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β_2-Agonists block tussive responses in guinea pigs *via* an atypical cAMP-dependent pathway

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ABSTRACT: β_2-Adrenoceptor agonists are the most effective bronchodilators currently available, and are used for symptom management in asthmatics. However, whether β_2-agonists are also antitussive is controversial. Identifying an antitussive role for β_2-agonists and dissecting the possible mechanism of action may help to explain the inconsistencies in the clinical literature and lead to the development of novel therapeutic agents. The aim of the present study was to determine whether or not β_2-agonists attenuate the tussive response in guinea pig and human models, and, if so, to identify the mechanism(s) involved.

Depolarisation of vagal sensory nerves (human and guinea pig) was assessed as an indicator of sensory nerve activity. Cough was measured in a conscious guinea pig model.

A β_2-agonist, terbutaline, dose-dependently inhibited the cough response to tussive agents in conscious guinea pigs. Terbutaline and another β_2-agonist, fenoterol, blocked sensory nerve activation *in vitro*. Using these mechanistic models, it was established that β_2-agonists suppress the tussive response *via* a nonclassical cyclic adenosine monosphosphate-dependent pathway that involves the activation of protein kinase G and, subsequently, the opening of large-conductance calcium-activated potassium channels.

In conclusion, β_2-adrenoceptor agonists are antitussive, and this property occurs due to a direct inhibition of sensory nerve activation. These findings may help to explain the confusion that exists in the clinical literature, and could be exploited to identify novel therapies for the treatment of cough, which is a significant unmet medical need.

KEYWORDS: Airway sensory nerves, β_2-adrenoceptor agonist, cough, cyclic adenosine monophosphate, large-conductance calcium-activated potassium channels, protein kinase G

The β_2-adrenoceptor agonists (β_2-agonists) are currently one of the most effective bronchodilators available; they have been used to alleviate bronchoconstriction in respiratory diseases, such as asthma, for many decades [1]. However, despite their prolific use, controversy remains as to whether or not they also possess antitussive properties. Clinical studies conducted to date have not managed to clearly establish whether or not β_2-agonists attenuate cough. Indeed, although some clinical studies in normal volunteers [2] and in chronic cough associated with allergic [3] or obstructive conditions [4–7] have found a beneficial effect of β_2-agonists, other trials have not found efficacy [8, 9]. This confusion may exist, in part, due to the subjective nature of symptom scoring in comparison with objective cough monitoring, which has only recently become available, and the fact that few double-blind randomised placebo-controlled studies have been performed with cough as a primary end-point. This is important given that patients (especially those with idiopathic cough) find it very difficult to make an accurate assessment of their cough. Furthermore, it is commonly believed that, if β_2-agonists do, indeed, suppress cough, bronchodilation is the likely mechanism of action [7, 8]. Currently the dose regimen/protocol for β_2-agonists in the clinic is routinely based around their relaxant properties and not geared to their antitussive activities (which may require higher doses), and we propose that this may be why a dominant antitussive property has hitherto not been discovered.

Cough is a protective reflex in healthy individuals [10]. However, chronic cough is associated with many inflammatory airway diseases, and is

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a very common and troublesome symptom, in particular in asthma or in chronic obstructive pulmonary disease [11, 12]. Cough is the most common respiratory complaint for which medical attention is sought (reviewed in [13]) However, currently, treatments for cough are very poor; most over-the-counter remedies are not effective and prescribed medications tend to work centrally, thereby rendering them inappropriate for most sufferers since, in many cases, they cause sedation. Clearly, understanding the mechanism behind any antitussive action of β_2-agonists could lead to new therapies that may impact upon this unmet clinical need.

We hypothesised that β_2-agonists possess antitussive properties through direct inhibition of sensory nerve activity, independent of bronchodilation. Confusion exists in the clinical literature surrounding this property of β_2-agonists, and this may be due to the fact that the signal transduction mechanisms that elicit bronchodilation are different from those that induce antitussive activity. Accordingly, the results of studies in which it was determined whether or not a β_2-agonist attenuated the *in vivo* cough response induced by clinically relevant tussive stimuli are reported here. The effect of β_2-agonists on guinea pig and human sensory nerve activity *in vitro* were also examined. This isolated preparation circumvents complicating issues, such as bronchodilation, and permits the signalling pathways that regulate cough due to changes in sensory nerve activity to be probed. These experiments were not aimed primarily at making a case for using β_2-agonists as antitussive therapies, but rather at uncovering an alternative signalling pathway, in which new targets could be revealed.

MATERIAL AND METHODS

Animals

Male Dunkin–Hartley outbred guinea pigs (300–500 g) were purchased (Harlan, Bicester, UK). Experiments were performed in accordance with the UK Home Office guidelines for welfare based on the Animals (Scientific Procedures) Act 1986. Unless specified otherwise, all chemicals were obtained from Sigma Aldrich (Poole, UK).

Measurement of cough in vivo *in the conscious guinea pig*

The measurement of cough in conscious guinea pigs has been described previously [14–17]. Terbutaline (0.05–3.0 mg·kg body weight^{-1} and 1 mL·kg body weight^{-1} intraperitoneally) or vehicle (0.5% methylcellulose/0.2% Tween 80 in saline) was administered 30 min prior to exposure to capsaicin (1×10^{-4}M; 5 min) or citric acid (0.3M; 10 min), and the number of coughs over a 10-min period was recorded.

Measurement of sensory nerve depolarisation of isolated vagus nerve preparations in vitro

In order to demonstrate an inhibitory action of β_2-agonists on sensory nerve activation, the fully characterised isolated vagal nerve preparation was used, as described in previous publications [14, 17]. Tussive agents, such as capsaicin and low-pH solutions, are known vagal sensory nerve stimulants and, as such, isolated guinea pig and human vagus nerve preparations have been shown to elicit nerve depolarisation responses to these stimulants [14, 17]. Furthermore, these agents are also known tussigenic agents in human and animal studies [16, 18]. These data suggest that the isolated vagus nerve is an ideal preparation for conducting comprehensive pharmacological assessments of agents that may activate or inhibit sensory nerve function and thus the cough reflex. Previous data also suggest that the information generated is broadly predictive of data generated in single afferent nerve fibre recording studies, further validating the use of the isolated vagus preparation as an *in vitro* indicator of the cough reflex [15].

Depolarisation was induced by superfusion of isolated nerves with capsaicin (1×10^{-6}M in guinea pig and 1×10^{-5}M in human tissue) or low-pH solution (pH 5) for 2 min. The nerves were then washed until the responses had returned to baseline. Following two reproducible depolarisations, the nerves were superfused with terbutaline (3×10^{-6}–3×10^{-4}M) or vehicle for 10 min. The responses to capsaicin or citric acid solution were then measured in the presence of terbutaline or its vehicle (0.1% dimethylsulfoxide). After washing, recovery was observed by again superfusing the nerves with the depolarising agents for 2 min. In some experiments, vagus nerves were pre-incubated (10 min) with antagonists, enzyme inhibitors or channel blockers before superfusion with terbutaline.

Human vagus nerves were obtained from donor tissue for heart/lung transplantation, with relevant approvals obtained from the Royal Brompton and Harefield Trust Ethics Committee (London, UK).

Measurement of cAMP levels in isolated guinea pig vagus nerve preparations in vitro

Desheathed vagus nerves were incubated in terbutaline (3×10^{-4}M) or vehicle (for 0–10 min at 37°C), and then frozen in liquid nitrogen and ground to a fine powder. Frozen tissues were weighed, homogenised in 10 volumes of 0.1M HCl and centrifuged at $600 \times g$. Cyclic adenosine monosphosphate (cAMP) levels were determined in the supernatants using a commercial enzyme immunoassay kit (cAMP EIA Kit; Biomol Research Laboratories, Inc., Plymouth Meeting, PA, USA) according to the manufacturer's instructions. cAMP levels were normalised to the frozen tissue weight. For each animal, one vagus nerve was treated with terbutaline while the second was used as a paired time-matched control.

Expression of results and statistical analysis

Data are presented as the mean$\pm$SEM of n independent observations. Nerve depolarisation is expressed in millivolts before and after drug addition, and presented as a percentage change. cAMP levels are expressed in picomoles per milligram of frozen tissue. Parametric data were analysed using a two-tailed unpaired t-test when comparing two groups, or by one-way ANOVA followed by a Newman–Keuls multiple comparison test, or followed by a Dunnett's multiple comparison test when comparing several treatment groups to the same control. Nonparametric data were analysed using a Kruskal–Wallis test followed by a Dunn's multiple comparison test. All data were analysed using GraphPad PRISM (Graphpad Software; San Diego, CA, USA). A p-value of <0.05 was considered significant.

RESULTS

Effect of terbutaline on cough in the conscious guinea pig

Capsaicin (1×10^{-4}M) induced cough in the conscious guinea pig, and this response was dose-dependently inhibited by

terbutaline, with a maximal inhibition of 84% at 0.3 mg·kg body weight^{-1} ($p<0.05$) (fig. 1a). Similarly, terbutaline dose-dependently inhibited cough elicited by a citric acid (low-pH) solution (0.3M), with a maximal inhibition of 85% at 1 mg·kg body weight^{-1} ($p<0.05$) (fig. 1b). Vehicle had no effect on cough induced by either capsaicin or by citric acid.

Effect of β_2-adrenoceptor agonists on depolarisation of the isolated vagus nerve

Capsaicin and citric acid solution elicited reproducible depolarisation of the guinea pig vagus nerve of 0.28 ± 0.05 and 0.18 ± 0.03 mV, respectively. Pretreatment of preparations with terbutaline inhibited the nerve depolarisation induced by both capsaicin (1×10^{-6}M) and low-pH solution (pH 5), in a concentration-dependent manner and with a maximal inhibition at 3×10^{-4}M ($p<0.001$ for both) (fig. 2a and b). Pretreatment of nerves with fenoterol (1×10^{-4}M), another β_2-adrenoceptor agonist, inhibited capsaicin-induced depolarisation of the guinea pig vagus nerve by 84% (fig. 2c). The effect of both terbutaline and fenoterol (1×10^{-4}M) on nerve depolarisation was abolished by the selective β_2-adrenoceptor antagonist ICI118551 (1 μM; $p<0.001$), showing involvement of the β_2-adrenoceptor (fig. 2c). Finally, the inhibitory effect of β_2-adrenoceptor agonists was also seen in the human vagus, since fenoterol (1×10^{-4}M) inhibited the depolarisation of isolated human vagus nerve preparations induced by capsaicin (fig. 2d).

All of the vehicles tested did not have any effect on vagus nerve depolarisation. Further experiments on depolarisation of isolated guinea pig vagus nerve preparations were then performed with capsaicin, using terbutaline at a submaximal concentration of 1×10^{-4}M.

Effect of increases in cAMP level on nerve depolarisation

Capsaicin-induced depolarisation of the guinea pig vagus nerve was inhibited in a concentration-dependent manner by pretreatment with 8-bromo-cAMP (8-Br-cAMP; a cAMP analogue), rolipram (a phosphodiesterase (PDE) 4 inhibitor) or forskolin (an activator of adenylyl cyclase), with maximal inhibition at 1×10^{-4}M ($p<0.05$ for all) (fig. 3a). 1,9-dideoxy-forskolin, an inactive analogue of forskolin, was inactive at all concentrations tested (fig. 3a). Terbutaline (3×10^{-4}M) produced a transient increase in cAMP levels in the guinea pig vagus nerve relative to time-matched controls, with a maximal two-fold increase after 20 s of treatment ($p<0.05$) (fig. 3b).

Involvement of adenylyl cyclase, protein kinase G and protein kinase A

Terbutaline-induced inhibition of capsaicin-elicited depolarisation of the guinea pig vagus nerve was abolished by the adenylyl cyclase inhibitor SQ 22536 (3×10^{-4}M; $p<0.05$) (fig. 4a), but unaffected by KT5720, a selective protein kinase (PK) A inhibitor (fig. 4b). In contrast, the inhibitory effect of terbutaline was blocked by the selective inhibitor of PKG KT5823 (87% inhibition; $p<0.05$) (fig. 4b).

Involvement of large-conductance calcium-activated potassium channels

The involvement of the various potassium channels was investigated using the following inhibitors: paxilline (1×10^{-6}M), to block large-conductance calcium-activated potassium channels (BK_{Ca}); clotrimazole (1×10^{-5}M), to block intermediate-conductance calcium-activated potassium channels; apamin (1×10^{-6}M), to block low-conductance calcium-activated potassium channels; and glibenclamide (10^{-5}M), to block ATP-activated potassium channels. Of the channel-blocking drugs tested, only paxilline reversed the inhibitory effect of terbutaline on capsaicin-induced depolarisation ($p<0.001$) (fig. 5a).

In separate experiments, depolarisation of the guinea pig vagus nerve was also induced by the endogenous activators prostaglandin (PG) E_2 (1×10^{-5}M) and bradykinin (1×10^{-5}M). These stimuli elicited nerve depolarisations of 0.12 ± 0.02 and 0.06 ± 0.01 mV, respectively. Pretreatment of the preparation with terbutaline inhibited both PGE_2- and bradykinin-induced nerve depolarisation by 95%, and this effect was prevented by paxilline ($p<0.001$) (fig. 5b).

1-(2′hydroxy-5′-trifluoromethylphenyl)trifluoro-methyl-2-[^{3}H]-benzimidazolone (NS1619; Research Biochemicals, Inc., St Albans, UK) has previously been shown to inhibit both the firing of single fibres in airway sensory nerves *in vitro* and

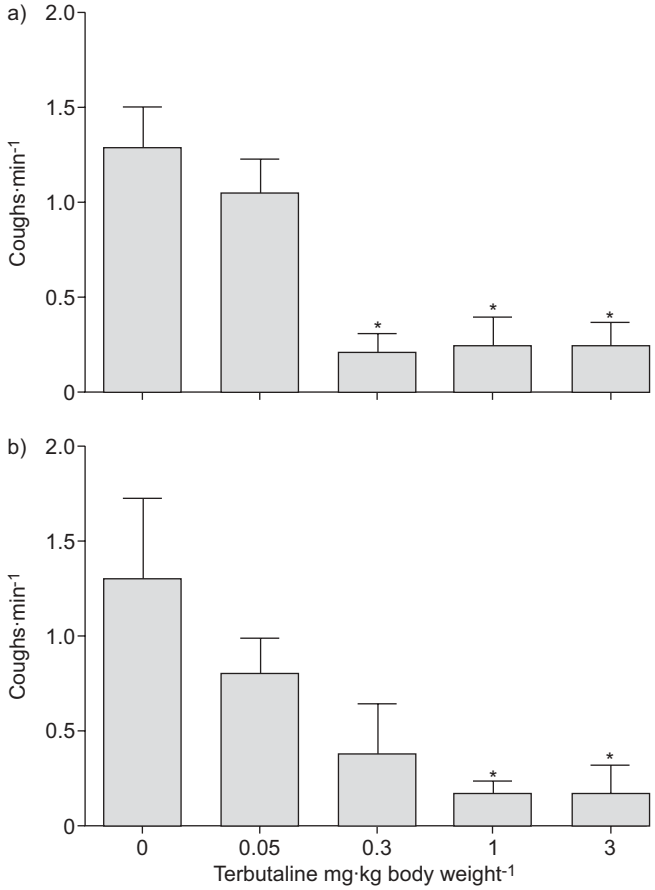

FIGURE 1. Effect of terbutaline on capsaicin- or low-pH-induced cough in the guinea pig *in vivo*. Terbutaline (0–3 mg·kg body weight^{-1}) was administered intraperitoneally 30 min prior to challenge with: a) aerosolised capsaicin (1×10^{-4} M); or b) citric acid (0.3 M). Data are presented as mean±SEM (n=8 per group). The β_2-agonist caused a dose-related inhibition of the cough response induced by both tussive agents. *: $p<0.05$.

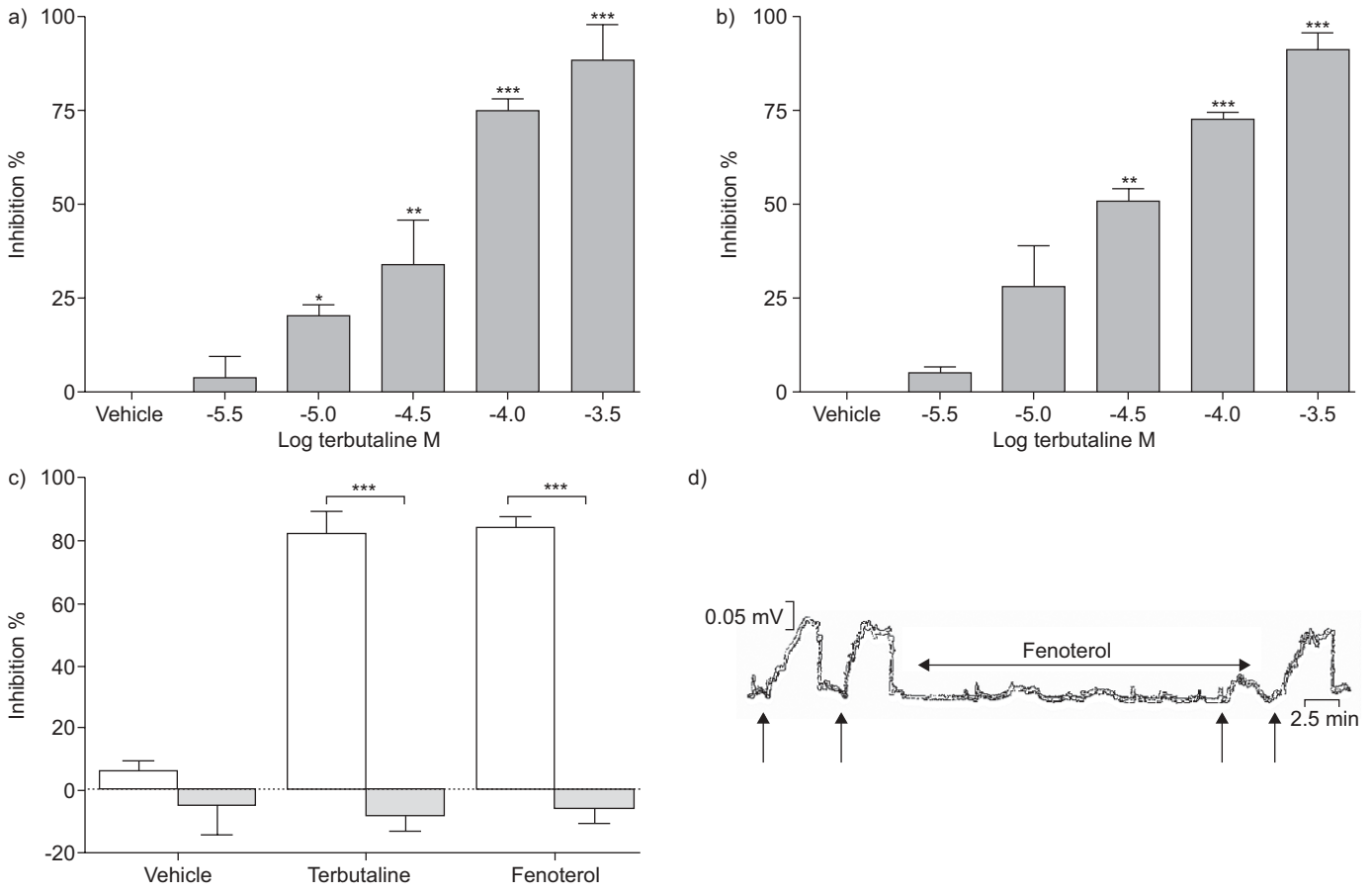

FIGURE 2. Effect of β_2-agonists on capsaicin- or citric-acid-induced depolarisation of the isolated vagus nerve. Terbutaline concentration-dependently inhibited depolarisation of the isolated guinea pig vagus nerve induced by: a) capsaicin (1×10^{-6}M; 0.28±0.05 mV); and b) low-pH solution (pH 5; 0.18±0.03 mV). c) A selective β_2-adrenergic receptor antagonist, ICI 118551 (1×10^{-6}M; ■), attenuated the blockade by terbutaline, and another β_2-agonist (fenoterol) of the capsaicin-induced depolarisation of the guinea pig vagus (□: vehicle). d) Trace from an experiment in which the effect of fenoterol (1×10^{-4}M) on capsaicin (1×10^{-5}M) challenge (vertical arrows)-induced depolarisation of the human (male transplant donor aged 45 yrs) vagus nerve was tested. Data are presented as mean±SEM (n=4). *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

guinea pig cough *in vivo*, through activation of BK_{Ca} [15]. NS1619 (3×10^{-5}M) was, therefore, tested on the guinea pig vagus nerve in order to validate the concept of inhibition of vagal activity through opening of BK_{Ca}, and was found to inhibit nerve depolarisation by 96% ($p<0.001$) (fig. 5c). Finally, paxilline (1×10^{-6}M) was also able to reverse the inhibitory effect of fenoterol (1×10^{-4}M) on capsaicin-induced depolarisation of the guinea pig vagus by 86% ($p<0.05$) (fig. 6a), and reversal of the fenoterol effect by paxilline was also demonstrated in an isolated human vagus preparation (fig. 6b).

DISCUSSION

In the present study, antitussive activity of β_2-agonists was demonstrated in a pre-clinical model of cough in the guinea pig *in vivo*, and it is here reported that this class of drugs directly inhibits sensory nerve activity *in vitro*. Moreover, the present findings were reproduced in the human vagus, implying that β_2-agonists may possess antitussive activity in humans. Mechanistically, β_2-agonists appear to inhibit depolarisation of the guinea pig vagus nerve by stimulating adenylyl cyclase, which leads to cAMP accumulation, activation of PKG and activation of BK_{Ca}, which, presumably, inhibit sensory nerve activation and, therefore, the cough reflex.

In the past, the antitussive activity of β_2-agonists, when found, was attributed to relaxation of the airway smooth muscle [9]. However, to date, clinical studies conducted to investigate the impact of β_2-agonists on the tussive response have led to contradictory results [1–5, 7–9], presumably due to the subjective nature of reporting in clinical trials prior to the advent of objective cough monitoring. Moreover, to our knowledge, the precise mechanism of action for this potential antitussive effect has not been studied. Herein, it is demonstrated that a β_2-agonist attenuates the cough response induced by capsaicin and citric acid. These tussive agents are commonly used in humans [19] and pre-clinical *in vivo* models, and this inhibitory effect of a β_2-agonist is in accordance with previous results observed in a similar model [20, 21].

In order to circumvent the potentially confounding bronchodilatory properties of β_2-agonists and to aid understanding of how the β_2-agonist inhibits cough, an isolated vagus preparation was used to study sensory nerve activity. This preparation has previously been characterised and shown to respond to a range of pre-clinical and clinical tussive agents. Moreover, this preparation provides the opportunity to conduct a comprehensive pharmacological assessment, to

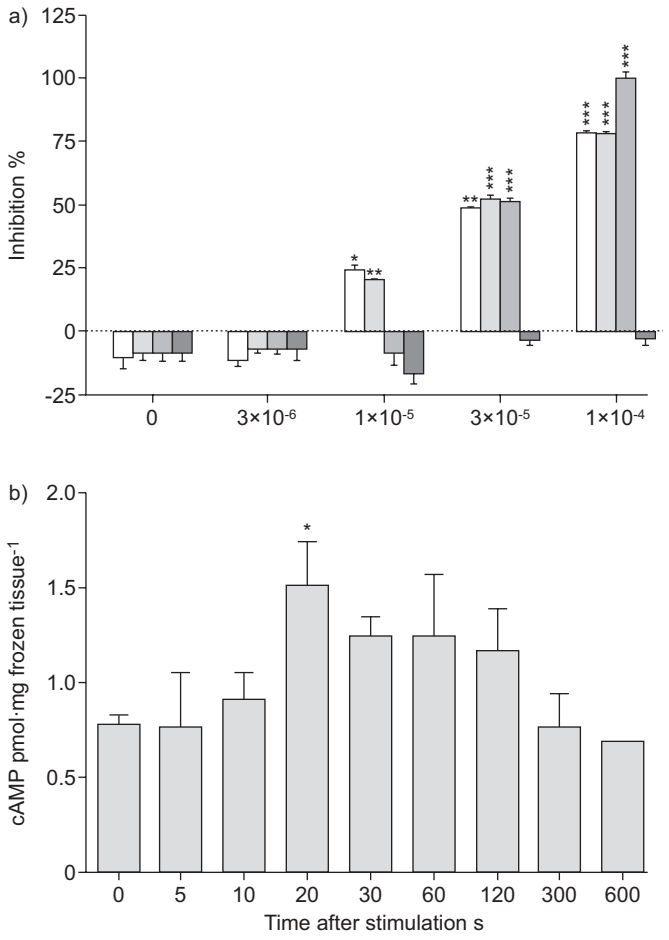

FIGURE 3. Involvement of cyclic adenosine monosphosphate (cAMP) in β_2-agonist-induced inhibition of guinea pig vagus nerve depolarisation. a) Effect of increasing cAMP levels in the guinea pig vagus nerve on capsaicin (1×10^{-6}M)-induced depolarisation. cAMP levels were altered using 8-bromo-cAMP (□; a cAMP analogue); rolipram (■; a phosphodiesterase 4 inhibitor); forskolin (■; an activator of adenylyl cyclase) and 1,9-dideoxyforskolin (■; an inactive analogue of forskolin). b) Increases in cAMP level in the guinea pig vagus nerve were measured after treatment with terbutaline (3×10^{-4}M). Data are presented as mean±SEM (n=3–4). *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

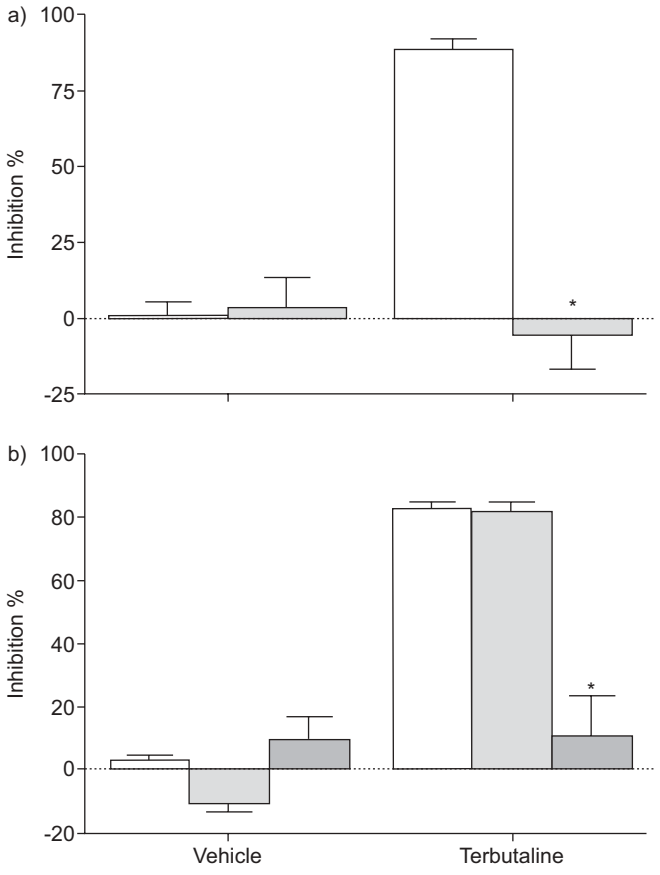

FIGURE 4. Effects of adenylyl cyclase, protein kinase (PK) A or PKG blockade on terbutaline-induced inhibition of guinea pig vagus nerve depolarisation. a) Effect of the selective adenylyl cyclase inhibitor SQ 22536 (3×10^{-4}M; ■) on the terbutaline-induced inhibition of depolarisation of the guinea pig vagus nerve elicited by capsaicin (1×10^{-6}M) (□: vehicle). b) Effect of the PKA inhibitor KT5720 (3×10^{-4}M; ■) or the PKG inhibitor KT5823 (3×10^{-4}M; ■) on the inhibitory action of terbutaline on capsaicin-induced depolarisation of the guinea pig vagus nerve (□: vehicle). Data are presented as mean±SEM (n=4). *: $p<0.05$.

assess the direct action of drugs without pharmacokinetics issues and to dissect the signalling pathways involved. However, data obtained using this model should be interpreted with some caution since the drugs are applied on the axon of the isolated vagus nerve preparation and not the nerve terminal. The depolarisation recorded is, therefore, the summation of changes in the membrane potentials of all of the axon due to activation of receptors on the axon itself, and these receptors may not reflect the receptors and signalling pathways at the peripheral endings [14, 17]. However, responses in guinea pig and human tissues are comparable, and the results appear to translate to *in vivo* models, thus suggesting that such preparations are amenable for studying the mechanisms involved in sensory nerve modulation [14, 17, 22].

Using this system, it was demonstrated that agonism of the β_2-receptor attenuated capsaicin- and citric acid-induced sensory nerve activation. Using a pharmacological approach, it was further established that both terbutaline and fenoterol were acting through the β_2-receptor. Moreover, by reproducing the effects in human vagal sensory tissue, it was demonstrated that this phenomenon may translate into humans. Finally, it was demonstrated that a β_2-agonist inhibits sensory nerve depolarisation elicited by PGE_2 or bradykinin, which are endogenous tussive mediators known to modulate sensory nerves in humans [23]. This suggests that β_2-agonists could be effective against a range of exogenous and endogenous tussive stimuli.

Terbutaline induced an elevation of cAMP concentration in the vagus nerve. Moreover, an inhibitor of adenylyl cyclase, SQ 22536, abolished terbutaline-induced inhibition of nerve depolarisation, whereas the cAMP analogue, 8-Br-cAMP, and other agents known to raise cAMP levels, such as forskolin or a PDE4 inhibitor, mimicked the effect of terbutaline. This finding is consistent with activation of the classical β_2-receptor signalling pathway that involves coupling of the β_2-receptor to adenylyl cyclase (reviewed in [24]). The present results, therefore, demonstrate a central role for cAMP in the signalling

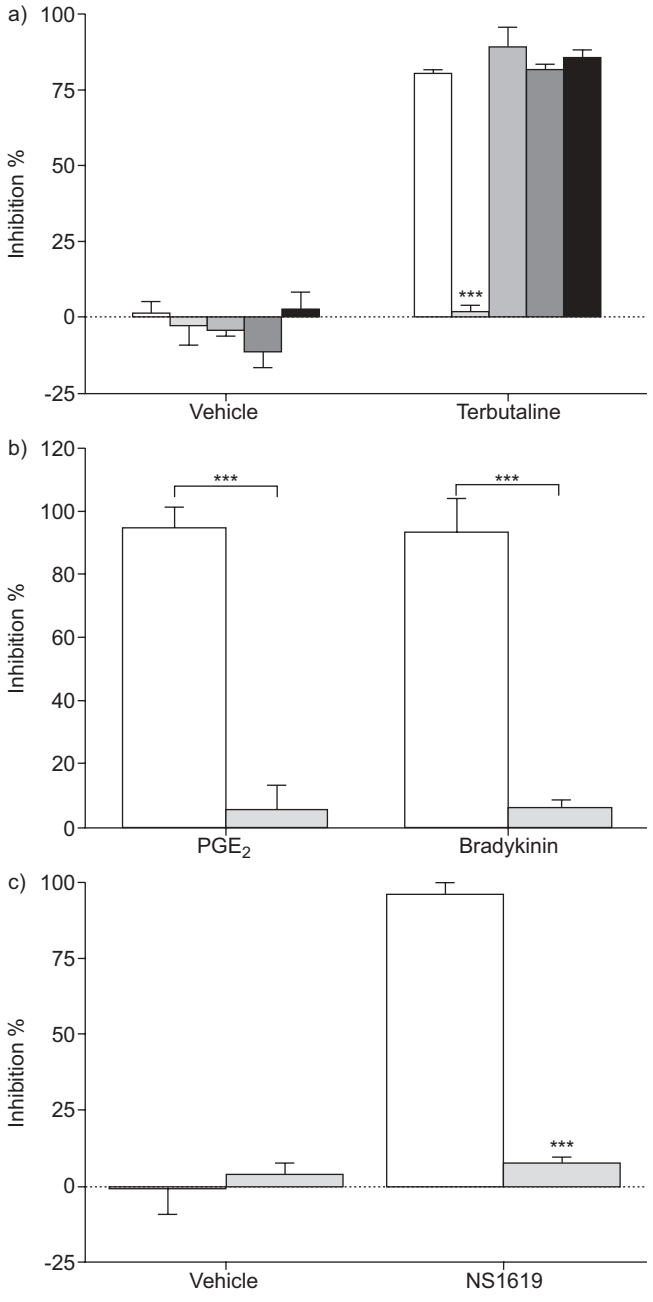

FIGURE 5. Involvement of large-conductance calcium-activated potassium channels (BK_{Ca}) in terbutaline-induced inhibition of guinea pig vagus nerve depolarisation. a) Effect of various channel blockers on the inhibitory action of terbutaline on capsaicin-induced depolarisation of the guinea pig vagus nerve. BK_{Ca} were blocked with paxilline (1×10^{-6}M; ■); low-conductance calcium-activated potassium channels with apamin (1×10^{-6}M; ■); ATP-activated potassium channels with glibenclamide (1×10^{-5}M; ■) and intermediate-conductance calcium-activated potassium channels with clotrimazole (1×10^{-5}M; ■) (□: vehicle). b) Inhibition by terbutaline (□) of depolarisation of the guinea pig vagus nerve induced by the known endogenous modulators prostaglandin (PG) E_2 (1×10^{-5}M) and bradykinin (1×10^{-5}M). These inhibitory effects were both reversed by paxilline (10^{-6}M; ■). c) Effect of 1-(2'hydroxy-5'-trifluoromethylphenyl)trifluoro-methyl-2-[^{3}H]benzimidazolone (NS1619; 3×10^{-5}M) on capsaicin-induced depolarisation of the guinea pig vagus nerve ((□: vehicle; ■: 1×10^{-6}M paxilline). Data are presented as mean±SEM (n=3–4). ***: $p<0.001$.

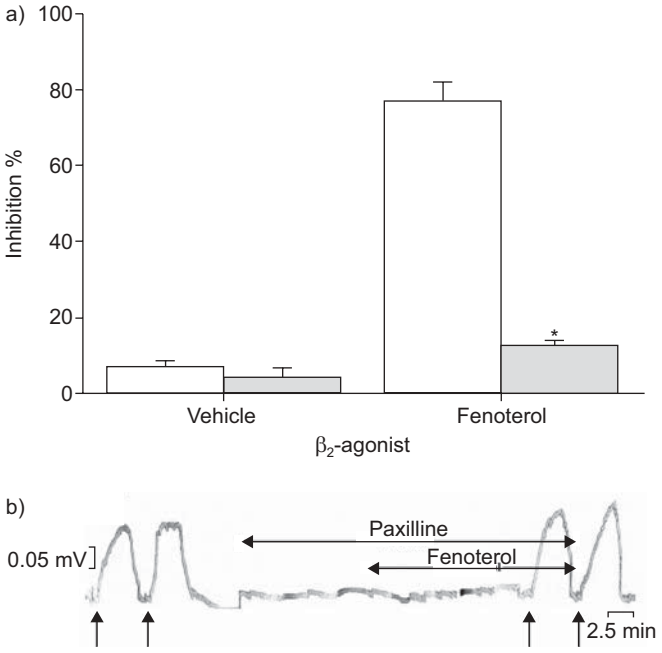

FIGURE 6. Effect of paxilline on β_2-agonist-induced inhibition of vagus nerve depolarisation. a) Effect of paxilline (1×10^{-6}M; ■) on the inhibitory action of fenoterol (1×10^{-4}M) on capsaicin-induced depolarisation of the guinea pig vagus nerve (□: vehicle). b) Trace from an experiment showing the effect of fenoterol (1×10^{-4}M) in the presence of paxilline (1×10^{-6}M) on capsaicin (1×10^{-5}M) challenge (vertical arrows)-induced depolarisation of the human (female transplant donor aged 53 yrs) vagus nerve. Data are presented as mean±SEM (n=3–4). *: $p<0.05$.

pathway activated by β_2-agonists that leads to the inhibition of sensory nerve activity.

The current dogma suggests that β_2-agonists relax airway smooth muscle *via* the cAMP-dependent activation of PKA [24, 25]. In the present study, however, terbutaline-induced inhibition of guinea pig vagus nerve depolarisation was unaffected by KT5720, an inhibitor of PKA, whereas KT5823, an inhibitor of PKG, abolished the effect of terbutaline. As the level of cyclic guanosine 3',5'-monophosphate was not elevated in the vagus (data not shown), these data suggest that cAMP cross-activates PKG. This phenomenon has been observed previously [26–28], leading to the suggestion that β_2-receptors can activate PKA-independent mechanisms to elicit functional responses [29–33]. If the bronchodilatory and antitussive activity of β_2-agonists were mediated by different signalling mechanisms, it could explain some of the discrepancies in the clinical literature (given that antitussive effects of β_2-agonists are not reported in every study in which this endpoint has been evaluated) since the dose regimen/protocol is routinely based around their relaxant properties and perhaps not geared to their antitussive activities.

In order to determine the potential direct or indirect target of PKG that could lead to inhibition of depolarisation in the vagus nerve, a range of ion-channel-blocking drugs were used. The results clearly demonstrate a role for BK_{Ca} since paxilline, a blocker of these channels, markedly inhibited the terbutaline

response. Moreover, NS1619, which activates BK_{Ca}, mimicked the effect of terbutaline. These results are in accordance with previous data [34–37]. Significantly, paxilline also reversed fenoterol-induced inhibition of capsaicin-induced depolarisation in the human vagus nerve, indicating that the signalling mechanism established in the guinea pig may be extrapolated to humans.

In conclusion, the present data show that β_2-adrenoceptor agonists are antitussive, and that this property occurs due to a direct inhibition of sensory nerve activation and not to the signalling pathways thought to be involved in bronchodilation. We suggest that these findings could explain the inconsistencies in the clinical literature regarding the variable antitussive activity of β_2-agonists. Currently, the dosing regimen/protocol for β_2-agonists in the clinic is routinely based around their relaxant properties and not geared to their antitussive activities (which, for example, may require higher doses), and we propose that this is why a dominant antitussive property has not been discovered in patients. In animal studies, there is no dose limitation in this regard and so clear antitussive activity, which is often missed in clinical studies, may be revealed. The message from the present study is not specifically to promote β_2-agonists as antitussive therapies but to uncover an alternative signalling pathway for exploitation in order to identify appropriate therapies for treating the unmet medical need of cough.

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STATEMENT OF INTEREST

None declared.

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REFERENCES

1 Dennis SM, Sharp SJ, Vickers MR, *et al.* Regular inhaled salbutamol and asthma control: the TRUST randomised trial. Therapy Working Group of the National Asthma Task Force and the MRC General Practice Research Framework. *Lancet* 2000; 355: 1675–1679.

2 Lowry R, Higenbottam T, Johnson T, *et al.* Inhibition of artificially induced cough in man by bronchodilators. *Br J Clin Pharmacol* 1987; 24: 503–510.

3 Ellul-Micallef R. Effect of terbutaline sulphate in chronic ''allergic'' cough. *BMJ (Clin Res Ed)* 1983; 287: 940–943.

4 Campbell M, Eliraz A, Johansson G, *et al.* Formoterol for maintenance and as-needed treatment of chronic obstructive pulmonary disease. *Respir Med* 2005; 99: 1511–1520.

5 Chong CF, Chen CC, Ma HP, *et al.* Comparison of lidocaine and bronchodilator inhalation treatments for cough suppression in patients with chronic obstructive pulmonary disease. *Emerg Med J* 2005; 22: 429–432.

6 Mulrennan S, Wright C, Thompson R, *et al.* Effect of salbutamol on smoking related cough. *Pulm Pharmacol Ther* 2004; 17: 127–131.

7 Pounsford JC, Birch MJ, Saunders KB. Effect of bronchodilators on the cough response to inhaled citric acid in normal and asthmatic subjects. *Thorax* 1985; 40: 662–667.

8 Chang AB, Phelan PD, Carlin JB, *et al.* A randomised, placebo controlled trial of inhaled salbutamol and beclomethasone for recurrent cough. *Arch Dis Child* 1998; 79: 6–11.

9 Smith CA, Adamson DL, Choudry NB, *et al.* The effect of altering airway tone on the sensitivity of the cough reflex in normal volunteers. *Eur Respir J* 1991; 4: 1078–1079.

10 Widdicombe JG. Neurophysiology of the cough reflex. *Eur Respir J* 1995; 8: 1193–1202.

11 Choudry NB, Fuller RW. Sensitivity of the cough reflex in patients with chronic cough. *Eur Respir J* 1992; 5: 296–300.

12 Karlsson JA, Sant'Ambrogio G, Widdicombe J. Afferent neural pathways in cough and reflex bronchoconstriction. *J Appl Physiol* 1988; 65: 1007–1023.

13 Morice AH, Fontana GA, Belvisi MG, *et al.* ERS guidelines on the assessment of cough. *Eur Respir J* 2007; 29: 1256–1276.

14 Belvisi MG, Patel HJ, Freund-Michel V, *et al.* Inhibitory activity of the novel CB2 receptor agonist, GW833972A, on guinea-pig and human sensory nerve function in the airways. *Br J Pharmacol* 2008; 155: 547–557.

15 Fox AJ, Barnes PJ, Venkatesan P, *et al.* Activation of large conductance potassium channels inhibits the afferent and efferent function of airway sensory nerves in the guinea pig. *J Clin Invest* 1997; 99: 513–519.

16 Lalloo UG, Fox AJ, Belvisi MG, *et al.* Capsazepine inhibits cough induced by capsaicin and citric acid but not by hypertonic saline in guinea pigs. *J Appl Physiol* 1995; 79: 1082–1087.

17 Patel HJ, Birrell MA, Crispino N, *et al.* Inhibition of guinea-pig and human sensory nerve activity and the cough reflex in guinea-pigs by cannabinoid (CB2) receptor activation. *Br J Pharmacol* 2003; 140: 261–268.

18 Laude EA, Higgins KS, Morice AH. A comparative study of the effects of citric acid, capsaicin and resiniferatoxin on the cough challenge in guinea-pig and man. *Pulm Pharmacol* 1993; 3: 171–175.

19 Morice AH, Kastelik JA, Thompson R. Cough challenge in the assessment of cough reflex. *Br J Clin Pharmacol* 2001; 52: 365–375.

20 Forsberg K, Karlsson JA, Zackrisson C, *et al.* Selective inhibition of cough and bronchoconstriction in conscious guinea pigs. *Respiration* 1992; 59: 72–76.

21 Lewis CA, Ambrose C, Banner K, *et al.* Animal models of cough: literature review and presentation of a novel cigarette smoke-enhanced cough model in the guinea-pig. *Pulm Pharmacol Ther* 2007; 20: 325–333.

22 Birrell MA, Crispino N, Hele DJ, *et al.* Effect of dopamine receptor agonists on sensory nerve activity: possible therapeutic targets for the treatment of asthma and COPD. *Br J Pharmacol* 2002; 136: 620–628.

23 Fox AJ, Lalloo UG, Belvisi MG, *et al.* Bradykinin-evoked sensitization of airway sensory nerves: a mechanism for ACE-inhibitor cough. *Nat Med* 1996; 2: 814–817.

24 Giembycz MA, Newton R. Beyond the dogma: novel β_2-adrenoceptor signalling in the airways. *Eur Respir J* 2006; 27: 1286–1306.

25 Shore SA, Moore PE. Regulation of β-adrenergic responses in airway smooth muscle. *Respir Physiol Neurobiol* 2003; 137: 179–195.

26 Jiang H, Colbran JL, Francis SH, *et al.* Direct evidence for cross-activation of cGMP-dependent protein kinase by cAMP in pig coronary arteries. *J Biol Chem* 1992; 267: 1015–1019.

27 Murthy KS, Makhlouf GM. Interaction of cA-kinase and cG-kinase in mediating relaxation of dispersed smooth muscle cells. *Am J Physiol* 1995; 268: C171–C180.

28 White RE, Kryman JP, El-Mowafy AM, *et al.* cAMP-dependent vasodilators cross-activate the cGMP-dependent protein kinase to stimulate BK_{Ca} channel activity in coronary artery smooth muscle cells. *Circ Res* 2000; 86: 897–905.

29 Koike K, Yamashita Y, Horinouchi T, *et al.* cAMP-independent mechanism is significantly involved in β_2-adrenoceptor-mediated tracheal relaxation. *Eur J Pharmacol* 2004; 492: 65–70.

30 Spicuzza L, Belvisi MG, Birrell MA, *et al.* Evidence that the anti-spasmogenic effect of the β-adrenoceptor agonist, isoprenaline, on guinea-pig trachealis is not mediated by cyclic AMP-dependent protein kinase. *Br J Pharmacol* 2001; 133: 1201–1212.

31 Staples KJ, Bergmann M, Tomita K, *et al.* Adenosine 3',5'-cyclic monophosphate (cAMP)-dependent inhibition of IL-5 from human T lymphocytes is not mediated by the cAMP-dependent protein kinase A. *J Immunol* 2001; 167: 2074–2080.

32 Tan KS, Nackley AG, Satterfield K, *et al.* β_2 Adrenergic receptor activation stimulates pro-inflammatory cytokine production in macrophages via PKA- and NF-κB-independent mechanisms. *Cell Signal* 2007; 19: 251–260.

33 Tanaka Y, Yamashita Y, Yamaki F, *et al.* MaxiK channel mediates β_2-adrenoceptor-activated relaxation to isoprenaline through cAMP-dependent and -independent mechanisms in guinea-pig tracheal smooth muscle. *J Smooth Muscle Res* 2003; 39: 205–219.

34 Jones TR, Charette L, Garcia ML, *et al.* Selective inhibition of relaxation of guinea-pig trachea by charybdotoxin, a potent Ca^{++}-activated K^+ channel inhibitor. *J Pharmacol Exp Ther* 1990; 255: 697–706.

35 Jones TR, Charette L, Garcia ML, *et al.* Interaction of iberiotoxin with β-adrenoceptor agonists and sodium nitroprusside on guinea pig trachea. *J Appl Physiol* 1993; 74: 1879–1884.

36 Kume H, Hall IP, Washabau RJ, *et al.* β-adrenergic agonists regulate KCa channels in airway smooth muscle by cAMP-dependent and -independent mechanisms. *J Clin Invest* 1994; 93: 371–379.

37 Miura M, Belvisi MG, Stretton CD, *et al.* Role of potassium channels in bronchodilator responses in human airways. *Am Rev Respir Dis* 1992; 146: 132–136.