in forced expiratory volume) (Hanania *et al.*, 2008, 2010) and have led to an ongoing double-blind, placebo-controlled multicentre clinical trial (ID# NCT01804218) testing the effects of chronic nadolol administration to mild asthmatics. However, subsequent to the pilot studies with nadolol, another pilot clinical study showed that chronic administration of another β_2-adrenoceptor inverse agonist, propranolol, was not effective in decreasing airway responsiveness to methacholine or histamine in a subset of asthmatics who were on inhaled corticosteroids (Short *et al.*, 2013a, b; Anderson *et al.*, 2014). These data highlighted a discrepancy in the effectiveness of β_2-adrenoceptor inverse agonists in asthma and, coupled with the results in murine models of asthma, necessitated a new hypothesis from that of inverse agonism at the canonical Gs-AC-cAMP pathway to explain the differential effects of β-blockers (Thanawala *et al.*, 2013, 2014).

Studies have shown that the β_2-adrenoceptor can signal through at least two independent pathways: the canonical Gs-AC-cAMP pathway and activation of MAPKs like ERK1/2 (Galandrin and Bouvier, 2006; Shenoy *et al.*, 2006; Wisler *et al.*, 2007; van der Westhuizen *et al.*, 2014). Several *in vitro* cell-based studies have consistently shown that while propranolol is an inverse agonist at the Gs-cAMP signalling pathway, it is also a partial agonist (relative to adrenaline or isoprenaline) at ERK1/2 activation through the β_2-adrenoceptor (Azzi *et al.*, 2003; Baker *et al.*, 2003; Galandrin and Bouvier, 2006; Wisler *et al.*, 2007; van der Westhuizen *et al.*, 2014). In contrast, nadolol does not activate either of these signalling pathways of the β_2-adrenoceptor (Galandrin and Bouvier, 2006; Wisler *et al.*, 2007; van der Westhuizen *et al.*, 2014). In the current study, we hypothesized the differential signalling profiles at the two β_2-adrenoceptor pathways could explain the discrepancy in the effectiveness of different β-blockers in murine asthma models and contribute to the understanding of the divergent clinical data (Thanawala *et al.*, 2014).

In order to characterize the signalling of the β_2-adrenoceptor ligands, we applied the three-state model of receptor activation (Leff *et al.*, 1997). In this model, there are two modes of operation: 'intact' and 'isolated'. In the intact mode of operation, the receptor equilibria driving the

two signalling pathways are linked, and the signalling through one pathway affects the signalling in the other. In the isolated mode, the equilibria are not linked, and the signalling pathways operate independently of one another. In the present study, we used computer simulations to determine which of these modes of operation the β_2-adrenoceptor ligands conformed to. Our results suggest that the β_2-adrenoceptor ligands behave in a manner consistent with the isolated mode of operation of the three-state model.

The PNMT$^{-/-}$ mice used in our studies provide a pharmacological system that for the first time allows the endogenous hormone to be 'replaced' with any exogenous ligand and therefore enables the inherent pharmacological effects of ligands to be studied *in vivo*. We had previously confirmed that the attenuation of the asthma phenotype was mediated by eliminating adrenaline activation of the β_2-adrenoceptor because WT mice treated with a reserpine dose that completely depleted both adrenaline and noradrenaline produced the same results as those in PNMT$^{-/-}$ mice (Thanawala *et al.*, 2013). PNMT$^{-/-}$ mice have non-detectable levels of serum adrenaline, but their levels of serum noradrenaline are similar to those in WT mice, thus ruling out any role for noradrenaline activation of the β_2-adrenoceptor in producing the asthma phenotype. The depletion of adrenaline in PNMT$^{-/-}$ mice also makes comparisons of *in vivo* and *in vitro* results pharmacologically more meaningful. Furthermore, unlike using model systems such as 'receptor activated solely by a synthetic ligand' or 'designer receptor exclusively activated by designer drugs', by using the PNMT$^{-/-}$ mice, we can study the effects of numerous synthetic ligands and their effects at the WT endogenous receptor (β_2-adrenoceptor) instead of a genetically modified receptor.

Our *in vivo* results show that β-blockers that activate ERK1/2 *in vitro*, such as alprenolol, carvedilol and propranolol, produce an asthma phenotype in ovalbumin sensitization and challenge (Ova S/C) PNMT$^{-/-}$ mice and do not attenuate the asthma phenotype produced by Ova S/C in wild-type (WT) mice. Conversely, in Ova S/C PNMT$^{-/-}$ mice, administration of nadolol, a β-blocker that does not activate ERK1/2 signalling, does not produce the asthma phenotype and prevents development of the asthma phenotype in Ova S/C WT mice. Our results thus show that despite the requirement of the β_2-adrenoceptor (Nguyen *et al.*, 2009) and the requirement that the receptor must be activated by a ligand (Thanawala *et al.*, 2013), the required activation is completely independent of the ligand's activity *in vitro* activity at the canonical Gs-AC-cAMP pathway. Furthermore, mathematical modelling of the two variants of the three-state model of receptor activation was also consistent with the independence of the two pathways. These findings are also consistent with the differential outcomes in the clinical trials with nadolol compared with propranolol (Hanania *et al.*, 2008, 2010; Short *et al.*, 2013a, b; Anderson *et al.*, 2014).

Methods

Animals

Male and female PNMT$^{-/-}$ mice 5 to 8 weeks old (mating pairs gifted by Steven Ebert, University of Central Florida) and their WT mice (Sv/129J) from Jackson Laboratories (Bar Harbor, ME, USA) were used for the study. PNMT$^{-/-}$ mice lack the enzyme PNMT to convert noradrenaline to adrenaline. PNMT$^{-/-}$ mice have no circulating plasma levels of adrenaline but have noradrenaline plasma levels comparable with WT mice (Thanawala *et al.*, 2013). All mice were genotyped for the PNMT gene and phenotyped for the circulating levels of adrenaline using HPLC (Thanawala *et al.*, 2013). Mice were randomized into treatment groups, and separate sets of mice were used to determine inflammation (inflammatory cell infiltration and mucous metaplasia) and AHR. The number of mice used in each group is indicated in the respective figure legends. All mice were housed under specific pathogen-free environment at an ambient temperature (22–24°C) and 48% humidity under a 12 h light/dark cycle. Food and water were provided *ad libitum*, and every effort was made to minimize any pain or discomfort to the animals. The University of Houston Institutional Animal Care and Use Committee approved all the procedures and protocols. All studies are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Treatment protocols and drugs

We used the Ova S/C model of murine asthma. Details of the treatment have been described previously and are shown in Figure 1 (Nguyen *et al.*, 2008). Briefly, mice were sensitized to 2 mg·kg^{-1}·day^{-1} ovalbumin with 2 mg alum i.p. on days

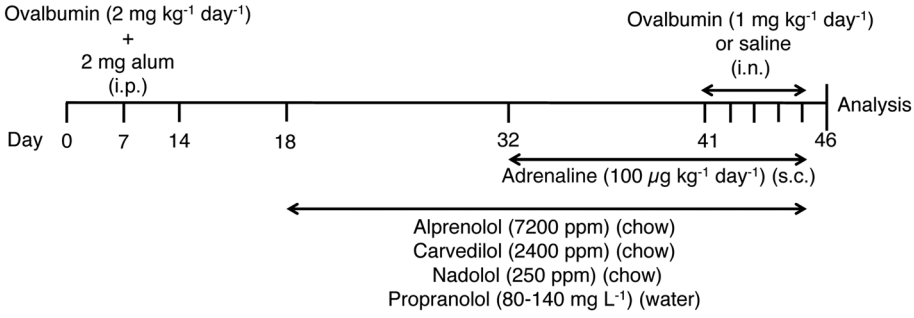

Figure 1
Treatment protocol.

0, 7 and 14, followed by once daily intranasal (i.n.) challenge with 1 mg·kg^{-1}·day^{-1} ovalbumin or saline on days 41–45. Mice were randomized to groups administered propranolol orally (80–140 mg·L^{-1}, Sigma-Aldrich, St Louis, MO, USA) in water or alprenolol (7200 ppm, Santa Cruz Biotechnologies, Dallas, TX, USA) or carvedilol (2400 ppm, Glenmark, Mumbai, India) or nadolol (250 ppm, Sigma-Aldrich) *ad libitum* mixed with chow for 28 days (Knowles *et al.*, 2001; Callaerts-Vegh *et al.*, 2004).

Additionally, a group of PNMT$^{-/-}$ mice was administered adrenaline bitartrate (equivalent to 100 μg·kg^{-1}·day^{-1} adrenaline, Sigma-Aldrich) dissolved in 0.2% ascorbic acid in saline via osmotic mini-pumps (#1002, Alzet, Cupertino, CA, USA). The adrenaline solution was bubbled with nitrogen to reduce oxidative degradation and passed through 0.45 μm filters before filling in the osmotic pumps. This dose of adrenaline was chosen because it resulted in plasma levels comparable with the average endogenous plasma levels of adrenaline observed in WT mice. The treatment time with adrenaline was shortened to 14 days (compared with 28 days with the other ligands) based on the stability of adrenaline because, even in the presence of ascorbic acid, ~25% adrenaline was degraded after 14 days (data not shown) (Terres *et al.*, 1989; Khasar *et al.*, 2003, 2005). On day 46, mice were studied for three features of the asthma phenotype as described previously (Thanawala *et al.*, 2013).

Mice were sensitized i.p. on days 0, 7 and 14 with 2 mg·kg^{-1}·day^{-1} ovalbumin and 2 mg alum. The mice were challenged on days 41–45 with 1 mg·kg^{-1}·day^{-1} i.n. ovalbumin or saline [control (CTL)]. Randomized groups of mice received adrenaline (100 μg·kg^{-1}·day^{-1} for 14 days via osmotic pumps), alprenolol (7200 ppm in chow), carvedilol (2400 ppm in chow), nadolol (250 ppm in chow) or propranolol (80–140 mg·L^{-1} in water) *ad libitum* for 28 days. At the end of the protocol, the mice were analysed for the asthma phenotype. For all the parameters described, the measurements were made in a 'blinded fashion', meaning that the investigator making the measurements had no knowledge of the treatment the mice had received.

Inflammatory cell infiltration

Mice were killed with 100 mg·kg^{-1} i.p. pentobarbital sodium, and the trachea was cannulated with an 18 G luer stub adapter. The left lobe of the lung was isolated using a haemostat, and the right lung lobe was lavaged with 400–500 μL of sterile saline to obtain the broncho-alveolar lavage fluid (BALF). BALF was then used to count the total number of cells in the airways using a haemocytometer. The remaining BALF was spun onto a charged slide using Cytospin® (Thermo Scientific, Waltham, MA, USA) and stained with Wright–Giemsa stain (Sigma-Aldrich) to allow visualization of the different granulocytes. Ova S/C is a eosinophilic model of asthma; therefore, the number of eosinophils was determined by light microscopy of the BALF from five random fields at 40× as a percentage of the total cells in each field and expressed as eosinophils mL^{-1} of BALF.

Mucous metaplasia

After obtaining BALF from the right lung lobe, the haemostat isolating the left lobe was removed. Both lobes of the lungs were then perfused and fixed with cold 4–10% formalin (Sigma-Aldrich) via the cannula for 24 h. After 24 h, the left lobe of the lung was sectioned transversely, dehydrated and embedded in paraffin blocks for sectioning. Five micrometre transverse sections of the lung were obtained using a microtome and stained with periodic acid fluorescent Schiff's (PAFS) stain. The sections were then excited using TritC or Texas Red filters (emission peak 628 nm) for glycoproteins (mucin) and GFP filter (emission peak 531 nm) for parenchymal cells to allow morphometric analysis of mucin content in the airways (Kim *et al.*, 2008; Piccotti *et al.*, 2012; Thanawala *et al.*, 2013).

Airway hyperresponsiveness

A separate set of mice was used for the measurement of AHR. AHR to increasing doses of methacholine was measured using the forced oscillation technique on the Flexivent® (Scireq, Montreal, Canada) with an in-line nebulizer. Mice were

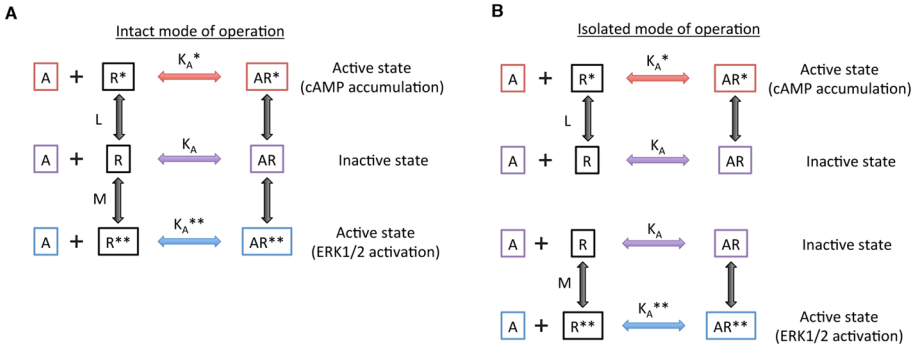

Figure 2

Schematic representation of the three-state model of receptor activation. In the model, the receptor exists in three conformations: two active states, *R** and *R***, and the inactive state, *R*. Ligands, denoted by *A*, interact with the receptor conformations with affinities K_A, K_A* and K_A**. Positive agonists have higher affinity for either or both of the active conformations (*R** and *R***); inverse agonists have higher affinity for the inactive state, *R*. *L* and *M* determine the ratios of receptor conformations in the absence of ligand and the extent of constitutive activity. Panels A and B of Figure 2 illustrate, respectively, the intact and isolated modes of operation of the model. In the intact mode, all the receptor conformations are in equilibrium with each other, and interactions in one pathway affect those in the other. In the isolated mode, the equilibria for the two pathways are unconnected, and the model operates as two independent two-state schemes.

anaesthetized with a mixture of ketamine (240 mg·kg^{-1}) and xylazine (48 mg·kg^{-1}), and booster doses were administered as needed. The trachea was cannulated using an 18 G cannula. Mice were maintained on a water-heated pad to avoid hypothermia due to anaesthesia during the entire procedure. If heart rate fell below 40 beats min^{-1} (ECG), the mouse was considered dead for the purpose of the experiment and killed immediately. The airway resistance to inhaled methacholine (1, 2.5, 10, 25 and 50 mg·mL^{-1}) was calculated by averaging the three peak resistance values for each dose. The airway sensitivity [provocative concentration of methacholine that results in a doubling of baseline airway resistance (PC_{100})] and reactivity were calculated by a non-linear regression analysis with exponential growth curve fit of the dose–response curve (Thanawala *et al.*, 2013).

Mathematical modelling

The three-state receptor model of Leff *et al.* (1997) was employed to interpret experimental data (Figure 2).

According to this model, the effects of ligands on the cAMP accumulation and ERK1/2 activation pathways are described by the following equations and using the following assumptions. For simplicity, it is assumed that response through the cAMP pathway is proportional to the fraction of receptors in the R^* state and that response through the ERK1/2 activation pathway is proportional to the fraction of receptors in the R^{**} state. The model can operate in basic modes. In the 'intact' mode, all the receptor equilibria are interconnected, and events in one pathway influence those in the other. In the 'isolated' mode, the two pathways operate independently of one another. The corresponding equations are as follows.

Intact three-state system:

$$f_{R^*} = \frac{\frac{1}{L} + \left[\frac{1}{L \cdot K_A^*}\right] \cdot [A]}{\left[1 + \frac{1}{L} + \frac{1}{M}\right] + \left[\frac{1}{K_A} + \frac{1}{L \cdot K_A^*} + \frac{1}{M \cdot K_A^{**}}\right] \cdot [A]}$$

$$f_{R^{**}} = \frac{\frac{1}{M} + \left[\frac{1}{M \cdot K_A^{**}}\right] \cdot [A]}{\left[1 + \frac{1}{L} + \frac{1}{M}\right] + \left[\frac{1}{K_A} + \frac{1}{L \cdot K_A^*} + \frac{1}{M \cdot K_A^{**}}\right] \cdot [A]}$$

Isolated three-state system:

$$f_{R^*} = \frac{\frac{1}{L} + \left[\frac{1}{L \cdot K_A^*}\right] \cdot [A]}{\left[1 + \frac{1}{L}\right] + \left[\frac{1}{K_A} + \frac{1}{L \cdot K_A^*}\right] \cdot [A]}$$

$$f_{R^{**}} = \frac{\frac{1}{M} + \left[\frac{1}{M \cdot K_A^{**}}\right] \cdot [A]}{\left[1 + \frac{1}{M}\right] + \left[\frac{1}{K_A} + \frac{1}{M \cdot K_A^{**}}\right] \cdot [A]}$$

where f_{R^*} is the fraction of receptors in the active state R^* (it is also indicative of the activity of the ligand at R*); $f_{R^{**}}$ the fraction of receptors in the active state R^{**} (it is also indicative of the activity of the ligand at R^{**}); $[A]$ the concentration of the ligand A; $[R]$ the concentration of the inactive state of the receptor, R; $[R^*]$ the concentration of the receptor active state 1, R^* (activates the cAMP pathway); $[R^{**}]$ the concentration of the receptor active state 2, R^{**} (activates the ERK1/2 activation pathway); L the equilibrium constant for the ratio of R to R^*; M the equilibrium constant for the ratio of R to R^{**}; K_A the equilibrium dissociation constant that determines affinity of the ligand for R; K_A^* the equilibrium dissociation constant that determines affinity of the ligand for R^*; and K_A^{**} the equilibrium dissociation constant that determines affinity of the ligand for R^{**}.

In order to characterize the pharmacological profiles of the β_2-adrenoceptor ligands used in this study, we analysed the data from Van der Westhuizen *et al.* (2014) with permission granted from the American Society for Pharmacology and Experimental Therapeutics and the data files provided by a coauthor. This data set represents a comprehensive study of ligand activities through the cAMP and ERK1/2 pathways (Tables 1 and 2). The values used for the mathematical modelling are shown in Table 3. Values of K_A, K_A^* and K_A^{**} were estimated from the EC_{50} and E_{max} values for the ligands and with knowledge of the properties of the model: for positive agonism through both pathways, $K_A^* < K_A$ and $K_A{**} < K_A$; for inverse agonism through both pathways $K_A^* > K_A$ and $K_A{**} > K_A$. Values of L and M were chosen based on the observed levels of constitutive activity and manifestation of inverse agonism through the two pathways. Arbitrary values of 2 and 6 were chosen for L and M, respectively, in order to allow for constitutive receptor activity and the manifestation of inverse agonism. In the three-state model, ligand bias is simply represented by the ratio between K_A, K_A^* and K_A^{**}. For example, bias towards the ERK1/2 activation pathway is given by K_A^*/K_A^{**}.

Statistical analysis

Prism® (version 4.0a; GraphPad, San Diego, CA, USA) was used for statistical testing of the data. Normally distributed data were

Table 1

Experimental data for accumulation of cAMP by β_2-adrenoceptor from Van der Westhuizen *et al.* for dose–response curves in Figure 7

No.	Ligand	E_{max}	pEC_{50}
1	Isoprenaline	100	8.23 ± 0.15
2	Salbutamol	111.3 ± 10.5	9.08 ± 0.13
3	Salmeterol	105.5 ± 6.11	8.63 ± 0.20
4	Labetalol	51.84 ± 3.73	7.82 ± 0.23
5	Bucindolol	62.07 ± 7.48	8.64 ± 0.38
6	Alprenolol	35.76 ± 3.70	9.81 ± 0.39
7	Pindolol	18.43 ± 2.51	9.50 ± 0.49
8	Propranolol	0	>4.00
9	Carvedilol	0	>4.00
10	ICI-118,551	−35.46 ± 5.39	7.95 ± 0.45
11	Nadolol	−34.07 ± 7.80	8.24 ± 0.37
12	Xamoterol	0	>4.00

Data are indicated as mean ± SEM.

Table 2

Experimental data for activation of ERK1/2 by β_2-adrenoceptor from Van der Westhuizen *et al.* for dose–response curves in Figure 7

No.	Ligand	E_{max}	pEC_{50}
1	Isoprenaline	100	7.95 ± 0.13
2	Salbutamol	87.16 ± 5.89	10.81 ± 0.28
3	Salmeterol	74.39 ± 5.28	10.14 ± 0.23
4	Labetalol	111.9 ± 7.52	10.28 ± 0.28
5	Bucindolol	107.2 ± 22.1	8.51 ± 0.41
6	Alprenolol	102.3 ± 5.71	9.54 ± 0.28
7	Pindolol	81.55 ± 19.86	8.90 ± 0.48
8	Propranolol	77.8 ± 7.18	7.87 ± 0.13
9	Carvedilol	88.55 ± 12.47	7.98 ± 0.12
10	ICI-118,551	0	>4.00
11	Nadolol	0	>4.00
12	Xamoterol	102.7 ± 8.63	6.58 ± 0.22

Data are indicated as mean ± SEM. Metoprolol, atenolol and bisoprolol do not activate ERK1/2 via the β_2-adrenoceptor, and based on the limitation of measuring constitutive activity at the ERK1/2 activation pathways, the EC_{50} for these ligands was not available.

analysed using one-way ANOVA with Tukey's *post hoc* test, whereas a Kruskal–Wallis test with Dunn's *post hoc* for non-normally distributed data for statistical significance was set at $P < 0.05$. PC_{100} and airway reactivity were calculated by non-linear regression analysis with exponential growth curve fit as described previously (Thanawala *et al.*, 2013). Microsoft Excel (version 14.4.7) was used for the three-state model of receptor activation mathematical modelling. Image J® (National Institutes of Health) was used for the morphometric quantification of the mucin volume density.

Results

Effect of β_2-adrenoceptor ligands on inflammatory cell infiltration

We studied the effect of five β_2-adrenoceptor ligands that have been shown to have different signalling profiles in several *in vitro* studies (Figure 3) (Galandrin and Bouvier, 2006; Wisler *et al.*, 2007; van der Westhuizen *et al.*, 2014). We studied the effects of these ligands on $PNMT^{-/-}$ and WT mice in an Ova S/C model of murine asthma using the protocol outlined in Figure 1. Ova S/C of $PNMT^{-/-}$ mice did not increase BALF total cellular or eosinophilic infiltration compared with CTL $PNMT^{-/-}$ mice (Figure 4A and B). Infusion of adrenaline to Ova S/C $PNMT^{-/-}$ mice resulted in an increase in the total and eosinophil cell infiltration in BALF compared with control and vehicle-treated (VEH) Ova S/C $PNMT^{-/-}$ mice (Figure 4A and B). The dose of adrenaline ($100\,\mu g \cdot kg^{-1} \cdot day^{-1}$ of adrenaline via osmotic mini-pumps) was chosen to produce plasma levels comparable with those observed in WT mice (plasma adrenaline levels were $80.06 \pm 7.96\,pg \cdot mL^{-1}$ in WT mice vs. $66.87 \pm 11.61\,pg \cdot mL^{-1}$ in $PNMT^{-/-}$ mice administered adrenaline). Of the β-blockers tested, alprenolol, carvedilol and propranolol treatments also increased the total and eosinophilic infiltration in Ova S/C $PNMT^{-/-}$ mice compared with CTL and Ova S/C VEH $PNMT^{-/-}$ mice (Figure 4A and B). However, nadolol treatment differed and did not cause an increase in the total and eosinophilic

Table 3

Receptor affinities for mathematical simulation of the three-state model for Figure 7

No.	Ligand	K_A	K_A*	K_A**	ERK bias (K_A*/K_A**)	log K_A*/K_A**
1	Isoprenaline	1×10^{-7}	2×10^{-9}	2.24×10^{-9}	0.9	−0.045
2	Salbutamol	5×10^{-9}	3×10^{-10}	1.58×10^{-11}	19.0	1.27
3	Salmeterol	4×10^{-9}	5.03×10^{-10}	6.29×10^{-11}	8.0	0.90
4	Labetalol	3.16×10^{-8}	1×10^{-8}	1×10^{-11}	1000	3
5	Bucindolol	3.16×10^{-8}	6.3×10^{-9}	3.16×10^{-10}	20.0	1.30
6	Alprenolol	1.25×10^{-9}	5.01×10^{-10}	2.28×10^{-11}	22.0	1.34
7	Pindolol	2×10^{-9}	1.3×10^{-9}	1.25×10^{-10}	10.4	1.01
8	Propranolol	5.62×10^{-8}	5.62×10^{-8}	3.98×10^{-9}	14.1	1.15
9	Carvedilol	5.62×10^{-8}	5.62×10^{-8}	2.81×10^{-9}	20	1.30
10	ICI-118,551	1.25×10^{-8}	3.16×10^{-8}	1.25×10^{-8}	2.6	0.41
11	Nadolol	1×10^{-8}	2.51×10^{-8}	1×10^{-8}	2.5	0.40
12	Xamoterol	1×10^{-5}	1×10^{-5}	5.62×10^{-8}	178.0	2.25

K_A: Affinity of the ligand for the inactive state of the receptor (*R*). K_A*: Affinity of the ligand for the active state of the receptor (*R**). K_A**: Affinity of the ligand for the active state of the receptor (*R***). The ratio K_A*/K_A** reflects ligand bias towards the ERK1/2 activation pathway. We also quote log values in order to allow comparisons of these ligand bias estimates with those made by the 'transducer ratio' method (Kenakin and Christopoulos, 2013).

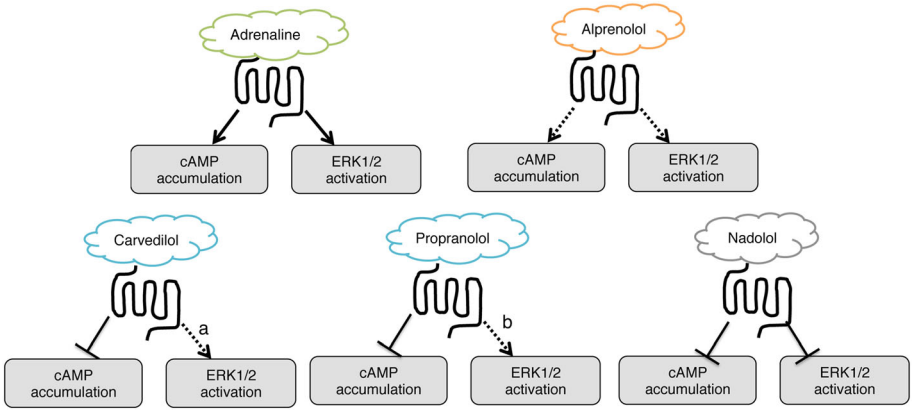

Figure 3

Schematic representation of the signalling profiles of different β_2-adrenoceptor ligands. The figures show the relative signalling profiles of different β_2-adrenoceptor ligands based on several *in vitro* studies. The endogenous ligand adrenaline is assigned by convention as unbiased and is a full agonist (solid arrows) at both the cAMP and ERK1/2 activation pathways. Alprenolol is a partial agonist (dotted arrows) at both signalling pathways in comparison with adrenaline, carvedilol and propranolol are both inverse agonists (solid T-line) at the cAMP accumulation pathway but partial agonists at the ERK1/2 activation pathway (dotted arrows). 'a' and 'b' indicate that carvedilol and propranolol activate ERK1/2 through different signalling cascades. Carvedilol activates ERK1/2 via an arrestin-dependent pathway, while propranolol-mediated ERK1/2 activation is arrestin independent. Nadolol is an inverse agonist at the cAMP accumulation pathway and an antagonist at the ERK1/2 activation pathway (Galandrin and Bouvier, 2006; Wisler *et al.*, 2007; van der Westhuizen *et al.*, 2014).

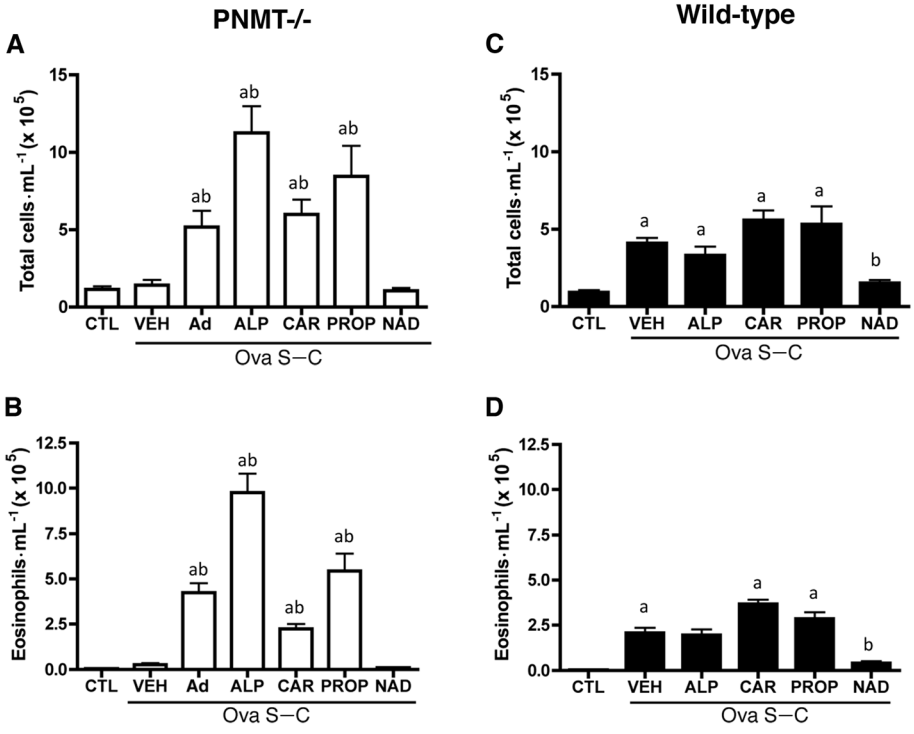

Figure 4

Effect of β-blockers on inflammatory cell infiltration in the airways. The graphs represent the total (A, C) and eosinophilic (B, D) cell infiltration into the airways of PNMT $^{-/-}$ and WT mice, respectively, as determined from the BALF. Randomized groups of mice were subjected to the Ova S/C model of asthma and received different treatments as indicated. Numbers in parentheses indicate *n* values of PNMT $^{-/-}$ and WT mice respectively. CTL mice ($n = 10, 11$) were sensitized to ovalbumin but challenged with saline. VEH ($n = 11, 9$) mice received regular food and water and Ova S/C. Adrenaline (Ad; $n = 9$, N/A), alprenolol (ALP; $n = 8, 10$), carvedilol (CAR; $n = 8, 9$), propranolol (PROP; $n = 6, 6$) and nadolol (NAD; $n = 11, 6$) were administered as discussed in the Methods section. Data are represented as mean ± SEM. The symbol 'a' means $P < 0.05$ compared with respective CTL mice and 'b' $P < 0.05$ compared with respective VEH mice.

inflammatory cell infiltration in Ova S/C PNMT$^{-/-}$ mice compared with the control and Ova S/C PNMT$^{-/-}$ mice (Figure 4A and B).

In WT mice, Ova S/C resulted in an increase in total and eosinophilic inflammatory cell in BALF (Figure 4C and D). Administration of the β-blockers alprenolol, carvedilol or propranolol did not change the numbers of total and eosinophilic cells infiltrating the airways of VEH Ova S/C WT (Figure 4C and D). However, administration of nadolol prevented the increase in numbers of total cellular and eosinophil infiltration observed in BALF from VEH Ova S/C WT mice (Figure 4C and D).

Effect of β_2-adrenoceptor ligands on mucin production

Similar to inflammatory cell infiltration, Ova S/C did not result in an increase in mucin production in PNMT$^{-/-}$ mice compared with control PNMT$^{-/-}$ mice (Figure 5A and B). Adrenaline, alprenolol, carvedilol and propranolol administration to Ova S/C PNMT$^{-/-}$ mice increased mucin production compared with control and Ova S/C VEH PNMT$^{-/-}$ mice (Figure 5A and B). However, nadolol did not increase mucin production compared with control or Ova S/C VEH PNMT$^{-/-}$ mice (Figure 5A and B).

In WT mice, Ova S/C resulted in an increase in mucin production compared with control WT mice (Figure 5C and D). Administration of alprenolol, carvedilol and propranolol also increased mucin production in Ova S/C WT mice compared with CTL WT mice, while nadolol administration to Ova S/C WT mice abolished the increased mucin production observed in Ova S/C VEH WT mice to levels not different from those in control WT mice (Figure 5C and D).

Effect of β_2-adrenoceptor ligands on AHR

Ovalbumin sensitization and challenge with or without adrenaline or alprenolol administration to PNMT$^{-/-}$ mice did not increase AHR (characterized by an increase in peak airway resistance, airway reactivity or lowering of the PC_{100}) to methacholine compared with control PNMT$^{-/-}$ mice (Figure 6A, C and D). Administration of propranolol and carvedilol to Ova S/C PNMT$^{-/-}$ mice increased peak airway resistance and lowered the PC_{100} concentration compared with control PNMT$^{-/-}$ mice (Figure 6A, C and D). Carvedilol administration to Ova S/C PNMT$^{-/-}$ mice lowered PC_{100} concentration compared with Ova S/C VEH PNMT$^{-/-}$ mice

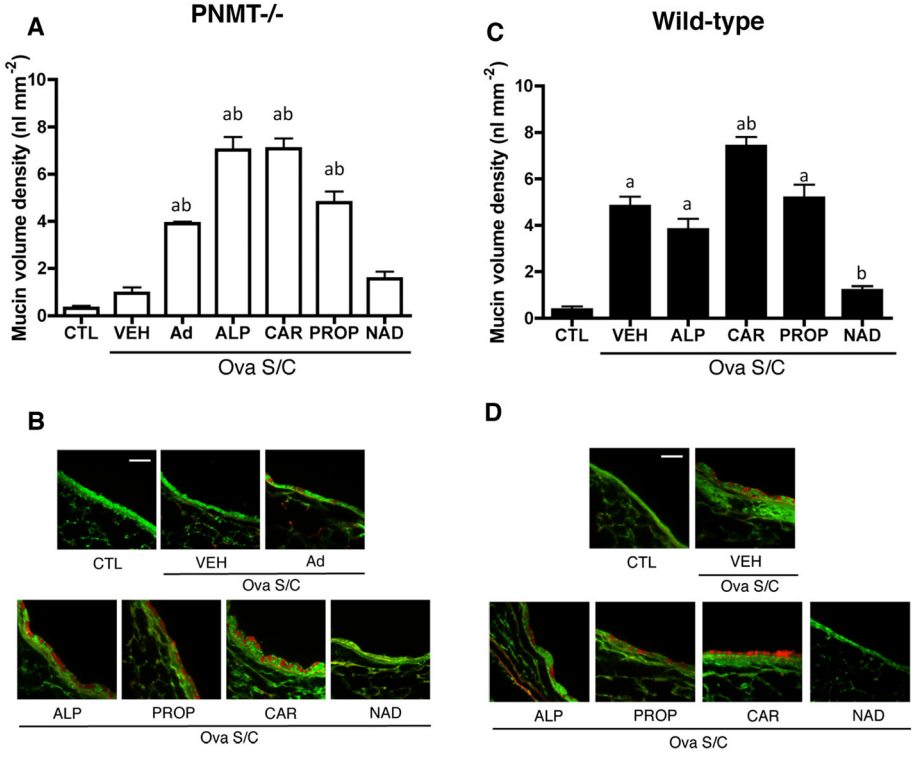

Figure 5

Effect of β-blockers on mucin production in the airways. The graphs represent the mucin volume density (A, C) and representative images (B, D) of PNMT$^{-/-}$ and WT mice, respectively, from PAFS. Mucin is seen as red globules among the green epithelium and parenchymal cells. Randomized groups of mice were subjected to the Ova S/C model of asthma and received different treatments as indicated. Numbers in parentheses indicate *n* values of PNMT$^{-/-}$ and WT mice respectively. CTL mice ($n = 10, 9$) were sensitized to ovalbumin but challenged with saline. VEH ($n = 10, 10$) mice received regular food and water and Ova S/C. Adrenaline ($n = 5$, N/A), alprenolol ($n = 6, 5$), carvedilol ($n = 9, 5$), propranolol ($n = 6, 6$) and nadolol ($n = 8, 5$) were administered as discussed in the Methods section. Scale bar is 100 μm. Data are represented as mean ± SEM. The symbol 'a' means $P < 0.05$ compared with respective CTL mice and 'b' $P < 0.05$ compared with respective VEH mice.

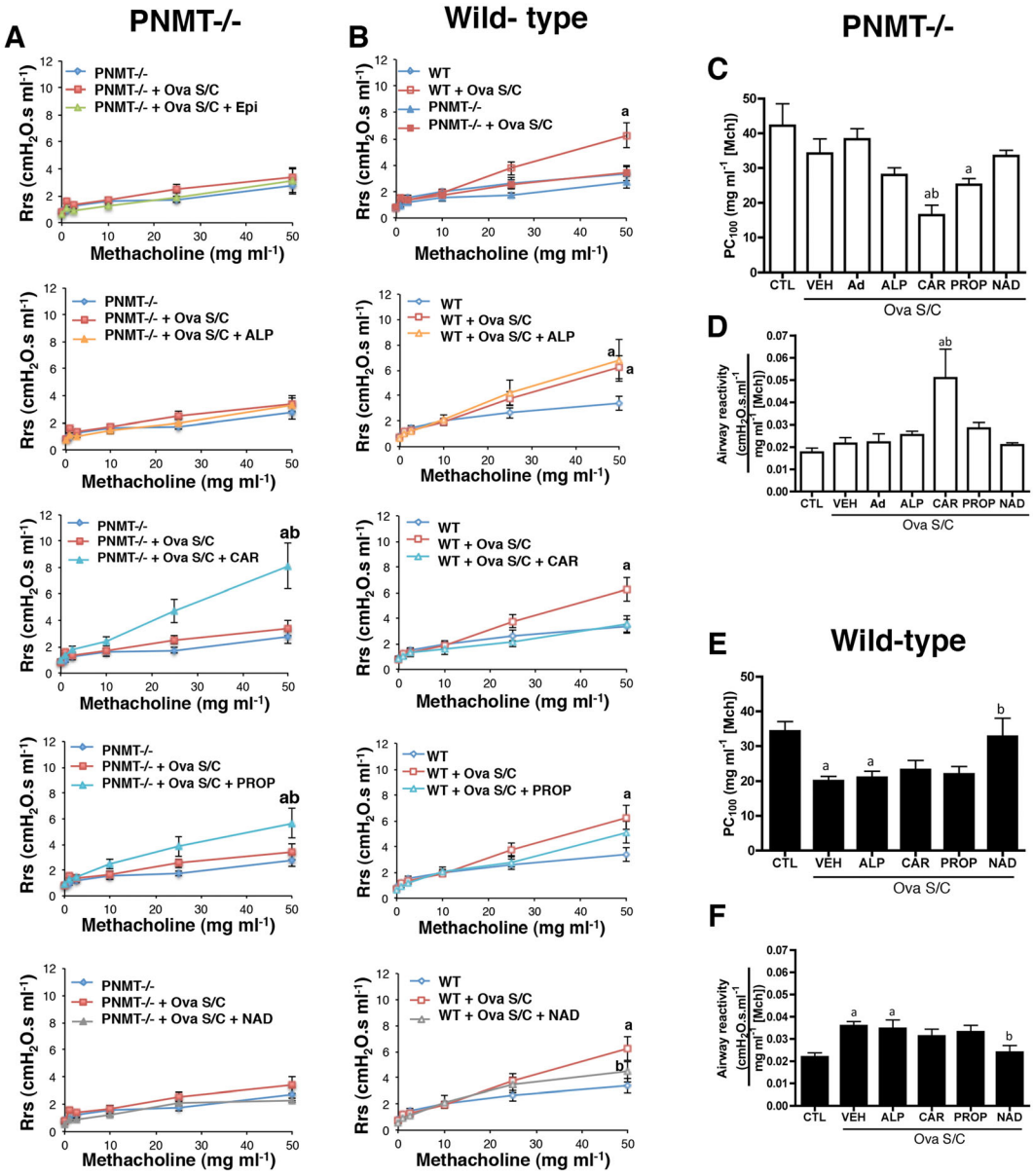

Figure 6

Effect of β-blockers on airway hyperresponsiveness. The graphs represent the airway resistance dose–response curves (A, B), PC_{100} (C, E) and airway reactivity (D, F) of PNMT $^{-/-}$ and WT mice, respectively, to inhaled methacholine measured using an invasive forced oscillation technique. An increased airway resistance and airway reactivity and a decrease in PC_{100} indicate an increase in AHR. Randomized groups of mice were subjected to the Ova S/C model of asthma and received different treatments as indicated. Numbers in parentheses indicate *n* values of PNMT $^{-/-}$ and WT mice respectively. CTL mice ($n = 5, 10$) were sensitized to ovalbumin but challenged with saline. VEH ($n = 5, 10$) mice received regular food and water and Ova S/C. adrenaline ($n = 5$, N/A), alprenolol ($n = 6, 6$), carvedilol ($n = 5, 5$), propranolol ($n = 6, 6$) and nadolol ($n = 5, 7$) were administered as discussed in the Methods section. Data are represented as mean ± SEM. The symbol 'a' mean $P < 0.05$ compared with respective CTL mice and 'b' $P < 0.05$ compared with respective VEH mice.

and increased airway reactivity compared with control and Ova S/C VEH PNMT$^{-/-}$ mice (Figure 6C and D). Nadolol administration to Ova S/C PNMT$^{-/-}$ mice did not increase any AHR compared with CTL and Ova S/C VEH PNMT$^{-/-}$ mice (Figure 6A, C and D).

In WT mice, Ova S/C increased AHR to methacholine compared with control mice (Figure 6B, E and F). On airway parameters in Ova S/C WT mice, there were subtle differences with administration of alprenolol compared with carvedilol and propranolol. Alprenolol did not alter peak airway resistance while carvedilol and propranolol decreased peak airway resistance compared with Ova S/C VEH WT mice (Figure 6B). Also, of these three ligands, only alprenolol significantly altered AHR or airway reactivity compared with CTL WT mice (Figure 6B, E and F). Administration of nadolol to Ova S/C WT mice resulted in airway

resistance values not different from those in CTL mice (Figure 6B, E and F).

β_2-Adrenoceptor ligand signalling is consistent with the isolated system of the three-state model of receptor activation

Van der Westhuizen *et al.* (2014) measured the cAMP accumulation and ERK1/2 activation of 12 β_2-adrenoceptor ligands in HEK293 cells overexpressing the β_2-adrenoceptor. The experimental data (Figure 7A and B) and the modelling data obtained for the isolated (Figure 7C and D) or intact (data not shown) systems were compared to determine whether the β_2-adrenoceptor ligands followed the isolated or intact system of the three-state model of receptor activation. The results of the computer simulations show that the experimental data accord closely with the isolated mode of the three-state model, in which both potency and efficacy orders of ligands can differ between the two pathways (Figure 7C and D), whereas in the intact mode, only efficacy, but not potency, orders can change. However, as illustrated by the potency orders for selected compounds at the cAMP accumulation and ERK1/2 activation pathways (indicated by the boxed numbers in Figure 7A and B), the data show clear potency reversals for several compounds.

Discussion

Many GPCRs, including the prototypical β_2-adrenoceptor, activate signalling via more than their canonical pathway (Luttrell *et al.*, 1999). Simultaneous with this discovery was the finding that different ligands for the same GPCR are capable of producing different activation or inactivation patterns for the multiple pathways. This latter finding was first termed ligand-directed trafficking of receptor signalling (Kenakin, 1995, 2009, 2012; Kenakin and Christopoulos, 2013) but is now widely referred to as biased signalling or ligand bias. For the β_2-adrenoceptor, the most studied signalling pathways are the canonical β_2-adrenoceptor-Gs-cAMP and ERK1/2 activation (Galandrin and Bouvier, 2006; Shenoy *et al.*, 2006; Wisler *et al.*, 2007; Whalen *et al.*, 2011; van der Westhuizen *et al.*, 2014).

Our previous studies have shown that (i) the β_2-adrenoceptor and its activation by the endogenous hormone, adrenaline, or another β_2-adrenoceptor agonist are required for development of the asthma phenotype in an allergen-driven (Ova S/C) murine asthma model (Nguyen *et al.*, 2009; Thanawala *et al.*, 2013), as well as for enhanced mucin production by NHBE cells in response to IL-13 (Al-Sawalha *et al*, 2015); and (ii) that chronic administration of some, but not all, β-blockers can attenuate the asthma phenotype

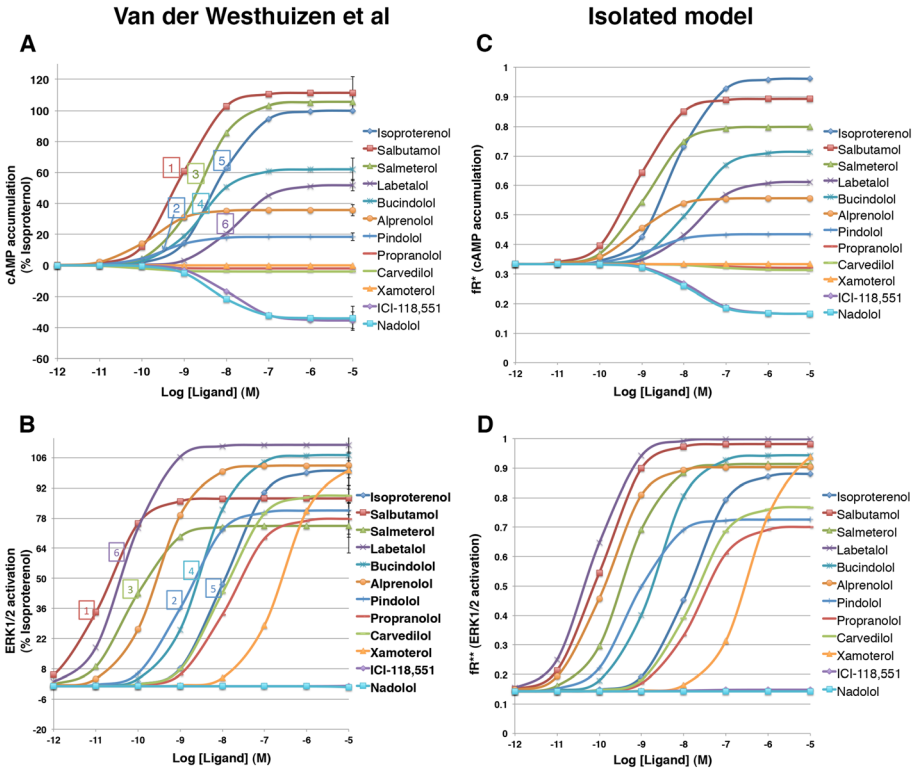

Figure 7

Mathematical modelling of different β_2-adrenoceptor ligands at cAMP and ERK1/2 signalling pathways. Panels A and B illustrate experimental concentration–effect curves for the ligands studies by Van der Westhuizen *et al.* (2014) acting through the cAMP and ERK1/2 pathways respectively. The EC_{50} values for six of the ligands (salbutamol, pindolol, salmeterol, bucindolol, isoprenaline and labetalol), indicated by the numbers in boxes, show clear potency order differences between the two pathways for some of the ligands. This observation is highlighted because the mathematical modelling of the intact mode of the three-state model does not allow potency reversals (discussed later). Panels C and D illustrate computer-generated curves using the model of Leff *et al.* (1997) operating in the isolated mode, with f_{R^*} representing cAMP generation and $f_{R^{**}}$ representing ERK1/2 activation.

(Callaerts-Vegh *et al.*, 2004; Lin *et al.*, 2008; Nguyen *et al.*, 2008). In the present study, we show that despite the requirement of the β_2-adrenoceptor and adrenaline, development of the asthma phenotype does not correlate with a ligand's ability to activate or inhibit the canonical β_2-adrenoceptor-Gs-cAMP pathway, and instead, the development of the asthma phenotype correlates with the β_2-adrenoceptor ligands that activate ERK1/2 in *in vitro* cell-based assays (Galandrin and Bouvier, 2006; Wisler *et al.*, 2007; van der Westhuizen *et al.*, 2014).

This is the first report of the effect of the four β-blockers on the asthma phenotype using PNMT$^{-/-}$ mice. These mice have no overt phenotype (Ebert *et al.*, 2004; Sun *et al.*, 2008; Sharara-Chami *et al.*, 2010; Ziegler *et al.*, 2011) and provide a valuable pharmacological tool because they allow replacing the endogenous hormone for the β_2-adrenoceptor with ligands with different signalling profiles and studying the effects of these ligands without any complicating interference from adrenaline. We tested four β-blockers, alprenolol, carvedilol, propranolol and nadolol, in both WT and PNMT$^{-/-}$ mice. The mice were administered the β-blockers for 28 days at doses previously used in murine models (Callaerts-Vegh *et al.*, 2004; Lin *et al.*, 2008; Nguyen *et al.*, 2008), and in PNMT$^{-/-}$ mice, we also infused adrenaline for 14 days at a dose that produced circulating levels similar to those measured in WT mice. Due to the short half-life and rapid degradation of adrenaline, the duration of administration was limited to 14 days via sub-cutaneous osmotic pumps (Terres *et al.*, 1989; Khasar *et al.*, 2003, 2005).

Several *in vitro* cell-based studies have reported the signalling profiles of these ligands for the two β_2-adrenoceptor pathways relative to an assigned reference ligand (usually adrenaline or isoprenaline; Tables 1 and 2 and Figure 3) (Galandrin and Bouvier, 2006; Wisler *et al.*, 2007; van der Westhuizen *et al.*, 2014). In these studies, alprenolol was a partial agonist at both pathways; carvedilol was an inverse agonist at the Gs-cAMP pathway and a partial agonist at ERK1/2 activation; propranolol was also an inverse agonist at the Gs-cAMP pathway and a partial agonist at ERK1/2; and nadolol was an inverse agonist at the Gs-cAMP pathway and an antagonist at the ERK1/2 pathway (Figure 3). Despite their similar signalling profiles and E_{max} values at ERK1/2 activation, we chose to study carvedilol and propranolol because there are data suggesting carvedilol activates ERK1/2 in an arrestin-dependent manner, while propranolol's activation of ERK1/2 is arrestin independent (Galandrin and Bouvier, 2006; Wisler *et al.*, 2007; van der Westhuizen *et al.*, 2014).

Our results show a consistent correlation between ligand *in vitro* activity at ERK1/2 activation and effect on the asthma phenotype *in vivo*. For example, administration of the ERK1/2 activators alprenolol, carvedilol, propranolol and adrenaline to Ova S/C PNMT$^{-/-}$ mice increased the total cells and eosinophils in BALF to levels observed in the BALF of VEH Ova S/C WT mice (Figure 4). Qualitatively, the same results were observed when the mucin production in the airways was quantified by morphometric analysis (Figure 6). On the other hand, administration of the *in vitro* ERK1/2 antagonist, nadolol, did not increase inflammatory cell numbers in the BALF or mucin production in Ova S/C PNMT$^{-/-}$ mice (Figures 4 and 5).

In WT mice, administration of alprenolol, carvedilol and propranolol had no effect on the increase in cells or mucin observed following Ova S/C, while nadolol reduced both parameters to that observed in CTL mice (Figures 4 and 5). nadolol attenuated the peak airway resistance and airway reactivity and increased the PC_{100} in the current study, providing attenuation of AHR in Ova S/C WT mice similar to our previous study (Callaerts-Vegh *et al.*, 2004). Studies with other strains of mice have shown that in addition to nadolol, metoprolol and ICI-118,551 also attenuate the asthma phenotype produced by Ova S/C, and these two ligands also have signalling profiles similar to nadolol (inverse agonists of the Gs-cAMP pathway and antagonist at the ERK1/2 pathway) (Wisler *et al.*, 2007; Lin *et al.*, 2008; Nguyen *et al.*, 2008; van der Westhuizen *et al.*, 2014). However, while the previous study using carvedilol (Callaerts-Vegh *et al.*, 2004) and the current study showed carvedilol reduced peak airway resistance, in the previous study, carvedilol produced an increase in AHR that was not observed in the current study where there was no change in AHR. This difference may be attributable to the strain differences between mice, as different mouse strains have shown quantitative differences in allergen-induced models (Zosky *et al.*, 2009).

The differing results on AHR with administration of nadolol compared with propranolol that were observed in this study are also consistent with the divergent findings of small pilot clinical trials using two β_2-adrenoceptor Gs-cAMP inverse agonists, nadolol and propranolol (Hanania *et al.*, 2008, 2010; Short *et al.*, 2013a, b; Anderson *et al.*, 2014). In two small trials, nadolol administration to mild asthmatics was shown to cause a decrease in AHR (as observed in this study Figure 6B, E and F), while propranolol (in a different subset of asthmatics) had no effect on AHR (again as observed in this study, Figure 6B, E and F) (Hanania *et al.*, 2008, 2010; Short *et al.*, 2013a, b; Anderson *et al.*, 2014). These divergent results are supportive of the hypothesis that only β-blockers that inhibit ERK1/2 activation *in vitro* may have therapeutic efficacy at reducing AHR in asthmatics, and the results add further support to the finding of a previous murine study that ruled out inverse agonism as the key drug property for producing attenuation of the asthma phenotype (Thanawala *et al.*, 2013, 2014).

There are also differences in the Ova S/C asthma phenotypes observed in WT mice compared with infusion of adrenaline in PNMT$^{-/-}$ mice. These differences can be attributed to the static infusion of adrenaline in PNMT$^{-/-}$ that cannot reproduce the physiological fluctuations in the levels of adrenaline in WT mice. A further support for this speculation is that we have previously shown that 14 days of treatment with a stable β_2-adrenoceptor agonist, formoterol, was sufficient time to restore the asthma phenotype (Thanawala *et al.*, 2013). The half-life of formoterol is in the same range (10 to 17 h) as the half-lives of the β-blockers used in this study.

Previously, we had shown that formoterol administration to PNMT$^{-/-}$ mice after the sensitization phase of the Ova S/C murine asthma model resulted in the development of asthma phenotype in PNMT$^{-/-}$ mice (Thanawala *et al.*, 2013). Moreover, nadolol attenuated the asthma phenotype in Ova S/C WT mice independent of whether the 28 day nadolol administration was during or after the sensitization phase of the Ova S/C model (Nguyen *et al.*, 2008). Together, these data and our current results indicate that although β_2-adrenoceptor signalling is required for the development

of the asthma phenotype, adrenergic stimulation during the sensitization phase does not play a role in development of the asthma phenotype. Given the requirement of β_2-adrenoceptor signalling for development of the asthma phenotype, it was a rather surprising finding that ligands having different and in some cases even opposing effects at the canonical β_2-adrenoceptor-Gs-cAMP pathway could have the same effect on inflammatory cell infiltration and mucin production. The effects at the β_2-adrenoceptor-Gs-cAMP pathway for these ligands range from the full agonist adrenaline to the partial agonist alprenolol and the inverse agonists carvedilol and propranolol. However, these ligands all restored the inflammatory cell infiltration and mucin production in PNMT$^{-/-}$ mice, consistent with the theory that only those ligands that activate ERK1/2 *in vitro* restore the asthma phenotype *in vivo* (Galandrin and Bouvier, 2006; Wisler *et al.*, 2007; van der Westhuizen *et al.*, 2014). Furthermore, activation of ERK1/2 appears to be the closest predictive readout to the receptor as it has been reported that while carvedilol activates ERK1/2 in an arrestin-dependent manner, propranolol does so in an arrestin-independent manner, yet both produce similar qualitative results *in vivo*.

To further test the hypothesis of pathway-independent effects produced by the *in vivo* data and to characterize the signalling profiles of the β_2-adrenoceptor ligands, we performed mathematical modelling using the three-state model of receptor activation (Leff *et al.*, 1997). Firstly, inspection of the experimental data from van der Westhuizen *et al.* (2014) demonstrated potency order reversals between the pathways, illustrated clearly by members of the set of six ligands whose rank order of EC_{50} values is indicated in boxed numbers in Figure 7A and B. The three-state model operating in its intact mode does not allow potency reversals because linking the equilibria between the two pathways means that ligands must express the same relative affinities at the two pathways. In contrast, the potency reversal of ligands at the two pathways can be observed by the model acting in its isolated mode. In this situation, the pathways operate independently, and ligand activities are determined solely by the affinity constants for the pathway measured. Figure 7C and D illustrates the simulation of the model in the isolated mode, demonstrating a good fit with the experimental data and confirming quantitatively the accordance of the ligand pharmacology with the predictions of the model. From these simulations, estimates of ligand bias towards the ERK1/2 activation pathway could be made from the ratio of K_A* and K_A** values as shown in Table 3. These values clearly correlated with the propensity of ligands to restore the asthma phenotype.

While both the *in vivo* and the modelling results suggest the independence of the pathways and the necessity of ligands to activate ERK1/2 in order for them to restore the asthma phenotype, it is worth noting that our *in vivo* results only show *correlation*. The signalling profiles from all the *in vitro* studies are measurements made within minutes of ligand exposure, whereas our *in vivo* data are only observed after weeks of treatment. Therefore, we are unable to make any comment on whether either or both pathways are activated, or inhibited, following chronic treatment with the ligands tested *in vivo*. Our results suggest the *in vitro* profiles appear predictive of the ligand's behaviour *in vivo* in a murine asthma model. There is however evidence supporting that inhibition of ERK1/2 activation could be the reason why, of the β-blockers tested, only nadolol attenuated the asthma phenotype. Studies carried out in arrestin3-knockout mice (βarrestin2 knockout), one of the upstream components that can activate ERK1/2, exhibit an attenuated asthma phenotype (Walker *et al.*, 2003). In addition, administration (albeit for a relatively acute administration of 5 days) of U0126, a MAPK kinase (MEK) inhibitor (MEK activates ERK1/2) to Ova S/C Balb/cj mice decreased the inflammatory markers and inflammatory phenotype (Duan *et al.*, 2004).

In conclusion, despite the requirement of a ligand-activated β_2-adrenoceptor for development of the asthma phenotype, our *in vivo* and mathematical modelling results suggest that the required activation is independent of the ligand's activity at the canonical β_2-adrenoceptor-Gs-cAMP pathway. Previously, we have shown that β_2-adrenoceptor-Gs-cAMP agonists such as formoterol (Thanawala *et al.*, 2013), or adrenaline in this study, are capable of restoring the asthma phenotype. However, we now show that even administration of 'β-blockers' that range from inverse agonists to partial agonists of the β_2-adrenoceptor-Gs-cAMP pathway also restores the asthma phenotype. The common property of carvedilol, propranolol and alprenolol, as well as formoterol and adrenaline, is their ability to activate ERK1/2 in *in vitro* signalling. This is the property that correlates with restoration of the asthma phenotype in Ova S/C PNMT$^{-/-}$ mice and a failure to decrease the asthma phenotype in Ova S/C WT mice. On the other hand, nadolol, a ligand that is similar to carvedilol and propranolol is an inverse agonist at the β_2-adrenoceptor-Gs-cAMP pathway but that does not activate ERK1/2 *in vitro*, does not produce the asthma phenotype in Ova S/C PNMT$^{-/-}$ mice and attenuates the phenotype in Ova S/C WT mice. Finally, the consistency of the *in vivo* results with the predictions of the three-state model operating in isolated mode is relevant pharmacologically and clinically, indicating the abilities of β_2-adrenoceptor ligands to independently activate or inhibit the pathways effectively as pharmacologically distinct receptors.

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Author contributions

R. A. B., V. J. T., M. B. and P. L. conceived and designed the experiments and analysed and interpreted the results. V. J. T., D. V., R. J., G. S. F. and S. P. performed and, along with B. J. K., contributed to the design of experiments.

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