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RESEARCH PAPER

The role of adenylyl cyclase isoform 6 in β-adrenoceptor signalling in murine airways

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BACKGROUND AND PURPOSE

Adenylyl cyclase (AC) is a key signalling enzyme for many GPCRs and catalyses the conversion of ATP to cAMP which, in turn, is a crucial determinant of many biological responses. β-Adrenoceptor agonists are prescribed as bronchodilators for asthma and chronic obstructive pulmonary disease, and it is commonly assumed that they elicit their actions via AC-dependent production of cAMP. However, empirical evidence in support of this is lacking and the exact mechanism by which these drugs acts remains elusive. This is partly due to the existence of at least 10 different isoforms of AC and the absence of any truly selective pharmacological inhibitors. Here, we have used genetically modified mice and model systems to establish the role of AC isoforms in the airway responses to β-adrenoceptor agonists.

EXPERIMENTAL APPROACH

Receptors mediating responses to β-adrenoceptor agonists in airway smooth muscle (ASM) and sensory nerve were identified in isolated tissue systems. Expression of mRNA for the AC isoforms in ASM and neurones was determined by qPCR. Functional responses were assessed in AC isoform KO mice and wild-type controls.

KEY RESULTS

Airway and vagal tissue expressed mRNA for various isoforms of AC. AC6 was the most prominent isoform. Responses to β-adrenoceptor agonists in tissues from AC6 KO mice were virtually abolished.

CONCLUSIONS AND IMPLICATIONS

AC6 played a critical role in relaxation of ASM to β_1-adrenoceptor agonists and in modulation of sensory nerves by $\beta_{1\text{-}3}$-adrenoceptor agonists. These results further unravel the signalling pathway of this extensively prescribed class of medicine.

Abbreviations

AC, adenylyl cyclase; *Adcy6*$^{-/-}$, AC6 KO mouse; *Adrb1*$^{-/-}$, *Adrb2*$^{-/-}$, *Adrb3*$^{-/-}$, β_1-, β_2-, β_3-adrenoceptor KO mouse; ASM, airway smooth muscle; CCh, carbachol

Tables of Links

TARGETS
Enzymes[a]
AC, adenylyl cyclase
GPCRs[b]
β_1-Adrenoceptors
β_2-Adrenoceptors
β_3-Adrenoceptors
Ion channels[c]
TRPV1

LIGANDS	
ACh, acetylcholine	Isoprenaline
BRL 37344	ICI 118551
Capsaicin	ONO-AE1-259
CCh, carbachol	PGE_2
CGP 20712	
Denopamine	
Fenoterol	
Formoterol	

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 ([a,b,c]Alexander *et al.*, 2013a,b,c).

Introduction

Adenylyl cyclase (AC) is believed to be a central component of signalling pathways downstream from many GPCRs. It catalyses the conversion of ATP to cAMP, an intracellular messenger which is involved in many biological processes including cell growth and differentiation, transcriptional regulation, apoptosis and various cellular functions (Patel *et al.*, 2001). The ability of this enzyme to be involved in such a diverse range of effects is due to its tissue distribution, the spatiotemporal compartmentalization of intracellular cAMP gradients, numerous regulatory mechanisms and, crucially, the existence of multiple AC isoforms, each with varying enzymic and pharmacological properties. Ten AC isoforms have been identified of which nine are transmembrane proteins activated by $G\alpha_s$, whereas AC10 is a soluble isoform that lacks the transmembrane domains and is insensitive to $G\alpha_s$ (see Sunahara *et al.*, 1996; Hanoune and Defer, 2001; Onda *et al.*, 2001; Patel *et al.*, 2001; Pavan *et al.*, 2009; Duan *et al.*, 2010; Brand *et al.*, 2013; Conley *et al.*, 2013).

Activation of β-adrenoceptors (β_2-adrenoceptors in humans, β_1-adrenoceptors in mice; Henry and Goldie, 1990; Henry *et al.*, 1990) on airway smooth muscle (ASM) increases cAMP (Kume *et al.*, 1994; Kotlikoff and Kamm, 1996; Billington *et al.*, 2013), and inhaled β_2-adrenoceptor agonists are used therapeutically as bronchodilators in patients with respiratory diseases such as asthma and COPD (Goldie *et al.*, 1986; Barnes, 1995; Johnson, 1998; Dennis *et al.*, 2000). β_2-adrenoceptor agonists also inhibit airway sensory nerve activation and cough in preclinical models (Freund-Michel *et al.*, 2010). Despite β-adrenoceptor agonists being commonly prescribed for numerous respiratory diseases, the exact mechanism by which they act is uncertain (Giembycz and Newton, 2006).

It is generally accepted, based on traditional second messenger signalling paradigms, that AC-linked cAMP production is central to the effects of β-adrenoceptor agonists, although direct evidence in support of this is minimal. Indeed some studies have found that AC inhibitors, while inhibiting cAMP production, do not inhibit β-adrenoceptor agonist-induced relaxation of ASM (Turcato and Clapp, 1999; Koike *et al.*, 2004). Furthermore, the AC isoform responsible for β-adrenoceptor agonist-induced increases in cAMP is yet to be determined and elucidating which isoform is responsible represents a vital step in improving our understanding of the mechanism(s) by which β-adrenoceptor agonists signal. To date, investigation into the role of AC isoforms has been hampered by the lack of available pharmacological tools. Although selective AC isoform inhibitors are becoming available, they currently lack the necessary potency and selectivity required (Iwatsubo *et al.*, 2006; Brand *et al.*, 2013; Conley *et al.*, 2013). In the present studies, the use of murine transgenic systems previously reported has allowed us to circumvent these issues by investigating functional responses in tissues from mice lacking individual AC isoforms.

Methods

Animals

All *in vivo* protocols were approved by Imperial College London ethical review process committee and we strictly adhered to the Animals (Scientific Procedures) Act 1986 UK Home Office guidelines. Experiments were performed under a Home Office project licence (PPL 70/7212). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 124 animals were used in the experiments described here.

Male wild-type mouse strains were originally obtained from Harlan UK Limited (Bicester, UK) and bred in our animal facility; food and water were supplied *ad libitum*. Genetically modified mice (knockout, KO) were back-crossed at least eight times and bred alongside the wild-type mouse strains (FVB/N or C57BL/6). The β-adrenoceptor KO mice (β_1, *Adrb1*$^{-/-}$; β_2, *Adrb2*$^{-/-}$; β_3, *Adrb3*$^{-/-}$) all on FVB/N backgrounds were kindly donated to us by Dr Brad Lowell. The AC6 KO mice (*Adcy6*$^{-/-}$) on a C57BL/6 background, were kindly donated to us by Drs Kirk Hammond and Tong Tang. As reported by Tang *et al.* (2008), the *Adcy6*$^{-/-}$ mice did not

appear to have any phenotypical differences from wild-type mice.

Role of β-adrenoceptor subtypes in mediating functional effects in murine tissues

Tracheal tissue was harvested from wild-type (18–24 g, FVB/N) or *Adrb1*$^{-/-}$, *Adrb2*$^{-/-}$, *Adrb3*$^{-/-}$ mice and placed into tissue baths as described by Buckley *et al.* (2011). Once the tissues had stabilized, they were challenged with ACh (1 mM) three times to confirm viability and to standardize responses. Carbachol (CCh; 1 μM) was used to induce increased tension in tissues over a 30 min period before the addition of test substances. Cumulative concentration–response curves were generated to the non-selective β-adrenoceptor agonist, isoprenaline (with ascorbic acid, 1 μM), and at the end of the experiment the tissues were challenged with papaverine to establish maximal relaxation. In parallel, other samples of trachea were exposed to PGE_2, which is known to relax mouse tracheal tissue via activation of the EP_2 receptor (Buckley *et al.*, 2011).

To confirm the data generated in tissue from genetically modified mice, selective β-adrenoceptor agonists/antagonists were used. Using the system described above, mouse trachea was exposed to cumulative concentrations of denopamine (p*K*i of 5.8 at the β_1-adrenoceptor, IUPHAR database, http://www.iuphar-db.org/) or fenoterol (p*K*i of 6.9 at the β_2-adrenoceptor, IUPHAR database) in the presence or absence of CGP 20712 (300 nM: pKi 8.5–9.2 at the β_1-adrenoceptor, IUPHAR database, ~10,000 less potent against the β_2-adrenoceptor; Dooley *et al.*, 1986) or ICI 118551 (100 nM: pKi 9.2–9.5 at the β_2-adrenoceptor, IUPHAR database, ~100 less potent against the β_1-adrenoceptor; Bilski *et al.*, 1983). These concentrations of antagonist were selected from published literature (Hall *et al.*, 1990). To confirm that the pharmacological tools were acting appropriately, fenoterol and ICI 118551 were tested in tracheal tissue collected from male Dunkin-Hartley guinea pigs, using the methods described previously (Buckley *et al.*, 2011).

Vagal tissue was harvested from wild-type (18–24 g, FVB/N), *Adrb2*$^{-/-}$ or *Adrb3*$^{-/-}$ mice and mounted in a grease-gap recording system as described previously (Maher *et al.*, 2009). Once a stable baseline was achieved, tissues were exposed to two challenges with a submaximal concentration of the TRPV1 agonist, capsaicin (1 μM), to induce control depolarization, and then incubated with vehicle (0.1% DMSO, v/v) or single concentrations of the selective β_1 (denopamine, 100 μM), β_2 (formoterol, 10 nM, p*K*i of 10.1 at the β_2-adrenoceptor, IUPHAR database) or β_3 (BRL 37344, 10 μM, p*K*i of 6.4–7.0 at the β_3-adrenoceptor, IUPHAR database) adrenoceptor agonists for 10 min. Formoterol (a clinically used long-acting β-adrenoceptor agonist; LABA) was used in these experiments to confirm earlier data with a short-acting β-adrenoceptor agonist (Freund-Michel *et al.*, 2010). Single concentrations were determined from concentration–response experiments and selectivity was confirmed with the respective, receptor-selective antagonists (data not shown). The tissue was then re-challenged with capsaicin in the presence of the vehicle or β-adrenoceptor agonist. Following a washout phase, capsaicin was reapplied to demonstrate tissue viability. Percentage inhibition was calculated by comparing the average depolarization to capsaicin before and after incubation with the vehicle or drug.

Expression of AC isoforms in ASM and neurones

The relative gene expression of individual AC isoforms was measured by real-time PCR, using TaqMan primers and probes, purchased from Applied Biosystems (Life Technology Limited, Paisley, UK). The following TaqMan assays (catalogue #) were used to measure the expression of each respective AC isoform: Adcy1: Mm01187829_m1, Adyc2: Mm00467874_m1, Adcy3: Mm00460371_m1, Adcy4: Mm00475491_m1, Adcy5: Mm00674122_m1, Adcy6: Mm00475772_m1, Adcy7: Mm00545780_m1, Adcy8: Mm00507722_m1, Adcy9: Mm00507743_m1, Adcy10: Mm00557236_m1. Prior to measuring the gene expression of AC isoforms in a multiplex system, each TaqMan assay was first validated to ensure equal amplification efficiency relative to the housekeeping gene, 18S rRNA, using cDNA from tissues (selected from a panel) that expressed the target AC isoform at high levels (data not shown). RNA (subsequently reverse transcribed to cDNA) was extracted by a standard guanidinium thiocyanate-phenol-chloroform method from ASM and vagal sensory ganglia (nodose/jugular ganglia) harvested from wild-type male C57BL/6 mice. Expression levels were determined as described previously (Wong *et al.*, 2009). Briefly, real-time PCR was performed using an ABI PRISM 7000 TaqMan machine (Applied Biosystems). The following amplification protocol was used: one cycle of 50°C for 2 min, one cycle of 95°C for 10 min and 40 cycles of 95°C for 15 s followed by 1 min at 60°C. Data were analysed using Sequence Detection Software (Applied Biosystems).

Role of AC6 in responses to β-adrenoceptor agonists in murine tissues

Tracheal tissue was harvested from wild-type (18–24 g, C57BL/6) or *Adcy6*$^{-/-}$ mice and placed into tissue baths as described above with a modification to circumvent any possible differences in responses to the muscarinic agonist (we found that reduced contraction to CCh was observed in the trachea from *Adcy6*$^{-/-}$ mice). Briefly, once the tissues had stabilized and had been challenged with ACh (1 mM) three times, they were incubated with vehicle (0.1% DMSO v/v) or the selective β_1-adrenoceptor agonist, denopamine (100 μM), for 10 min. The tissues were then exposed to ACh in increasing concentrations to generate cumulative concentration–response curves and the effects of the β_1-adrenoceptor agonist on responses to ACh in tissue from wild-type mice and *Adcy6*$^{-/-}$ mice were compared.

In parallel experiments, the effect of an EP_2 receptor agonist, ONO AE1 259 (10 μM) (Birrell *et al.*, 2013), was determined in tracheal tissue harvested from wild-type and *Adcy6*$^{-/-}$ mice.

Vagal tissue was collected from wild-type (18–24 g) or *Adcy6*$^{-/-}$ mice and mounted in a grease-gap recording system as described above. Once a stable baseline was achieved, tissues were exposed to two challenges with capsaicin (1 μM) to induce control depolarizations, and then incubated with vehicle (0.1% DMSO) or selective β_1 (denopamine, 100 μM), β_2 (formoterol, 10 nM) or β_3 (BRL 37344, 10 μM)

adrenoceptor agonists for 10 min. The concentrations of agonists used in these experiments were selected from previous concentration ranging studies. At these concentrations the ligands achieved around 80% of their maximal effect. The tissue was then re-challenged with capsaicin in the presence of the vehicle or β-adrenoceptor agonist. Following a wash phase, capsaicin was reapplied to show tissue viability. To determine if the downstream signalling was still intact in the *Adcy6*$^{-/-}$ mice, we first tried to mimic the effect of the β-adrenoceptor agonist with cAMP analogues that can activate PKA and PKG (8 Br-cAMPS and 8-pCPT-cGMPS, respectively (30 μM; selected from Butt *et al.*, 1992; Freund-Michel *et al.*, 2010). Having established that PKA activation mimicked the effect of a β-adrenoceptor agonist on capsaicin responses in wild-type tissues, the experiment was repeated in tissues from *Adcy6*$^{-/-}$ mice.

Data analysis

ASM tone experiments. Monophasic agonist concentration–effect curves were fitted by least-squares, non-linear iterative regression following the form of the Hill equation (Prism 5®, GraphPad Software Inc., San Diego, CA, USA).

Vagal depolarization experiments. Data are presented as mean ± SEM of *n* independent observations. Inhibition of agonist responses in the isolated vagus nerve preparation were analysed by two-tailed paired *t*-test, comparing responses to agonist in the absence and presence of antagonist in the same piece of nerve. $P < 0.05$ was taken to indicate significant differences between group means.

Materials

The EP_2 receptor agonist (ONO-AE1-259) was a gift from Ono Pharmaceuticals (Osaka, Japan). It was prepared in DMSO (10 mM stock) and stored at –20°C until required. PGE_2 was purchased from Cayman Europe (Tallinn, Estonia) and stock solutions of 10 mM were made in ethanol. Papaverine was purchased from Sigma Aldrich (Poole, UK) and dissolved in distilled water at 100 mM. Krebs salts were obtained from BDH (Dorset, UK) and all other chemicals and reagents were

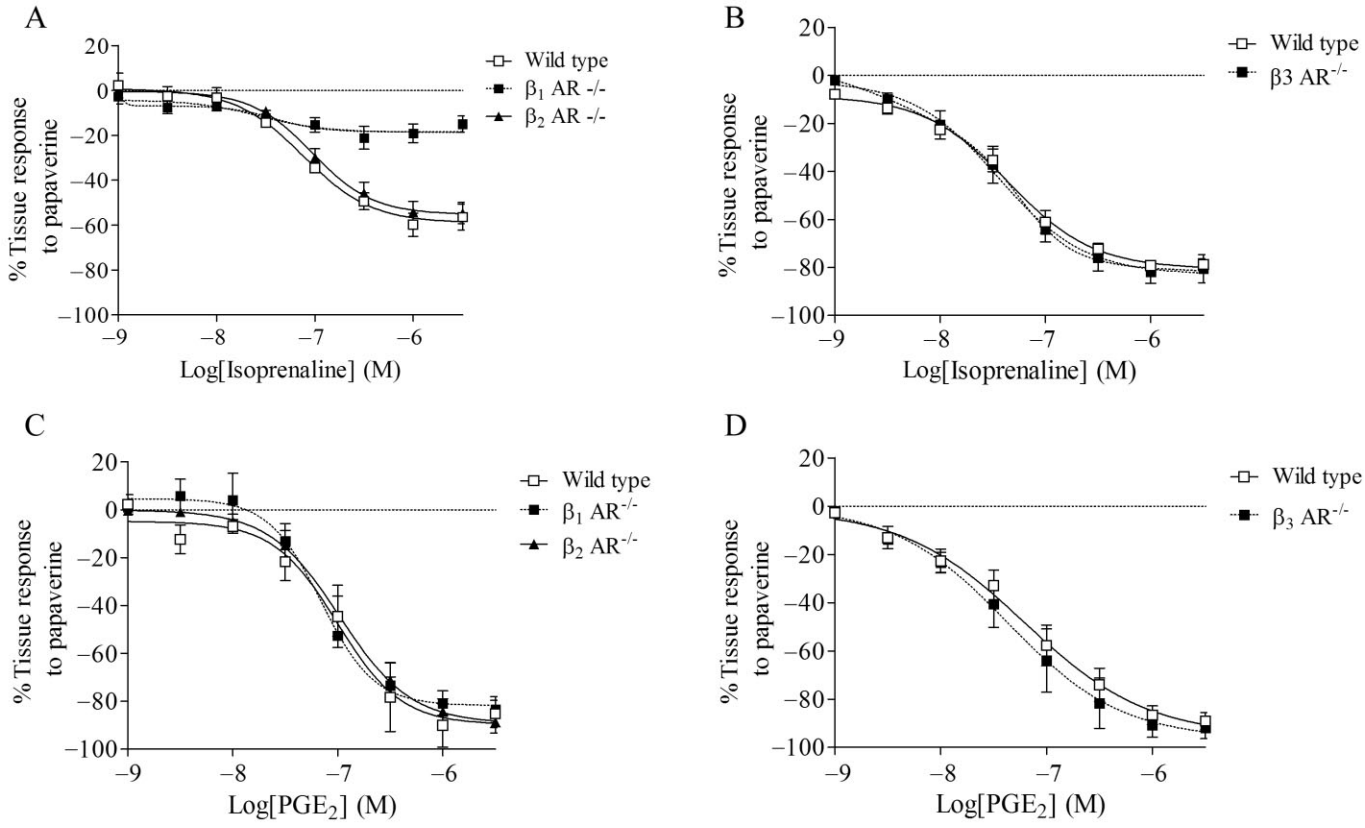

Figure 1

Effect of β-adrenoceptor activation on isolated tracheal tissue from wild–type and KO mice. Tracheal tissue from wild-type, age-matched control mice and KO mice (*Adrb1*$^{-/-}$, *Adrb2*$^{-/-}$ or *Adrb3*$^{-/-}$), placed in tissue baths and the effect of cumulative concentrations of isoprenaline or PGE_2 assessed on tissues pre-contracted with CCh (1 μM). Panel A compares agonist responses in tissues from wild-type (FVB/N) mice with those in tissues from *Adrb1*$^{-/-}$ ('β_1 AR$^{-/-}$') and from *Adrb2*$^{-/-}$ ('β_2 AR$^{-/-}$') mice. Panel B compares responses to the β-adrenoceptor agonist in tissues from wild-type (FVB/N) mice with those in tissues from *Adrb3*$^{-/-}$ ('β_3 AR$^{-/-}$') mice. Panel C compares PGE_2 responses in tissues from wild-type (FVB/N) mice with those in tissues from *Adrb1*$^{-/-}$ ('β_1 AR$^{-/-}$') and *Adrb2*$^{-/-}$ ('β_2 AR$^{-/-}$') mice. Panel D compares PGE_2 responses in tissues from wild-type (FVB/N) mice with those in tissues from *Adrb3*$^{-/-}$ ('β_3 AR$^{-/-}$') mice. The data shown are the percentage of papaverine relaxation and are presented as mean ± SEM; *n* = 4–6 animals in each group. The concentration-response curve was fitted using GraphPad Prism.

purchased from Sigma Aldrich. ACh and CCh were purchased from Sigma Aldrich and dissolved in Krebs solution at 100 and 1 mM respectively. Capsaicin was purchased from Sigma Aldrich and dissolved in DMSO at 1 mM. Denopamine, fenoterol, formoterol and BRL 37344 were purchased from Sigma Aldrich and dissolved in DMSO at 100, 1 and 100 mM respectively. ICI 118551 and CGP 20712 were from Sigma Aldrich. 8 Br-cAMPS and 8-pCPT-cGMPS were bought from Sigma Aldrich and dissolved in DMSO at 30 mM.

Results

Identifying the β-adrenoceptor associated with functional responses in murine airway tissue

The non-selective β-adrenoceptor agonist, isoprenaline, caused a concentration-dependent relaxation of wild-type tracheal smooth muscle tissue that was not different in the trachea harvested from *Adrb2*$^{-/-}$ and *Adrb3*$^{-/-}$ mice (Figure 1A and B). The tracheal tissue from the *Adrb1*$^{-/-}$ mice failed to relax to isoprenaline. In parallel tissues, the PGE_2 response appeared not to be altered (Figure 1C and D). These data confirm observations by Henry and Goldie (1990), using pharmacological tools that activation of the β_1-, and not the β_2-adrenoceptor causes relaxation of murine ASM.

The β_1-adrenoceptor selective agonist, denopamine, caused concentration-dependent relaxation of the mouse trachea with the potency similar to reported values (Figure 2A). The β_1–adrenoceptor selective antagonist, CGP 20712, caused a rightward shift in the denopamine response (Figure 2). The β_2-adrenoceptor agonist, fenoterol, also caused concentration-dependent relaxation of the mouse trachea but with potency far below that expected in a β_2-adrenoceptor-driven system (Figure 2B). Indeed, the data shown in Figure 2C, using the guinea pig trachea, a tissue known to be driven by β_2-adrenoceptors (Spicuzza *et al.*, 2001), demonstrate the expected potency of fenoterol.

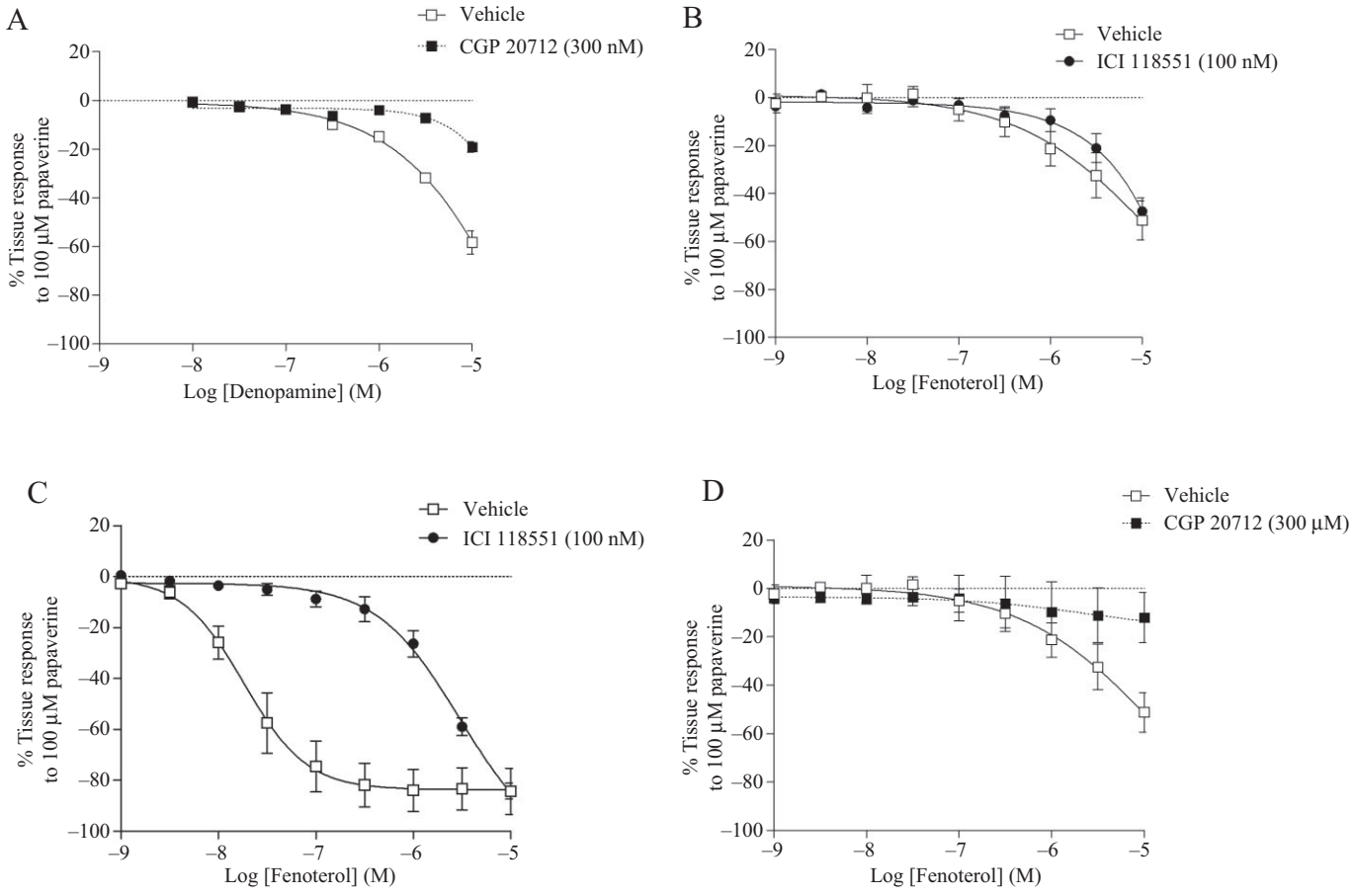

Figure 2

Effect of β-adrenoceptor activation on isolated murine tracheal tissue, using pharmacological tools. Tracheal tissues from male mice, or guinea pigs were placed in tissue baths and the effect of cumulative concentrations of the β_1-or β_2-adrenoceptor agonists, denopamine or fenoterol, assessed on tissues pre-contracted with CCh (1 µM). Panel A shows responses to denopamine ± the β_1-adrenoceptor antagonist, CGP 20712, in mouse tissue. Panel B shows responses to fenoterol ± the β_2-adrenoceptor antagonist, ICI 118551, in mouse tissue. Panel C shows responses to fenoterol ± the β_2-adrenoceptor antagonist, ICI 118551, in guinea pig tissue. Panel D shows responses to fenoterol ± the β_1-adrenoceptor antagonist, CGP 20712, in mouse tissue. The data shown are the percentage of papaverine relaxation and are presented as mean ± SEM; n = 4–6 animals in each group. The concentration-response curve was fitted using GraphPad Prism.

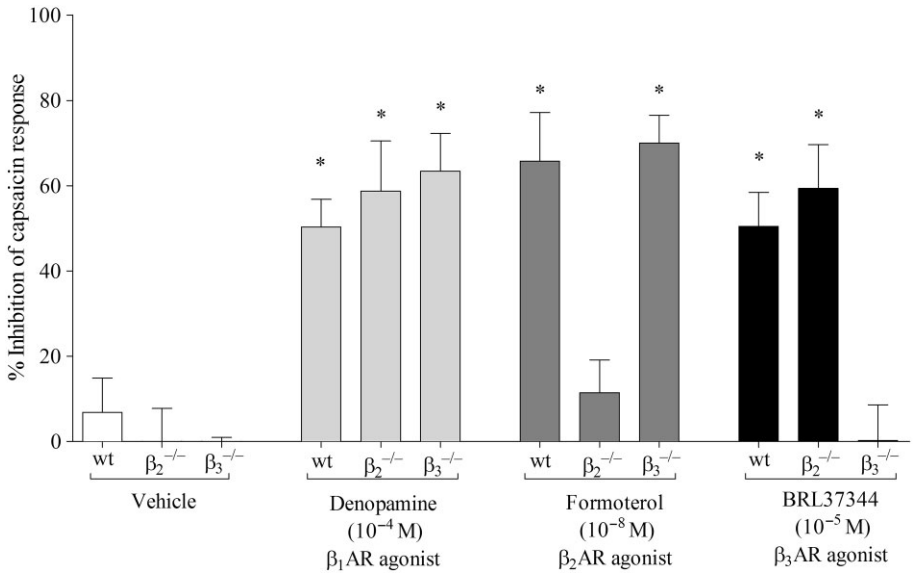

Figure 3

Effect of β-adrenoceptor activation on isolated murine vagal tissue. Vagal tissue from wild-type, age-matched control mice and KO mice (*Adrb2*$^{-/-}$;'$\beta_2^{-/-}$' or *Adrb3*$^{-/-}$;'$\beta_3^{-/-}$') was placed in a grease-gap recording system. The effect of selective β_1, β_2 and β_3-adrenoceptor agonists (denopamine: 100 μM, formoterol: 10 nM, BRL 37344: 10 μM) on capsaicin (1 μM) induced nerve depolarization in each strain was determined. The data (% inhibition of capsaicin responses) are presented as mean ± SEM; $n = 4$ animals in each group. *$P < 0.05$, significant inhibition of the capsaicin responses; paired Student's *t*-test.

Further, in the mouse trachea, the fenoterol-induced relaxation was suppressed by the β_1-adrenoceptor selective antagonist and much less so with the β_2-adrenoceptor selective antagonist (Figure 2B and D). However in the guinea pig trachea, the β_2-selective antagonist caused a marked rightward shift in the concentration-response curve with a pA_2 value of around the reported value, 9.25 (Figure 2C).

All of the selective β–adrenoceptor agonists modulated the responses to capsaicin in vagal tissue from wild-type mice (Figure 3). The β_2- and β_3-adrenoceptor selective agonists failed to modulate capsaicin responses in vagal tissue from their respective KO mice (Figure 2). The β_1-adrenoceptor selective agonist was still effective in the vagus from *Adrb2*$^{-/-}$ and *Adrb3*$^{-/-}$ mice, a finding consistent for a role of the β_1–adrenoceptor receptor. Recovery capsaicin responses were not altered in these experiments (data not shown).

AC isoform expression in ASM and vagal sensory ganglia

The data generated using the specific and validated RT-PCR assays demonstrated that AC6 was the dominant isoform expressed both in the mouse ASM and in the vagal sensory ganglia (Figure 4). Although mRNA expression is not necessarily indicative of protein expression or enzyme activity, in the absence of any available selective AC6 antibodies for Western blotting/immunohistochemistry, we focussed on the functional role of AC6 in our further investigations. To this end, *Adcy6*$^{-/-}$ mice were acquired, a colony established and male mice used for further studies.

Role of AC6 in β–adrenoceptor agonist responses in murine tissues

Maximal contractile responses to ACh were not reduced in trachea from *Adcy6*$^{-/-}$ mice compared with wild type (140.3 ± 45.8 mg, $\log EC_{50} = -4.9$ compared with 205.5 ± 50.9 mg and a $\log EC_{50} = -4.8$, respectively, $n = 6$, $P > 0.05$). Pre-incubation with the β_1–adrenoceptor agonist, denopamine caused a rightward shift in the concentration–response curve to ACh in tissue from wild-type mice typical of a bronchodilator drug of this class (Figure 5). In the tissues from the *Adcy6*$^{-/-}$ mice, denopamine failed to alter the ACh concentration-dependent contractile responses (Figure 5B). This suggests that the AC6 isoform is crucial for to β-adrenoceptor agonist-induced relaxation of murine ASM. The EP_2 receptor agonist, ONO AE1 259, also evoked a rightward shift in the concentration–response curve to ACh, which was not changed in tissue from the *Adcy6*$^{-/-}$ mice (Figure 5C and D). This suggests that, unlike the β–adrenoceptor agonist, in this system, PGE_2 acts independently of the AC6 isoform.

Vagal depolarization to capsaicin was not reduced in tissues from the *Adcy6*$^{-/-}$ mice compared with wild type (0.256 ± 0.04 and 0.311 ± 0.05 mV, respectively, $n = 6$, $P < 0.05$). As previously demonstrated β_1-, β_2- and β_3-adrenoceptor selective agonists attenuated capsaicin-induced vagal depolarization in tissues from wild-type mice (Figure 6). These effects were lost in vagal tissue from *Adcy6*$^{-/-}$ mice, suggesting that the AC6 isoform was also crucial to the responses of mouse sensory nerves to β-adrenoceptor agonists (Figure 6). To determine if signalling downstream of the β-adrenoceptor was still functional in the *Adcy6*$^{-/-}$ mice, we tested a cAMP analogue (8-Br-cAMPS) that is reported to activate PKA at a

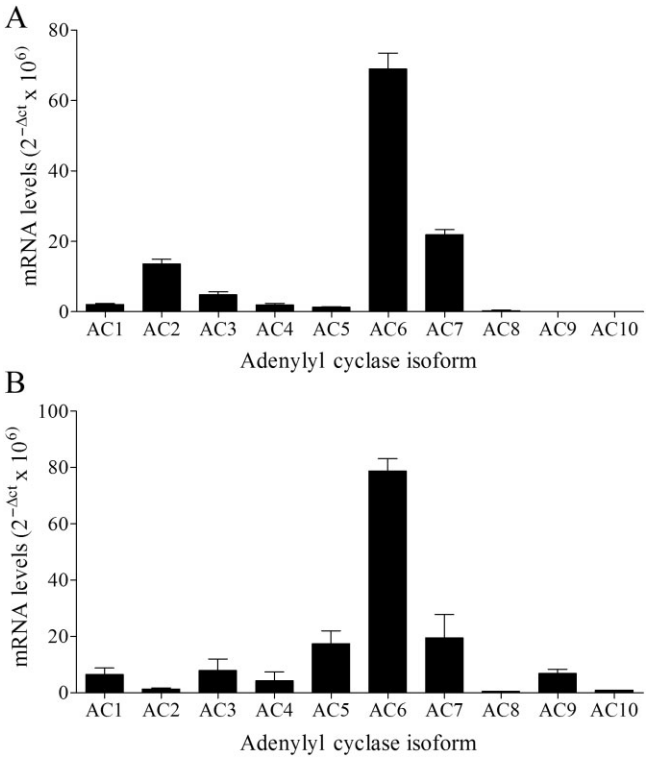

Figure 4

AC isoform mRNA expression in murine ASM and nodose/jugular ganglia. Tracheal ASM and nodose/jugular ganglia were harvested from male C57BL/6 mice. mRNA was extracted and cDNA prepared for real-time RT-PCR. Validated assays were used to measure the expression profiles of the AC isoforms in ASM (panel A) and ganglia (panel B). The data represented as mean ± SEM; $n = 4$ animals in each group.

specific range of concentrations, while not activating PKG. This analogue was able to mimic the effect of β-adrenoceptor agonists (Figure 6A). Then we used the same tool in vagal tissue from mice missing functional *Adcy6*$^{-/-}$ and demonstrated that PKA activation modulates capsaicin responses in *Adcy6*$^{-/-}$ mice to the same extent as tissue from wild-type mice (Figure 7B).

Discussion and conclusions

The β-adrenoceptor agonists are one of the most commonly prescribed medicines for patients with respiratory diseases such as asthma and COPD. They are thought to achieve their clinical benefit via AC-dependent production of cAMP but the exact mechanism by which this class of drug acts remains elusive. This is partly due to the fact that there are several AC isoforms and there are currently no truly selective pharmacological inhibitors. The aim of this study was to use tissue from genetically modified mice to establish the role of AC isoforms in β-adrenoceptor agonist responses in the airway.

Before investigating the signalling pathways, the aim was to establish or confirm the functional role of the three β-adrenoceptors (β_1, β_2 and β_3) in the murine tissue based systems. Using tissue from β-adrenoceptor KO mice and pharmacological ligands, we confirmed previous findings that, in the mouse airway, it was activation of β_1-adrenoceptors, and not the β_2-adrenoceptor (as it is in humans), that led to relaxation of ASM. Although certain experimental conditions might reveal a role for the β_2-adrenoceptor (Lin *et al.*, 2012), our data suggest the β_1-adrenoceptor plays a dominant role in mediating relaxation of murine tracheal ASM by β-adrenoceptor agonists, consistent with the findings of Henry and Goldie (1990). Interestingly, in a separate publication the same authors demonstrated that the lack of β_2-adrenoceptor agonist mediated relaxation was not due to a lack of receptor expression in murine ASM (Henry and Goldie, 1990; Henry *et al.*, 1990). Finally, in these experiments PGE_2 still induced relaxation (via activation of the EP_2 receptor) in tracheal tissue from mice lacking the β_1-adrenoceptor, suggesting that the ability to relax ASM was not compromised by genetic modification in this strain. Having established that the relaxation of ASM in murine trachea was dependent on β_1-adrenoceptor activation, further experiments investigated which β-adrenoceptor was involved in another functional response related to airway function. Earlier, we reported that β_2-adrenoceptor agonists inhibited sensory nerve activation in guinea pig and human vagal tissue and the cough reflex in a conscious guinea pig model (Freund-Michel *et al.*, 2010). Using tissue from genetically modified mice and pharmacological tools, these results were confirmed but it was also apparent that this property was also shared by the other β-adrenoceptors, namely, the β_1 and β_3 receptors. These data corroborate previously published data in which agonists of each β-adrenoceptor caused a concentration-related inhibition of capsaicin-induced depolarization in guinea pig, mouse and human vagus. This inhibition was reversed only by respective β_1, β_2 or β_3-adrenoceptor antagonists in guinea pig and human vagus (Wortley *et al.*, 2013).

To investigate which isoform of AC could be central to these β-adrenoceptor agonist-driven responses, we first assessed AC mRNA expression profiles in ASM and sensory nerve cell bodies (contained in the nodose and jugular ganglia). Using validated assays, we found that AC6 was the predominant isoform expressed in both types of tissue . Although mRNA expression is not necessarily indicative of actual protein expression, or the activity of the isoform, it is strongly suggested that AC6 is present in the cell types we were investigating. Although one study describes the presence of AC isoforms (including AC6) in rat dorsal root ganglia (Zhu and Oxford, 2011) and others have presented data from whole murine lung tissue (Sunahara *et al.*, 1996; Hanoune *et al.*, 1997), we are not aware of other reports of AC isoform expression in these specific murine cell types. However, in human ASM, several publications have indicated a similar mRNA expression profile to that observed in the murine tissues presented here, with dominant expression of AC6 (Billington *et al.*, 1999; Xu *et al.*, 2001; Bogard *et al.*, 2011). This would suggest that our data on AC isoform expression in mouse tissue are indicative of that seen in human cells (Billington *et al.*, 1999; Bogard *et al.*, 2011). At this time, the

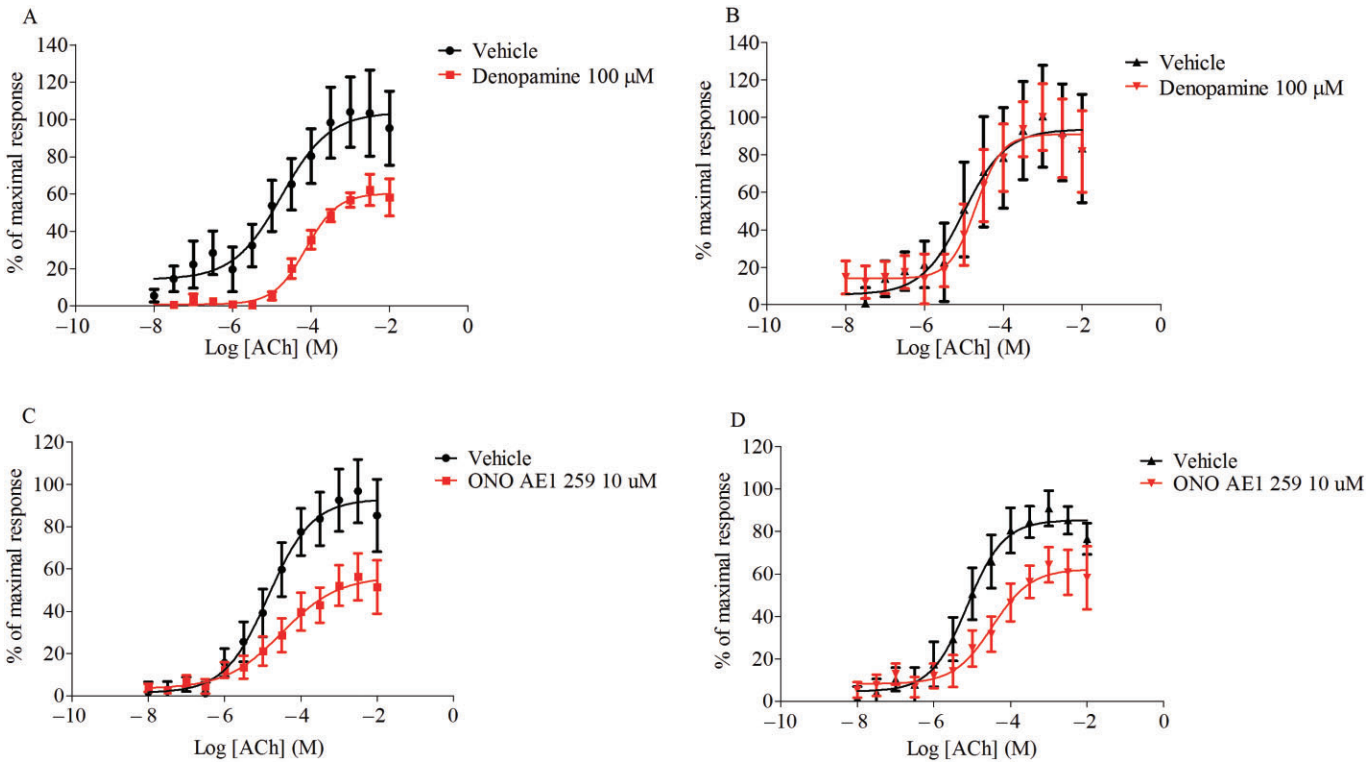

Figure 5

A role for AC6 isoform in β-adrenoceptor-mediated responses in mouse trachea. Tracheal tissue from wild-type, age-matched control mice and KO mice (*Adcy6*$^{-/-}$;'AC6$^{-/-}$') was placed in tissue baths. Vehicle (0.1% DMSO), a β_1-adrenoceptor agonist, denopamine (100 μM) or an EP_2 receptor agonist, ONO AE1 259 (10 μM), were incubated with the tissue for 10 min prior to the addition of cumulative concentrations of ACh. Panel A compares the effect of vehicle and β_1-adrenoceptor agonist on ACh responses in tissues from wild-type mice whereas panel B compares the same responses in tissues from *Adcy6*$^{-/-}$ ('AC6$^{-/-}$') mice. Panel C compares the effect of vehicle and ONO AE1 259 ACh responses in tissues from wild-type mice whereas panel D compares the same responses in tissues from *Adcy6*$^{-/-}$ mice. The data shown are the percentage of initial supra-maximal ACh, and were curve fitted using GraphPad Prism and are presented as mean ± SEM; *n* = 6–7 animals in each group.

lack of truly selective antibodies prevents us from confirming that the mRNA expression profile is reproduced at the protein level.

In these studies, it was established that AC6 was a prime candidate for mediating the post β-adrenoceptor signalling cascade and functional responses seen in murine tissues. In the absence of selective inhibitors, we performed comparative functional experiments with wild-type tissue and tissues harvested from AC6 KO mice (Tang *et al.*, 2008). The data clearly impy a central role for the AC6 isoform in β_1 receptor-relaxation of murine ASM mediated by β_1-adrenoceptors and attenuation of sensory nerve activity mediated by β_1, β_2 and β_3-adrenoceptors. To our knowledge this is the first study to provide clear evidence that AC activity is actually involved in the relaxation of ASM by β-adrenoceptor agonists and that it is the AC6 isoform which is key. Furthermore, it would seem that in murine sensory nerves the three β receptors share a common step in their signalling cascade, that is, AC6. Finally, it would seem that activating PKA (with an cAMP analogue), and not PKG, can mimic the effect of the β-adrenoceptor agonists and that AC6 is upstream of PKA as the PKA activator was still functional in the tissue from the *Adcy6*$^{-/-}$ mice. A previous publication utilizing the *Adcy6*$^{-/-}$ mice reported that these mice also had lower levels of AC5, but that the apparent reduction was not sufficient to generate the phenotype usually observed in *Adcy5*$^{-/-}$ mice, suggesting that any reduction in expression was not functionally relevant (Tang *et al.*, 2008). Interestingly, in the same system, relaxation of murine ASM triggered by EP_2 receptor agonists was not dependent on the AC6 isoform. This may suggest that other G_s-coupled receptors, such as the EP_2 receptor, may preferentially couple to other AC isoforms in the same cell, which has interesting implications for compartmentalized signalling, its regulation and targeting. These data build on a recent publication which suggested that in cultured human ASM cells overexpressing AC6, production of cAMP was enhanced by β-adrenoceptor agonists, but not by EP receptor agonists (Bogard *et al.*, 2011).

In summary, our data demonstrate that AC6 plays a key role in the relaxation of ASM and modulation of sensory nerves induced by β-adrenoceptor agonists. Mice are often the species of choice for preclinical modelling of respiratory diseases and thus our data may help to design and interpret relevant future studies in this field. Furthermore, these data represent a vital step in understanding the mechanism of action of this extensively prescribed class of medicine and leads the way for the next phase of research and determining the role of AC isoforms in human systems.

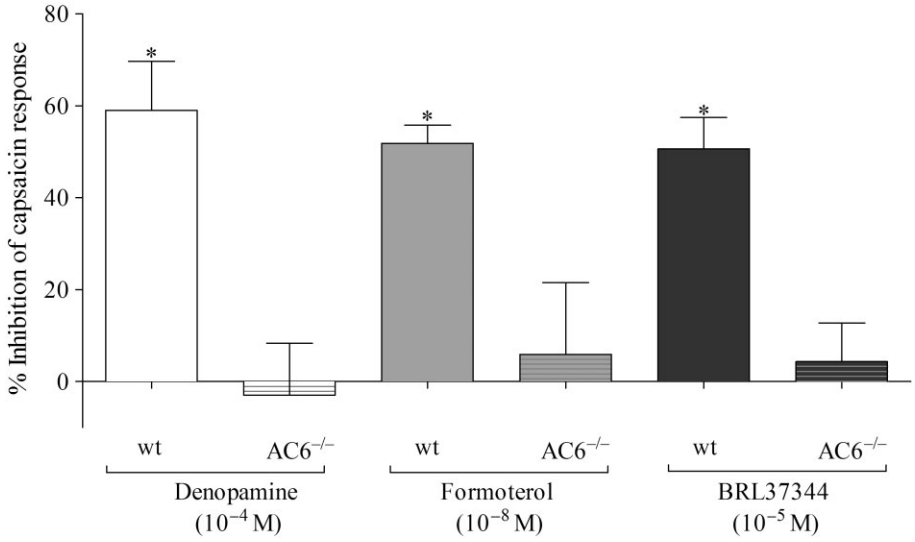

Figure 6

A role for AC6 in β-adrenoceptor responses in mouse vagus. Vagal tissue from wild-type, age-matched control mice and KO mice (*Adcy6*$^{-/-}$) was placed in a grease-gap recording system. The effect of specific β_1, β_2 and β_3-adrenoceptor agonists (denopamine: 100 μM, formoterol: 10 nM, BRL 37344: 10 μM) on capsaicin-induced nerve depolarization were compared in tissue from wild-type and *Adcy6*$^{-/-}$ ('AC6$^{-/-}$') mice. The data (% inhibition of capsaicin responses) are presented as mean ± SEM; $n = 4$ animals in each group. *$P < 0.05$, significant inhibition of the capsaicin responses; paired Student's *t*-test.

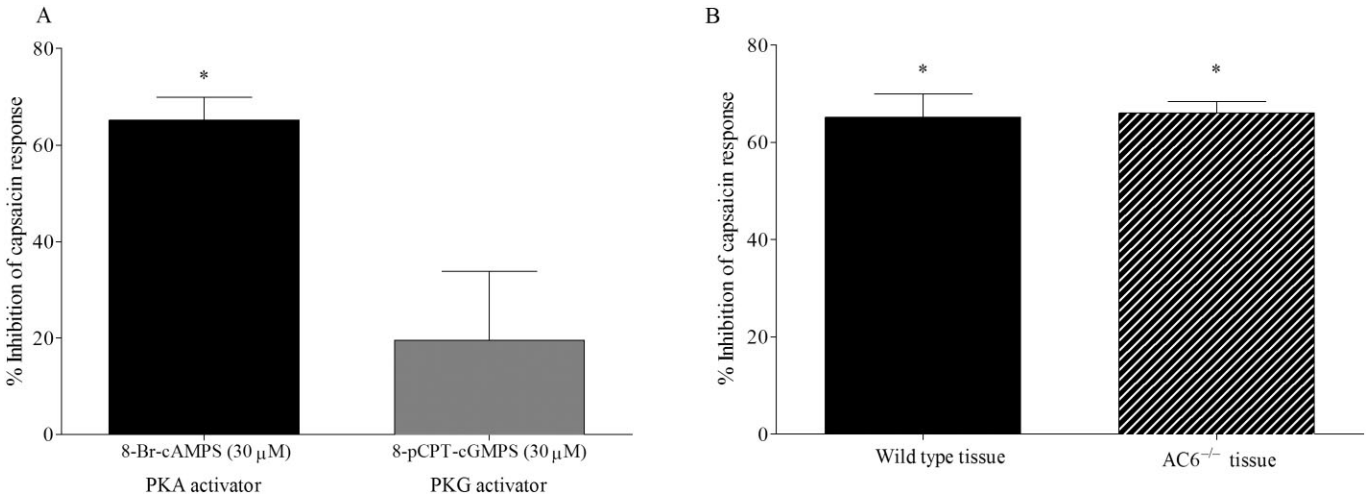

Figure 7

Role for PKA/PKG in *Adcy6*$^{-/-}$ mouse vagus. Vagal tissue from wild-type, age-matched control mice and KO mice (*Adcy6*$^{-/-}$) was placed in a grease-gap recording system. The effect of cAMP analogues that can activate either PKA or PKG (both 30 μM) on capsaicin-induced nerve depolarization in nerve tissue from wild-type mice was determined (panel A). The effect of the cAMP analogue that can activate PKA on capsaicin-induced depolarization was then compared in vagal tissue from wild-type and *Adcy6*$^{-/-}$ ('AC6$^{-/-}$') mice (panel B). The data (% inhibition of capsaicin responses) are presented as mean ± SEM; $n = 4$ animals in each group. *$P < 0.05$, significant inhibition of the capsaicin responses; paired Student's *t*-test.

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Author contributions

M. A. B. and M. G. B. devised the experiment and wrote the manuscript; S. J. B., J. B. and N. D. performed and analysed the tissue bath experiments; M. W. performed and analysed

the vagal experiments, L. Y.-B. and S. A. M. performed the genotyping and RT-PCR.

Conflict of interest

None.

References

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL Spedding M *et al* (2013a). The Concise Guide to PHARMACOLOGY 2013/14: Enzymes. Br J Pharmacol 170: 1797–1867.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL Spedding M *et al* (2013b). The Concise Guide to PHARMACOLOGY 2013/14: G Protein-Coupled Receptors. Br J Pharmacol 170: 1459–1581.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Catterall WA *et al* (2013c). The Concise Guide to PHARMACOLOGY 2013/14: Ion Channels. Br J Pharmacol 170: 1607–1651.

Barnes PJ (1995). Beta-adrenergic receptors and their regulation. Am J Respir Crit Care Med 152: 838–860.

Billington CK, Hall IP, Mundell SJ, Parent JL, Panettieri RA, Benovic JL *et al*. (1999). Inflammatory and contractile agents sensitize specific adenylyl cyclase isoforms in human airway smooth muscle. Am J Respir Cell Mol Biol 21: 597–606.

Billington CK, Ojo OO, Penn RB, Ito S (2013). cAMP regulation of airway smooth muscle function. Pulm Pharmacol Ther 26: 112–120.

Bilski AJ, Halliday SE, Fitzgerald JD, Wale JL (1983). The pharmacology of a beta 2-selective adrenoceptor antagonist (ICI 118,551). J Cardiovasc Pharmacol 5: 430–437.

Birrell MA, Maher SA, Buckley J, Dale N, Bonvini S, Raemdonck K *et al*. (2013). Selectivity profiling of the novel EP2 receptor antagonist, PF-04418948, in functional bioassay systems: atypical affinity at the guinea pig EP2 receptor. Br J Pharmacol 168: 129–138.

Bogard AS, Xu C, Ostrom RS (2011). Human bronchial smooth muscle cells express adenylyl cyclase isoforms 2, 4, and 6 in distinct membrane microdomains. J Pharmacol Exp Ther 337: 209–217.

Brand CS, Hocker HJ, Gorfe AA, Cavasotto CN, Dessauer CW (2013). Isoform selectivity of adenylyl cyclase inhibitors: characterization of known and novel compounds. J Pharmacol Exp Ther 347: 265–275.

Buckley J, Birrell MA, Maher SA, Nials AT, Clarke DL, Belvisi MG (2011). EP4 receptor as a new target for bronchodilator therapy. Thorax 66: 1029–1035.

Butt E, Nolte C, Schulz S, Beltman J, Beavo JA, Jastorff B *et al*. (1992). Analysis of the functional role of cGMP-dependent protein kinase in intact human platelets using a specific activator 8-para-chlorophenylthio-cGMP. Biochem Pharmacol 43: 2591–2600.

Conley JM, Brand CS, Bogard AS, Pratt EPS, Xu R, Hockerman GH *et al*. (2013). Development of a high-throughput screening paradigm for the discovery of small-molecule modulators of adenylyl cyclase: identification of an adenylyl cyclase 2 inhibitor. J Pharmacol Exp Ther 347: 276–287.

Dennis SM, Sharp SJ, Vickers MR, Frost CD, Crompton GK, Barnes PJ *et al*. (2000). Regular inhaled salbutamol and asthma control: the TRUST randomised trial. Lancet 355: 1675–1679.

Dooley DJ, Bittiger H, Reymann NC (1986). CGP 20712 A: a useful tool for quantitating beta 1- and beta 2-adrenoceptors. Eur J Pharmacol 130: 137–139.

Duan B, Davis R, Sadat EL, Collins J, Sternweis PC, Yuan D *et al*. (2010). Distinct roles of adenylyl cyclase VII in regulating the immune responses in mice. J Immunol 185: 335–344.

Freund-Michel VC, Birrell MA, Giembycz MA, Hele DJ, Haj-Yahia S, Belvisi MG (2010). Beta(2)-agonists block tussive responses in guinea pigs via an atypical cAMP-dependent pathway. Eur Respir J 35: 647–654.

Giembycz MA, Newton R (2006). Beyond the dogma: novel beta2-adrenoceptor signalling in the airways. Eur Respir J 27: 1286–1306.

Goldie RG, Spina D, Henry PJ, Lulich KM, Paterson JW (1986). In vitro responsiveness of human asthmatic bronchus to carbachol, histamine, beta-adrenoceptor agonists and theophylline. Br J Clin Pharmacol 22: 669–676.

Hall JA, Kaumann AJ, Brown MJ (1990). Selective beta 1-adrenoceptor blockade enhances positive inotropic responses to endogenous catecholamines mediated through beta 2-adrenoceptors in human atrial myocardium. Circ Res 66: 1610–1623.

Hanoune J, Defer N (2001). Regulation and role of adenylyl cyclase isoforms. Annu Rev Pharmacol Toxicol 41: 145–174.

Hanoune J, Pouille Y, Tzavara E, Shen T, Lipskaya L, Miyamoto N *et al*. (1997). Adenylyl cyclases: structure, regulation and function in an enzyme superfamily. Mol Cell Endocrinol 128: 179–194.

Henry PJ, Goldie RG (1990). Beta 1-adrenoceptors mediate smooth muscle relaxation in mouse isolated trachea. Br J Pharmacol 99: 131–135.

Henry PJ, Rigby PJ, Goldie RG (1990). Distribution of beta 1- and beta 2-adrenoceptors in mouse trachea and lung: a quantitative autoradiographic study. Br J Pharmacol 99: 136–144.

Iwatsubo K, Okumura S, Ishikawa Y (2006). Drug therapy aimed at adenylyl cyclase to regulate cyclic nucleotide signaling. Endocr Metab Immune Disord Drug Targets 6: 239–247.

Johnson M (1998). The beta-adrenoceptor. Am J Respir Crit Care Med 158: S146–S153.

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). NC3Rs Reporting Guidelines Working Group. Br J Pharmacol 160: 1577–1579.

Koike K, Yamashita Y, Horinouchi T, Yamaki F, Tanaka Y (2004). cAMP-independent mechanism is significantly involved in beta2-adrenoceptor-mediated tracheal relaxation. Eur J Pharmacol 492: 65–70.

Kotlikoff MI, Kamm KE (1996). Molecular mechanisms of beta-adrenergic relaxation of airway smooth muscle. Annu Rev Physiol 58: 115–141.

Kume H, Hall IP, Washabau RJ, Takagi K, Kotlikoff MI (1994). Beta-adrenergic agonists regulate KCa channels in airway smooth muscle by cAMP-dependent and -independent mechanisms. J Clin Invest 93: 371–379.

Lin R, Degan S, Theriot BS, Fischer BM, Strachan RT, Liang J *et al*. (2012). Chronic treatment in vivo with β-adrenoceptor agonists induces dysfunction of airway β(2)-adrenoceptors and exacerbates lung inflammation in mice. Br J Pharmacol 165: 2365–2377.

Maher SA, Birrell MA, Belvisi MG (2009). Prostaglandin E2 mediates cough via the EP3 receptor: implications for future disease therapy. Am J Respir Crit Care Med 180: 923–928.

McGrath J, Drummond G, Kilkenny C, Wainwright C (2010). Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol 160: 1573–1576.

Onda T, Hashimoto Y, Nagai M, Kuramochi H, Saito S, Yamazaki H *et al.* (2001). Type-specific regulation of adenylyl cyclase. Selective pharmacological stimulation and inhibition of adenylyl cyclase isoforms. J Biol Chem 276: 47785–47793.

Patel TB, Du Z, Pierre S, Cartin L, Scholich K (2001). Molecular biological approaches to unravel adenylyl cyclase signaling and function. Gene 269: 13–25.

Pavan B, Biondi C, Dalpiaz A (2009). Adenylyl cyclases as innovative therapeutic goals. Drug Discov Today 14: 982–991.

Pawson AJ, Sharman JL, Benson HE, Faccenda E, Alexander SP, Buneman OP *et al.*; NC-IUPHAR (2014). The IUPHAR/BPS Guide to PHARMACOLOGY: an expert-driven knowledge base of drug targets and their ligands. Nucl Acids Res 42 (Database Issue): D1098–1106.

Spicuzza L, Belvisi MG, Birrell MA, Barnes PJ, Hele DJ, Giembycz MA (2001). Evidence that the anti-spasmogenic effect of the beta-adrenoceptor agonist, isoprenaline, on guinea-pig trachealis is not mediated by cyclic AMP-dependent protein kinase. Br J Pharmacol 133: 1201–1212.

Sunahara RK, Dessauer CW, Gilman AG (1996). Complexity and diversity of mammalian adenylyl cyclases. Annu Rev Pharmacol Toxicol 36: 461–480.

Tang T, Gao MH, Lai NC, Firth AL, Takahashi T, Guo T *et al.* (2008). Adenylyl cyclase type 6 deletion decreases left ventricular function via impaired calcium handling. Circulation 117: 61–69.

Turcato S, Clapp LH (1999). Effects of the adenylyl cyclase inhibitor SQ22536 on iloprost-induced vasorelaxation and cyclic AMP elevation in isolated guinea-pig aorta. Br J Pharmacol 126: 845–847.

Wong S, Belvisi MG, Birrell MA (2009). MMP/TIMP expression profiles in distinct lung disease models: implications for possible future therapies. Respir Res 10: 72.

Wortley MA, Birrell MA, Bonvini SJ, Maher SA, Dubuis ED, Grace MS *et al.* (2013). An investigation into the anti-tussive properties of β1-, β2-, and β3-adrenoceptor agonists. Am J Respir Crit Care Med 187: A4930.

Xu D, Isaacs C, Hall IP, Emala CW (2001). Human airway smooth muscle expresses 7 isoforms of adenylyl cyclase: a dominant role for isoform V. Am J Physiol Lung Cell Mol Physiol 281: L832–L843.

Zhu W, Oxford GS (2011). Differential gene expression of neonatal and adult DRG neurons correlates with the differential sensitization of TRPV1 responses to nerve growth factor. Neurosci Lett 500: 192–196.