British Journal of Pharmacology

British Journal of Pharmacology (2018) **175** 3162–3174 3162

RESEARCH PAPER

Central adenosine A_1 receptors inhibit cough *via* suppression of excitatory glutamatergic and tachykininergic neurotransmission

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Received 1 November 2017; **Revised** 2 March 2018; **Accepted** 10 April 2018

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BACKGROUND AND PURPOSE

The adenosine A_1 receptor is reported to mediate several excitatory effects in the airways and has inhibitory effects in the CNS. In this study, we investigated the role of peripheral and central A_1 receptors in regulating cough and airway obstruction.

EXPERIMENTAL APPROACH

Drugs were administered to guinea pigs *via* inhalation or i.c.v. infusion. Following the administration of different drugs, cough was induced by exposing guinea pigs to aerosolized 0.4 M citric acid. An automated analyser recorded both cough and airway obstruction simultaneously using whole-body plethysmography.

KEY RESULTS

The A_1 receptor agonist, cyclopentyladenosine (CPA, administered by inhalation), dose-dependently inhibited cough and also inhibited airway obstruction. Similarly, CPA, administered i.c.v., inhibited both the citric acid-induced cough and airway obstruction; this was prevented by pretreatment with the A_1 receptor antagonist DPCPX (i.c.v.). Treatment with DPCPX alone dose-dependently enhanced the citric acid-induced cough and airway obstruction. This effect was reversed following treatment with either the glutamate GluN1 receptor antagonist D-AP5 or the neurokinin NK_1 receptor antagonist FK-888.

CONCLUSIONS AND IMPLICATIONS

These findings suggest that activation of either peripheral or central adenosine A_1 receptors inhibits citric acid-induced cough and airway obstruction. The data also suggest that tonic activation of central adenosine A_1 receptors serves as a negative regulator of cough and airway obstruction, secondary to inhibition of excitatory glutamatergic and tachykininergic neurotransmission.

Abbreviations

ACSF, artificial CSF; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AP, anteroposterior; AVPN, airway-related vagal preganglionic neuron; CHS, cough hypersensitivity syndrome; CPA, cyclopentyladenosine; D-AP5, DL-2-amino-5-phosphonovaleric acid; DPCPX, cyclopentyl-1,3-dipropylxanthine; DV, dorsoventral; GluN1, glutamate ionotropic receptor NMDA type subunit 1; ML, mediolateral; NK_1, neurokinin 1; nTS, nucleus tractus solictarus; PE-20, polyethylene tubing; Penh, enhanced pause

DOI:10.1111/bph.14360

Introduction

Cough has been classically viewed as a simple brainstem defensive reflex in response to increased airway sensory input resulting from inhalation of foreign particles, mucus, aspiration of gastric content or due to sensitization of sensory airway nerves (Ohi *et al.*, 2005; Canning and Mori, 2010; Mazzone *et al.*, 2015). However, whilst the brainstem plays a critical role in regulating the reflex cough (Bonham *et al.*, 2006; Mutolo *et al.*, 2008; Canning and Mori, 2011; El-Hashim *et al.*, 2013), recent evidence shows that higher brain regions may also play an important role (Ando *et al.*, 2016; Eccles, 2006). For example, the urge to cough can be consciously suppressed in humans (Hegland *et al.*, 2011; Mazzone *et al.*, 2011). Also, a placebo effect has been demonstrated to be responsible for a significant component of the action of anti-tussive drugs (Eccles, 2002; Leech *et al.*, 2012).

Chronic cough accounts for a large proportion of respiratory outpatients seen, but treatment options are not only limited but are also ineffective (Irwin *et al.*, 1981; Schroeder and Fahey, 2002). One reason for the lack of efficacy of these drugs is that they are not thought to affect neuronal sensitization – an important determinant of chronic cough. Indeed, the term 'cough hypersensitivity syndrome' (CHS) has been recently coined and reflects both the mechanisms underlying chronic cough and the nature of its triggers (Morice *et al.*, 2014b; Keller *et al.*, 2017). These mechanisms include neuronal activation, sensitization and/or dysfunction, and the triggers are usually low threshold thermal, mechanical or chemical exposures (El-Hashim and Jaffal, 2017; Morice *et al.*, 2014a). Whilst much attention has been given to the sensitization of airway nerves, CHS is also thought to be driven by enhanced sensitization of certain regions in the CNS involved in the regulation of cough (Lee *et al.*, 2003; El-Hashim and Jaffal, 2017; Morice *et al.*, 2014b).

Glutamatergic and substance P-dependent excitatory signalling pathways have been identified as being important in the CNS processing of cough-inducing stimuli (Mutolo *et al.*, 2007; Smith *et al.*, 2012). Despite these pathways being demonstrated to have this role in preclinical animal models (Canning, 2009; El-Hashim *et al.*, 2004b), antagonism of these pathways has failed to show any significant benefit in clinical studies (Fahy *et al.*, 1995; Dicpinigaitis *et al.*, 2015). In addition to possible pharmacokinetic issues, it may be that both excitatory pathways need to be simultaneously blocked in order to achieve significant inhibition of cough. Alternatively, the mechanisms underlying CHS may be associated with a dysfunction in the inhibitory inputs, rather than increased excitatory inputs, in the neural circuits of the cough centre. Recent evidence shows that a dysfunction of the opioidergic and GABAergic systems, both of which are known to be important inhibitory pathways for cough (Bolser *et al.*, 1993; Barnabe *et al.*, 1995; Dicpinigaitis *et al.*, 1998; Poliacek *et al.*, 2010), may contribute to CHS (Ando *et al.*, 2016). This may explain why blockade of the excitatory systems has not been very successful in effectively treating cough.

Increased levels of **adenosine** have long been known to mediate pulmonary effects associated with lung diseases such as inflammation, airway hyperresponsiveness and airway obstruction (Polosa and Holgate, 2006; Burnstock *et al.*, 2012), possibly through activation of airway sensory C-fibres and cholinergic nerves (Hong *et al.*, 1998; Chuaychoo *et al.*, 2006; Reynolds *et al.*, 2008). Adenosine activates the GPCRs, **A_1**, A_{2A}, A_{2B} and A_3 receptors (also known as P1 receptors; Burnstock *et al.*, 2012). In the CNS, adenosine has been reported to exert both inhibitory and stimulatory effects *via* adenosine A_1 and A_{2A} receptors respectively (Ralevic and Burnstock, 1998; Burnstock and Ralevic, 2014). Activation of the A_1 receptors in the nucleus tractus solictarus (nTS), the putative cough centre, inhibits regional sympathetic responses evoked by activation of cardiopulmonary chemoreflex (Ichinose *et al.*, 2012). In mice, an enhanced expiration response with some similarities to cough has been shown to be inhibited following activation of central adenosine A_1 receptors (Kamei *et al.*, 1994).

In this study, we investigated (i) whether airway and/or central adenosine A_1 receptors play a role in regulating cough and airway obstruction in response to inhaled citric acid and (ii) the mechanisms by which CNS adenosine A_1 receptors may modulate cough and airway obstruction in response to **citric acid**.

Methods

Animals

In-house-bred Dunkin–Hartley guinea pigs (400–600 g) of either sex were maintained under temperature-controlled conditions with a 12 h light/dark cycle with free access to standard chow and water *ad libitum*. Animals were arbitrarily assigned to control and experimental groups. All experimental protocols were approved by the Animal Welfare and Use of Laboratory Animals Committee in the Health Sciences Centre, Kuwait University, and complied with the BJP Guidelines and were carried out in accordance with the EU Directive 2010/63/EU for animal experiments and the National Institutes of Health guide for the care and use of laboratory animals (National Institutes of Health Publications No. 8023, revised 1978). Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015).

Measurement of cough response and enhanced pause

Cough and enhanced pause (Penh; a correlate of airway obstruction) were measured simultaneously in conscious unrestrained guinea pigs using whole-body plethysmography (Buxco, Troy, NY, USA). In brief, coughs and Penh were recorded using the Buxco system analyser that differentiates coughs from other events like sneezes and has a >99.0% correlation with manual counting. Penh is a dimensionless value that reflects changes in the waveform of the box pressure signal and combines a timing comparison factor of early versus late expiration (Pause). Penh has been reported to be an index of airway function, and increases in Penh correlate with lower airway obstruction in a number of animal models including mice, guinea pigs and dogs (Hamelmann *et al.*, 1997; Chong *et al.*, 1998; Lewis *et al.*, 2007; Agrawal *et al.*, 2008; Hirt *et al.*, 2008; El-Hashim *et al.*, 2011).

Implantation of chronic i.c.v. cannula

A cannula was implanted in the cerebroventricles as described previously (El-Hashim *et al.*, 2013). Guinea pigs were anaesthetized with a combination of ketamine (50 mg·kg^{-1}; i.m.) and xylazine (5 mg·kg^{-1}; i.m.). The depth of anesthesia was assessed by checking the toe-pinch reflex. Supplemental anesthesia was administered in case of any reflex response. After 20 min, each animal was injected with the antibiotic enrofloxacin (0.25 mg·kg^{-1}; s.c.). The fur above the skull was shaved, and the animal was placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). A 2 cm midline incision was made in the skin above the skull with a sharp surgical blade – no. 10 (World Precision Instruments, Sarasota, FL, USA) – and the skull was cleaned with 3% hydrogen peroxide. A 20-gauge stainless steel guide cannula and its suitable dummy cannula, HTX-20T and HTX-25R stainless steel hypo tubes (Small Parts, Inc., Logansport, IN, USA), were placed in the lowering arm of the stereotaxic apparatus. The tip of the guide cannula was placed directly above the bregma, and this was taken as the zero coordinate of the animal. The cannula was then moved in three dimensions: anteroposterior (AP), mediolateral (ML) and dorsoventral (DV). The coordinates used in our protocol (2.0 mm AP, 1.8 mm ML and 4.8 mm DV relative to bregma), which corresponded to the lateral ventricles, were based on previously published reports (Mazzone *et al.*, 2005; El-Hashim *et al.*, 2011). The calculated coordinates were determined, and a small burr hole was drilled in the skull. Two additional holes were drilled for two anchor screws. The guide cannula (with the dummy cannula inside) was lowered gradually into the brain using the stereotaxic lowering arm according to the predetermined coordinates, and the cannula was cemented to the skull with screws. Surgical suturing was performed using stainless steel surgical needles with 36 mm cutting edge (1/2 circle) and silk non-absorbable surgical sutures. Animals were treated with tramadol (1 mg·kg^{-1}; i.m.) and enrofloxacin (0.25 mg·kg^{-1}; s.c.) once a day for three consecutive days.

Intracerebroventricular drug administration

As described previously (El-Hashim *et al.*, 2011), prior to cough assessment, the dummy cannula was removed from the guide cannula. The infusion cannula, connected *via* polyethylene tubing (PE-20) (Small Parts, Inc.) to a Hamilton syringe pump model (Harvard Apparatus, Holliston, MA, USA), was inserted into the guide cannula. The animals were infused with 15 μL of any drug over 50 min. The cannula was kept in place for an additional 15 min to prevent backflow leakage and for the drug to equilibrate. The accuracy of the placement was checked by injecting methylene blue through the guide cannula of randomly chosen guinea pigs at the end of the experiment.

At the end of the experiment, the guinea pigs were killed by CO_2 asphyxiation. CO_2 flow rate was adjusted to 5 L·min^{-1} and continued until breathing had completely stopped. After that, cervical dislocation was performed to ensure death. The total number of guinea pigs used in the study was 263.

Exposure to drug via inhalation

Aerosols were generated using a DeVilbiss aerogen ultrasonic nebulizer (DeVilbiss, Somerset, PA, USA), which had an aerodynamic mass median diameter range of 1–5 μm (manufacturer's indication). Animals were allowed to acclimatize for a period of 5 min in the whole-body plethysmograph, and baseline airway function (Penh) was recorded for 2 min prior to aerosol administration. All animal groups were exposed to citric acid (0.4 M) aerosol for 10 min during which both cough and Penh were recorded and for a 5 min period thereafter (total 15 min). In one study, animals in four groups were exposed to **cyclopentyladenosine (CPA**; 0.3, 0.6 and 1 mg·mL^{-1}) or aerosolized distilled water (vehicle).

Preparation of buffers and drugs

A stock solution of CPA for aerosol delivery was prepared by dissolving it in distilled water with subsequent dilutions of 0.3, 0.6 and 1 mg·mL^{-1} made in distilled water. CPA for i.c.v. administration (1.8 and 3 nmol·mL^{-1}) was made by initially dissolving CPA in distilled water and then diluting with artificial CSF (ACSF) to yield final dilutions of drug in 99% ACSF. **DPCPX** was made by initially dissolving in ethanol with subsequent dilutions (0.6, 1, 3.3 and 33 nmol·mL^{-1}) made in ACSF to yield final dilutions of drug in 5% ethanol. **FK-888** (0.1 and 1 nmol·mL^{-1}) was made by initially dissolving in ethanol and diluting with distilled water to yield a final dilution of drug in 5 and 13% ethanol respectively. **D-AP5** (15 nmol·mL^{-1}) was made by initially dissolving in distilled water and diluting with ACSF to yield a final dilution of drug in 99% ACSF. Lastly, **CGP 55845** (6 nmol·mL^{-1}) and **naloxone hydrochloride** (2 pmol·mL^{-1}) were made by initially dissolving in distilled water and diluting with ACSF to yield a final dilution in 99% ACSF. Citric acid (0.4 M) was dissolved in PBS. All drugs were freshly prepared for each experiment.

Experimental protocol and design (in accordance with BJP guidelines)

Effect of inhaled A_1 receptor agonist CPA on citric acid-induced cough and airway obstruction. Animals were arbitrarily divided into four groups. Group 1 (n = 10) was treated with the vehicle of CPA (99% ACSF). Groups 2 (n = 6), 3 (n = 6) and 4 (n = 10) were treated with 0.3, 0.6 and 1 mg·mL^{-1} CPA respectively. The drug was nebulized over a 10 min period followed by exposure to aerosolized citric acid (0.4 M) for 10 min. Cough and airway obstruction were assessed during the citric acid challenge and for 5 min thereafter.

Effect of the A_1 receptor agonist CPA (i.c.v.) on citric acid-induced cough and airway obstruction. Animals were arbitrarily divided into four groups. Group 1 (n = 14) was treated with the vehicle of CPA (99% ACSF). Groups 2 (n = 12) and 3 (n = 8) were treated with 1.8 and 3 nmol·mL^{-1} CPA respectively. The fourth group was pretreated with the adenosine A_1 receptor antagonist, DPCPX (33 nmol·mL^{-1}, n = 12), and 15 min after the infusion of DPCPX, animals were treated with CPA (3 nmol·mL^{-1}). Fifteen minutes after infusion of CPA, all animals were exposed to aerosolized citric acid (0.4 M) for 10 min. Cough and airway obstruction were assessed during the citric acid challenge and 5 min thereafter.

Effect of the adenosine A_1 receptor antagonist DPCPX (i.c.v.) on citric acid-induced cough and airway obstruction. Animals

were arbitrarily divided into five groups. Group 1 (n = 20) was pretreated with the vehicle of DPCPX (5% ethanol in ACSF). Group 2 (n = 9), group 3 (n = 12), group 4 (n = 15) and group 5 (n = 17) were treated with 0.6, 1, 3.3 and 33 nmol·mL^{-1} of DPCPX respectively. After 15 min, all treatment groups were exposed to aerosolized citric acid (0.4 M) for 10 min. Cough and airway obstruction were assessed during the citric acid challenge and 5 min thereafter.

Effect of blockade of the GluN1 receptor on DPCPX (i.c.v.)-enhanced citric acid-induced cough and airway obstruction. Animals were arbitrarily divided into four groups. Group 1 (n = 8) was pretreated with the vehicle of DPCPX. Fifteen minutes after the infusion of vehicle of DPCPX, animals were treated with the vehicle of D-AP5 (5% water in ACSF). Group 2 (n = 11) was pretreated with the vehicle of DPCPX. Fifteen minutes after the infusion of the vehicle, animals were treated with D-AP5 (15 nmol·mL^{-1}). Group 3 (n = 9) was pretreated with 3.3 nmol·mL^{-1} of DPCPX. Fifteen minutes after the infusion of DPCPX, animals were treated with vehicle of D-AP5. Group 4 (n = 12) was pretreated with 3.3 nmol·mL^{-1} of DPCPX, and 15 min later, animals were treated with the glutamate **GluN1 receptor** antagonist D-AP5 (15 nmol·mL^{-1}). Fifteen minutes after i.c.v. infusion of D-AP5 or its vehicle, all treatment groups were exposed to aerosolized citric acid (0.4 M) for 10 min. Cough and airway obstruction were assessed during the citric acid challenge and 5 min thereafter.

Effect of blockade of the NK_1 receptor on DPCPX (i.c.v.)-enhanced citric acid-induced cough and airway obstruction. Animals were arbitrarily divided into five groups. Group 1 (n = 13) was treated with the vehicle of DPCPX (5% ethanol in ACSF). Fifteen minutes after infusion with the vehicle of DPCPX, animals were treated with the vehicle of FK-888 (5% ethanol in ACSF). Group 2 (n = 4) was pretreated with the vehicle of DPCPX. Fifteen minutes after infusion with the vehicle of DPCPX, animals were treated with the **NK_1 receptor** antagonist FK-888 (1 nmol·mL^{-1}). Group 3 (n = 14) was pretreated with DPCPX (3.3 nmol·mL^{-1}), and 15 min later, they were treated with the vehicle of FK-888 (13% ethanol in ACSF). Group 4 (n = 9) was pretreated with 3.3 nmol·mL^{-1} of DPCPX and then treated 15 min later with FK-888 (0.1 nmol·mL^{-1}). Group 5 (n = 5) was pretreated with 3.3 nmol·mL^{-1} of DPCPX and then treated 15 min later with FK-888 (1 nmol·mL^{-1}). Fifteen minutes after i.c.v. infusion of FK-888 or its vehicle, all treatment groups were exposed to aerosolized citric acid (0.4 M) for 10 min. Cough and airway obstruction were assessed during the citric acid challenge and 5 min thereafter.

Effect of blockade of $GABA_B$ and opioid μ receptors on citric acid-induced cough and airway obstruction. Animals were arbitrarily divided into three groups. Group 1 (n = 10) was pretreated with the vehicle of CGP 55845 and the opioid **μ receptor** antagonist naloxone hydrochloride (99% ACSF). Group 2 (n = 8) and group 3 (n = 9) were treated with CGP 55845 (6 nmol^{-1}; **$GABA_B$ receptor** antagonist) and naloxone (2 pmol^{-1}; μ-opioid receptor antagonist) respectively. Fifteen minutes after i.c.v. infusion of drugs or their vehicle, all treatment groups were exposed to aerosolized citric acid (0.4 M) for 10 min. Cough and airway obstruction were assessed during the citric acid challenge and 5 min thereafter.

Statistical analyses

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2018). For cough experiments, data are expressed as mean number of coughs during a 15 min period ± SEM. Airway obstruction is presented as an absolute change in Penh and expressed as mean ± SEM. Differences in the degree of airway obstruction between different groups were determined as the mean AUC for the Penh values for 15 min for each animal. Data analysis was performed blind. All treatment groups were initially tested for normality and the appropriate statistical test applied depending on the distribution of the data. Normally distributed data were analysed using an ANOVA test followed by Bonferroni *post hoc* test, and non-normally distributed data were analysed using Kruskal–Wallis test. All statistical analyses were performed using the Sigmaplot, version 12.3, Systat Software, Inc. SigmaPlot for Windows. In all cases, differences were considered significant at $P < 0.05$. The difference in group sizes was due to two factors: (i) inconsistency in supply of our in-house bred guinea pigs and (ii) death of animals at various times after anaesthesia or even removal of the cannula. However, all groups were always time matched.

Materials

Chemicals used were PBS tablets, citric acid (Sigma-Aldrich, St. Louis, MO, USA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), N^6-cyclopentyladenosine (CPA) and DL-2 aminuteso-5-phosphonopentanoic acid (D-AP5) (Sigma-Aldrich), FK-888, CGP 55845, naloxone hydrochloride (Tocris; Cookson Ltd., Langford, UK), tramadol (Grünenthal, Aachen, Germany), enrofloxacin (Baytril®; Bayer AG, Leverkusen, Germany), KCl (BDH Laboratory, Poole, UK), NaH_2PO_4 (Sigma-Aldrich), NaCl (Merck, Darmstadt, Germany), D(+)-glucose anhydrous, $NaHCO_3$ (Merck), $MgCl_2$ (Fluka; BioChemika, Messerschmitt, Switzerland), $CaCl_2$ (Surechem, Suffolk, UK), ketaminutese (Hikma Pharmaceuticals, Amman, Jordan), xylazine (Sigma-Aldrich), stainless steel surgical needles with 36 mm cutting edge (1/2 circle) (Mani, Inc., Tochigi, Japan), silk non-absorbable surgical sutures (Look Surgical Specialties Corporation, Wyomissing, PA, USA), surgical blades (Feather Safety Razar Co., Osaka, Japan), dual syringe pump-model 11 Plus (Harvard Apparatus), polyethylene tubes (PE-20) and HTX-20T and HTX-25R stainless steel hypo tubes (Small Parts, Inc.). HTX-20T and HTX-25R hypo tubes were cut to form 20-gauge guides and infusion cannulae respectively.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to the corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017,b).

Results

Effect of inhaled A_1 receptor agonist CPA on citric acid-induced cough and airway obstruction

Inhalation of CPA dose-dependently inhibited the citric acid-induced cough response (Figure 1A). Although inhalation of CPA did not cause any immediately noticeable changes in Penh (data not shown), it dose-dependently inhibited the citric acid-induced airway obstruction (Figure 1B, C).

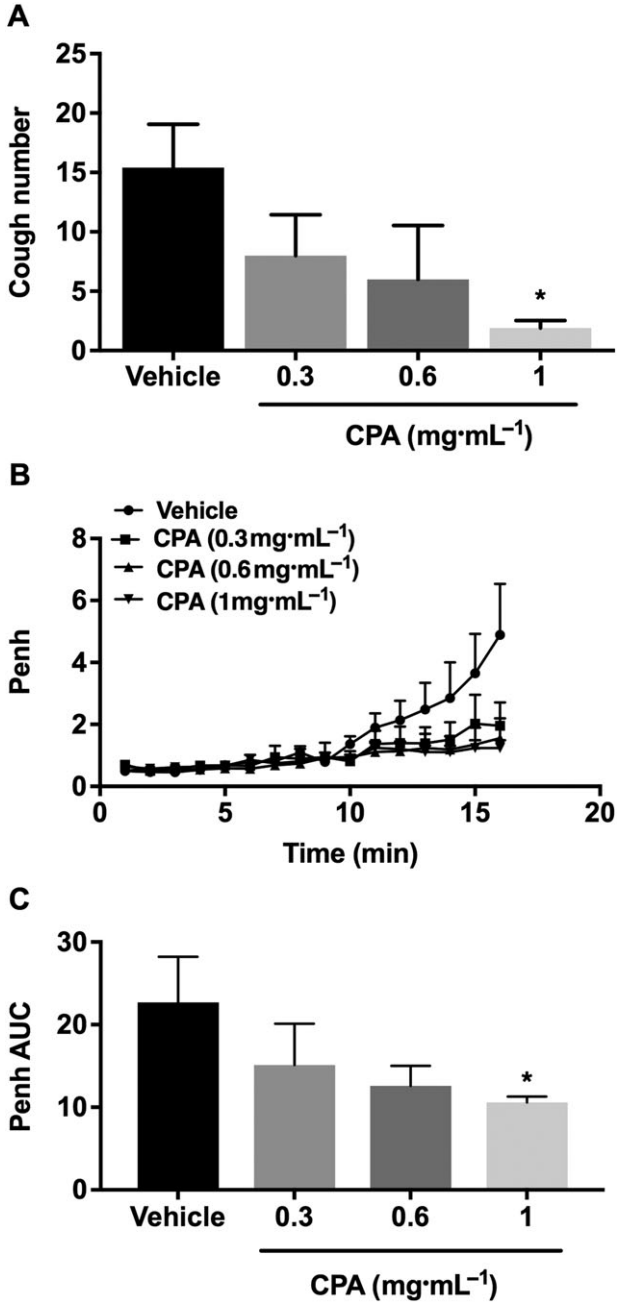

Figure 1

Effect of inhaled A_1 receptor agonist, CPA, 0.3, 0.6 and 1 mg·mL^{-1} (n = 6–10) on citric acid-induced cough (A), changes in Penh (B) and Penh AUC (C). Values represent means + SEM; *P<0.05, compared to vehicle-treated animals.

Effect of CPA (i.c.v.) on citric acid-induced cough and airway obstruction

CPA, at 3 but not at 1.8 nmol·mL^{-1}, significantly reduced the citric acid-induced cough by more than 60% compared with the vehicle group (Figure 2A). This effect was reversed in animals pretreated with DPCPX and was comparable with the vehicle-treated group (Figure 2A). CPA, at both 1.8 and 3 nmol·mL^{-1}, significantly reduced the citric acid-induced airway obstruction compared with the vehicle-treated group (Figure 2B, C). Again, treatment with DPCPX completely reversed the CPA (at 3 nmol·mL^{-1})-induced reduction of the

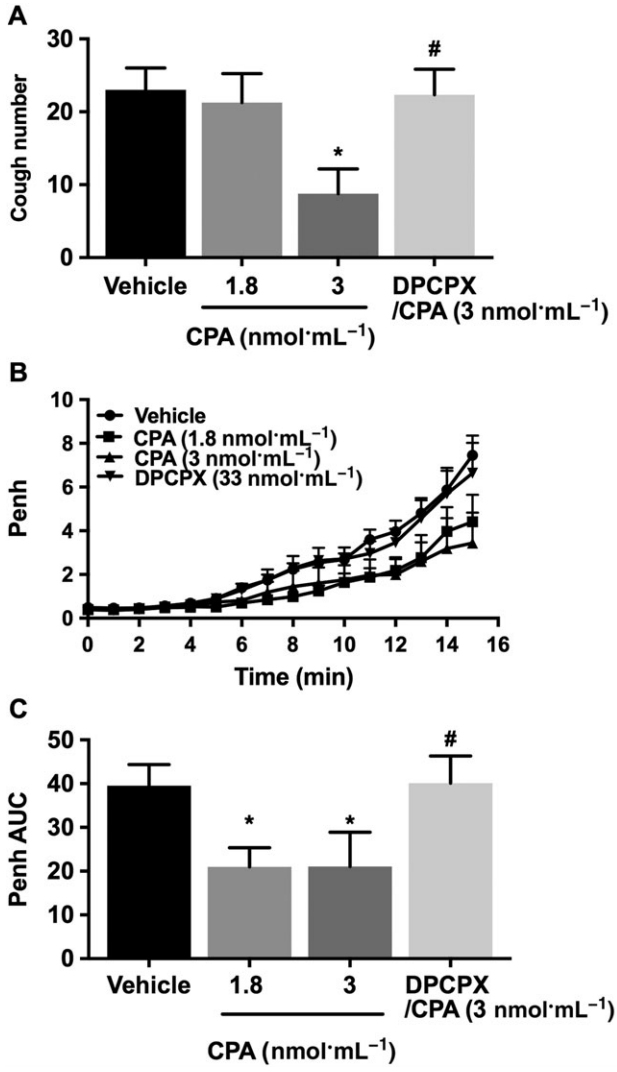

Figure 2

Effect of treatment with CPA (i.c.v.) 1.8 nmol·mL^{-1} (n = 12) and 3-nmol·mL^{-1} (n = 8) and pretreatment with DPCPX (33 nmol·mL^{-1}; n = 12) on citric acid-induced cough (A), changes in Penh (B) and Penh AUC (C). Values represent means + SEM. *$P < 0.05$, significant difference compared with vehicle-treated animals. #$P < 0.05$, significant difference compared with CPA (3 nmol·mL^{-1})-treated animals.

citric acid-induced airway obstruction compared with vehicle (Figure 2B, C).

Effect of the adenosine A_1 receptor antagonist DPCPX (i.c.v.) on citric acid-induced cough and airway obstruction

During the DPCPX infusion, no cough was induced, and the average Penh values, immediately after the infusion, were not significantly different between the groups (data not shown). However, treatment of animal with DPCPX (0.6, 1, 3.3 and 33 nmol·mL^{-1}) dose-dependently enhanced the citric acid-induced cough response compared with control animals (Figure 3A). This was significant at doses of 3.3 and 33 nmol·mL^{-1}. Similarly, treatment with DPCPX (0.6, 1, 3.3 and 33 nmol·mL^{-1}) dose-dependently enhanced the citric acid-induced airway obstruction compared with vehicle treatment (Figure 3B, C). In subsequent experiments, we used DPCPX at 3.3 mol·mL^{-1} as this dose resulted in a maximum enhancement of the parameters measured (mean cough number increased by 116.0%, and mean Penh AUC increased by 95.2%).

Effect of blockade of the GluN1 receptor on DPCPX (i.c.v.)-enhanced citric acid-induced cough and airway obstruction

Treatment with **the GluN1 receptor** antagonist D-AP5 (15 nmol·mL^{-1}) significantly inhibited the DPCPX (3.3 nmol·mL^{-1})-enhanced citric acid-induced cough compared with vehicle pretreated animals (Figure 4A) and also significantly reduced the DPCPX-enhanced citric acid-induced airway obstruction compared with vehicle treatment (Figure 4B, C). Treatment with D-AP5, alone, did not have any significant effects on either baseline cough or Penh compared with vehicle-treated guinea pigs ($P > 0.05$; Figure 4A–C).

Effect of blockade of the NK_1 receptor on DPCPX (i.c.v.)-enhanced citric acid-induced cough and airway obstruction

Treatment with the NK_1 receptor antagonist FK-888 (at 0.1 and 1 nmol·mL^{-1}) dose-dependently reduced the DPCPX (3.3 nmol·mL^{-1}) enhancement of citric acid-induced cough compared with vehicle; this was a significant 72% reduction at the 1 nmol·mL^{-1} dose (Figure 5A). Similarly, treatment with FK-888 dose-dependently reduced the DPCPX enhancement of citric acid-induced airway obstruction; this was a significant 74.2% (Figure 5B) reduction at the 1 nmol·mL^{-1} dose. Treatment with FK-888 (1 nmol·mL^{-1}) did not have any significant effect on either baseline cough or Penh compared with vehicle-treated guinea pigs ($P > 0.05$; Figure 5A–C).

Effect of blockade of the $GABA_B$ and opioid μ receptors on citric acid-induced cough and airway obstruction

Treatment of guinea pigs with the $GABA_B$ receptor antagonist, CGP 55845 (6 nmol·mL^{-1}, i.c.v.), or the μ receptor antagonist, naloxone (2 pmol·mL^{-1}, i.c.v.), did not affect the citric acid-induced cough (Figure 6A) nor the citric acid-induced airway obstruction (Figure 6B, C).

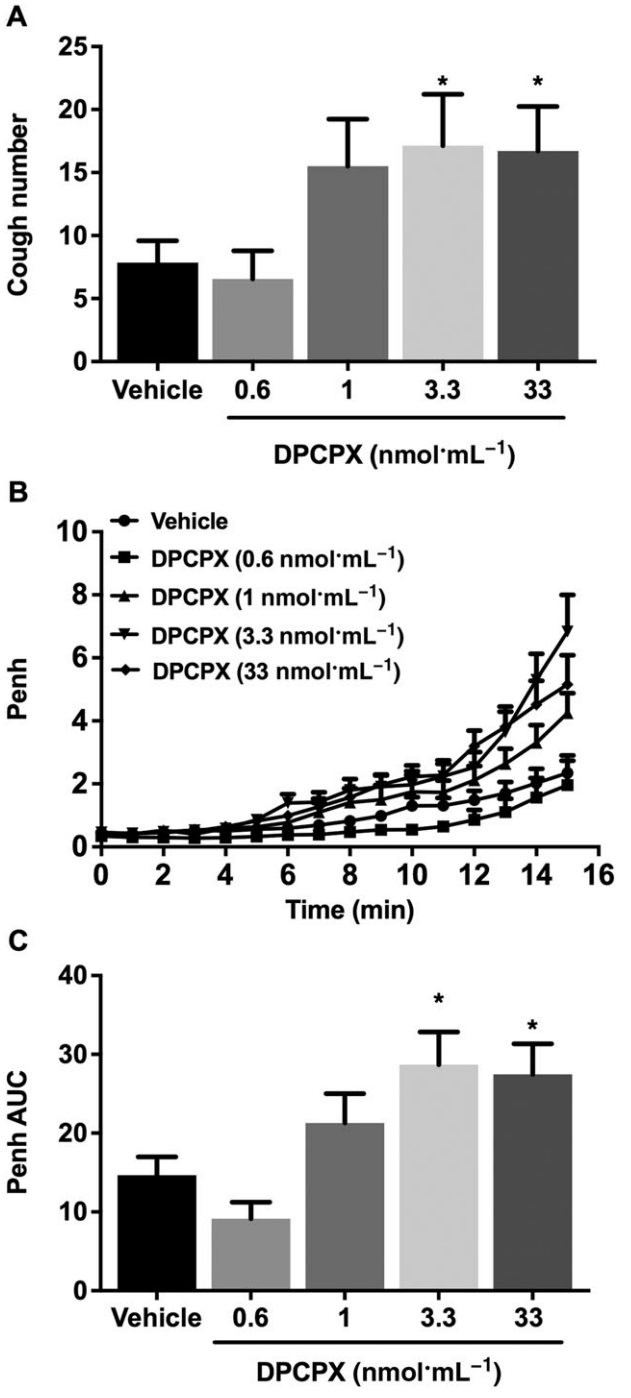

Figure 3

Effect of DPCPX (0.6, 1, 3.3 and 33 nmol·mL^{-1}; i.c.v., n = 9–20) treatment on citric acid-induced cough (A), changes in Penh (B) and Penh AUC (C). Values represent means + SEM. $^*P < 0.05$, significant difference compared with vehicle-treated animals.

Discussion

The classical inhibitory pathways that are thought to regulate the cough reflex are the opioidergic and GABAergic, in addition to the more recently described cannabinergic neurotransmission (Dicpinigaitis and Dobkin, 1997; Kotzer

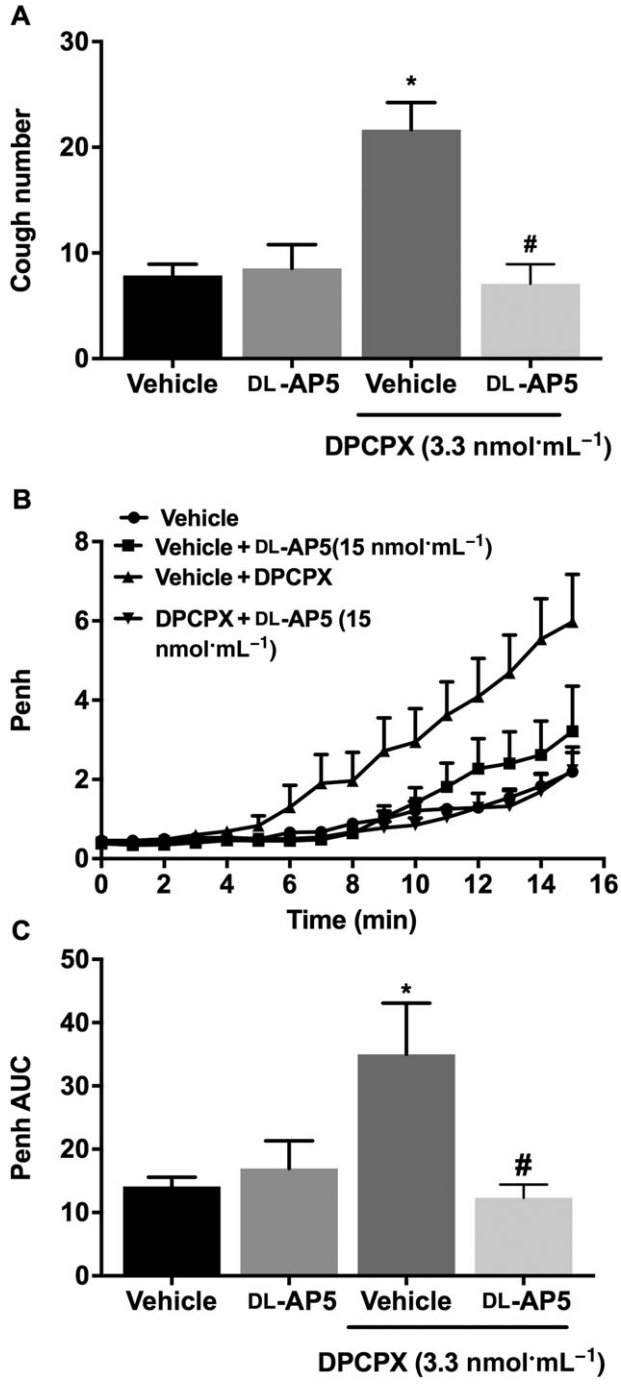

Figure 4

Effect of pretreatment with D-AP5 (15 nmol·mL^{-1}; i.c.v., $n = 12$) on DPCPX-enhanced citric acid-induced cough (A), changes in Penh (B) and Penh AUC (C). Values represent means + SEM. $^{*}P < 0.05$, significant difference compared with vehicle alone ($n = 8$) and D-AP5 alone ($n = 11$)-treated animals. $^{\#}P < 0.05$, significant difference compared with DPCPX (3.3 nmol·mL^{-1})-treated animals.

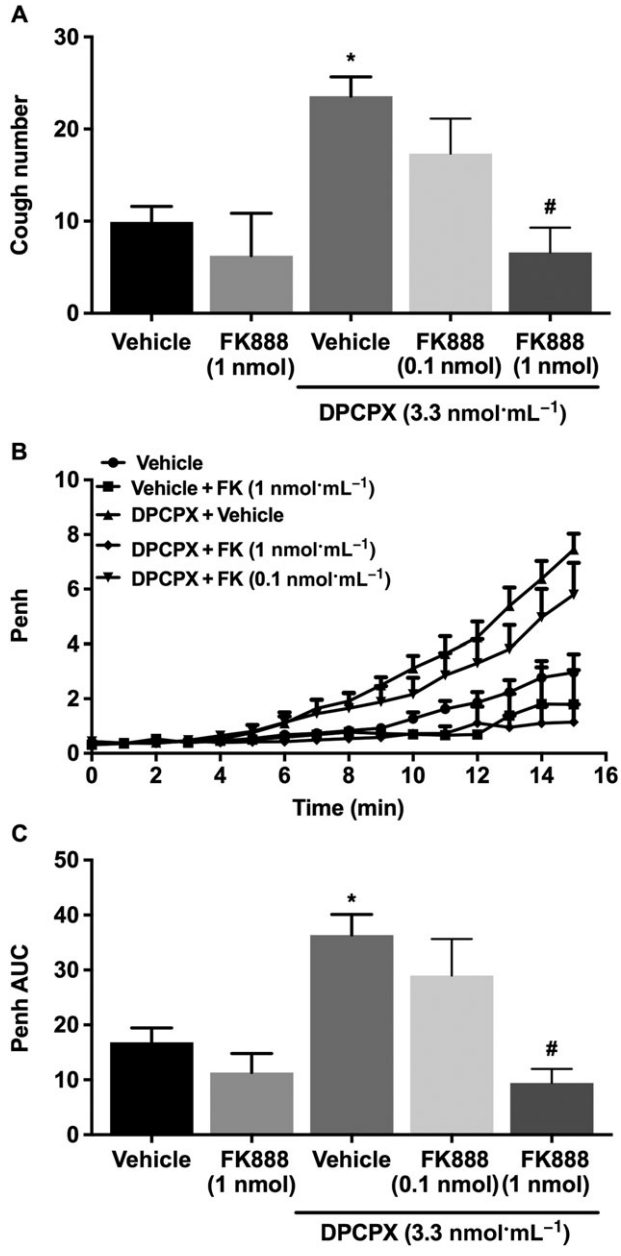

Figure 5

Effect of pretreatment with FK-888 (0.1 nmol·mL^{-1}; i.c.v., $n = 9$ and 1.0 nmol·mL^{-1}, $n = 5$) on DPCPX-enhanced citric acid-induced cough (A), changes in Penh (B) and Penh AUC (C). Values represent means + SEM. $^{*}P < 0.05$, significant difference compared with vehicle alone ($n = 13$) and FK-888 alone ($n = 4$)-treated animals. $^{\#}P < 0.05$, significant difference compared with DPCPX (3.3 nmol·mL^{-1})-treated animals.

et al., 2000; McLeod *et al.*, 2002; Patel *et al.*, 2003). In this study we showed, for the first time, that activation of the adenosine A_1 receptor, both in the airways and centrally, inhibits cough and airway obstruction. We also showed that tonic activation of the central adenosine A_1 receptors acts as 'a braking mechanism' to limit cough and airway obstruction *via* inhibition of excitatory glutamatergic and tachykininergic transmission.

Ex vivo and in vivo studies have shown that adenosine, *via* the A_1 receptor, can activate airway C-fibre terminals, specifically those arising from the nodose ganglia but not those of jugular origin (Hong *et al.*, 1998; Chuaychoo *et al.*, 2006).

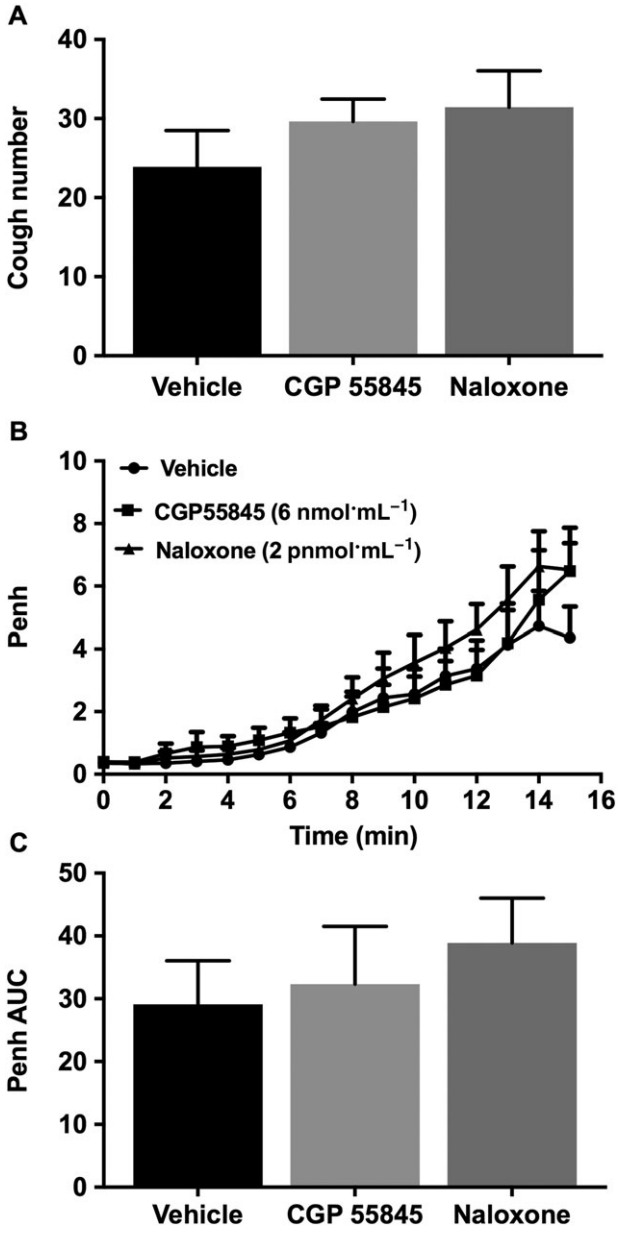

Figure 6

Effect of CGP 55845 (6 nmol·mL^{-1}; i.c.v., n = 8) and naloxone (2 pmol·mL^{-1}; i.c.v., n = 9) on citric acid-induced cough (A), changes in Penh (B) and Penh AUC (C). Values represent means + SEM.

Stimulation of airway A_1 receptors in allergic animals and patients with respiratory diseases such as asthma also results in bronchoconstriction, whereas adenosine has no effects in naïve animals or normal individuals (el-Hashim *et al.*, 1996; Polosa and Holgate, 2006; Chou *et al.*, 2008). On the other hand, adenosine has also been reported to induce relaxation of precontracted tracheal spirals (Darmani and Broadley, 1986). Therefore, whether adenosine constricts or relaxes airways is not entirely clear. Our data show that CPA, an A_1 receptor agonist, administered by aerosol, dose-dependently inhibited the citric acid-induced cough. This is in line with findings from a very recent study showing that adenosine inhibited coughing evoked by different stimuli in both anaesthetized and conscious models of cough (Chou *et al.*, 2017). Our results are also consistent with data from a clinical study showing that exposure to AMP (adenosine precursor) decreased the cough sensitivity to capsaicin (Basoglu *et al.*, 2017).

Inhaled CPA did not result in any changes in Penh. This is consistent with the data from both clinical and non-clinical studies showing that neither naïve animals nor healthy non-asthmatic individuals do not respond to adenosine (el-Hashim *et al.*, 1996; Basoglu *et al.*, 2017). In contrast to the lack of direct effect on Penh, exposure to CPA dose-dependently reduced the citric acid-induced airway obstruction. This is consistent with the inhibitory effects of CPA on citric acid-induced cough and is in agreement with findings from earlier studies showing that adenosine induces relaxation of guinea pig, isolated tracheal spirals and airway-perfused lungs from naïve animals constricted with carbachol (Darmani and Broadley, 1986; Thorne and Broadley, 1992). Therefore, the bronchoconstrictor versus bronchodilator response to adenosine in animal models of asthma or asthmatic patients compared with that of naïve animals, respectively, could be partly due to the differences in the expression of adenosine receptor subtypes between diseased and normal airways.

Given that the inhaled adenosine A_1 receptor agonist CPA suppresses cough and that adenosine has inhibitory actions in several CNS regions (caudal regions of the nTS, the hypoglossal nucleus and the ventrolateral) (Bisserbe *et al.*, 1985; St Lambert *et al.*, 1996), we sought to determine whether CPA, administered centrally, can also modulate the citric acid-induced cough and airway obstruction. I.c.v. administration of CPA dose-dependently inhibited both responses; this was prevented by pretreatment with the adenosine A_1 receptor antagonist DPCPX. A previous study, using mice, showed that i.c.v. injection of the adenosine A_1 receptor antagonist, N^6-cyclohexyladenosine, reduced enhanced expiration – a reflex with some similarities to cough (Kamei *et al.*, 1994). Although this was described as a 'cough response', it is generally accepted that mice and rats do not possess a cough reflex but rather an expiration reflex originating from the larynx (Belvisi and Bolser, 2002). Nonetheless, the suppressive effects on the breathing response highlight the inhibitory role of the adenosine A_1 receptors in the brainstem. Our findings are also generally consistent with studies showing that inhibitory effects of adenosine A_1 receptors are dominant in most central structures (Ralevic and Burnstock, 1998; Tupone *et al.*, 2013). The nTS is considered to be an important site for termination of vagal afferent nerves and thus a key site for control of autonomic breathing and sympathetic activities, both during resting conditions and during an acute hypoxic response similar to the situation following citric acid challenge and reflexes such as cough. Our findings are also in line with other studies, which have reported inhibition of neural and cardiopulmonary chemoreflex responses following activation of adenosine A_1 receptors in nTS – an effect blocked by treatment with an A_1 receptor antagonist (Ginsborg and Hirst, 1972; Eldridge *et al.*, 1985). Although the nTS is the most studied CNS region in the context of cough, the paratrigeminal nucleus (Pa5), located in the dorsalateral pole of the medullary spinal trigeminal tract, with ascending pathways that terminate broadly throughout the CNS, has been

shown to receive primary afferent inputs from the airways (Driessen *et al.*, 2015). Hence, the involvement of regions in CNS, other than the nTS, in the adenosine A_1 receptor – mediated modulation of reflexes such as cough, cannot be ruled out.

Our data also show that CPA (i.c.v.) dose-dependently inhibited citric acid-induced airway obstruction. This suggests that activation of central A_1 receptors plays a role in limiting the airway response to bronchoconstrictor stimuli such citric acid. It is well known that full expression of not only the cough reflex but also bronchoconstriction requires sensory fibres to ascend in the vagus nerve and enter the brain stem through the solitary tract, making their first synapse with the nTS second-order neurons (Bonham and Joad, 1991; Haxhiu *et al.*, 1997). Indeed, the anatomical convergence of visceral afferent nerve fibre subtypes in the nTS may explain why different organ reflexes such as cough, bronchospasm and bradycardia tend to occur at the same time under similar physiological conditions (Mazzone and Canning, 2002a). In the nTS, the afferent vagal terminals responsible for transmitting information from the airway sensory receptors, related to airway tone, form a distinct wiring organization with specific second-order neurons that project to airway-related vagal preganglionic neurons (AVPNs) (Haxhiu *et al.*, 2005). Several studies have reported an important role for the brainstem in controlling the airway calibre and show that the net airway motor response results not only from local interaction of airway afferent and efferent nerves but also due to interaction at the brainstem of airway sensory vagal projections (Solomon, 1998; Haxhiu *et al.*, 2000; Canning *et al.*, 2004).

Based on the findings that adenosine A_1 receptor activation suppresses both citric acid-induced cough and airway obstruction, we hypothesized that tonic activation of the A_1 receptor may 'switch off' a cough response. This hypothesis was tested by blocking the A_1 receptor with a competitive antagonist, DPCPX. Our data showed that DPCPX dose-dependently enhanced the citric acid-induced cough and airway obstruction. This suggests that during activation of airway responses, such as cough and airway obstruction, adenosine release in brain regions that regulate cough, such as the nTS, may be increased thereby enhancing adenosine A_1 receptor-mediated purinergic transmission. The effect of this is to limit the frequency and intensity of cough and airway obstruction, thus acting as 'a braking system'. These data concur with previous studies showing that blockade of the adenosine A_1 receptor in the nTS significantly changes the pattern of autonomic responses in pathological conditions such as hypoxia and ischaemia (Park *et al.*, 1988; Scislo and O'Leary, 2006). Indeed, a recent study has shown that the cardiopulmonary reflex-mediated inhibition of renal and adrenal sympathetic activity is significantly attenuated during severe haemorrhage. This was shown to be due to increased adenosine release as the effect was reversed by blockade of adenosine A_1 receptors in the caudal nTS (Minic *et al.*, 2014).

To address the issue of how central A_1 receptors could control these airway protective reflexes, we assessed whether blockade of GluN1 receptors would affect the DPCPX-enhanced citric acid-induced cough and airway obstruction. The potent GluN1 antagonist D-AP5 (Jafari-Sabet *et al.*, 2005) completely blocked the DPCPX-enhanced cough. This effect was not due to inhibition of baseline cough, since treatment with D-AP5 alone had no effect. The lack of effect on baseline cough was surprising, since central GluN1 receptors were previously shown to have a role in the mediation of cough (Canning, 2009). This may have been due to an already low cough response, possibly because of the use of ethanol as a solvent/vehicle to dissolve DPCPX. Ethanol has been reported to have inhibitory effects in the CNS possibly mediated through increased adenosine release, increased GABAergic transmission or inhibition of GluN1 receptors (Peoples and Stewart, 2000; Theile *et al.*, 2008; Ruby *et al.*, 2010). Another possible explanation may be the medium dose of the citric acid used in this study (0.4 M), not the usual higher doses of 0.6 to 1 M, which was chosen so as to not to induce maximal cough and airway response stimulation. Whether any of these factors, singly or in combination, may have contributed to the decrease in the cough response in controls with ethanol remains to be determined.

The interaction between the A_1 receptor-mediated purinergic signalling system and glutamate/GluN1 receptors is likely taking place in the nTS (see Figure 7), where adenosine A_1 receptors and glutamate/GluN1 receptors are co-expressed (Bisserbe *et al.*, 1985; St Lambert *et al.*, 1996; Haxhiu *et al.*, 2005). Interestingly, an interaction between adenosine A_1 receptor-mediated purinergic signalling system and glutmate/GluN1 receptors, in several other brain regions, has also been reported to control glutamate-induced neuronal excitability and excitotoxicity, primarily *via* presynaptic A_1 receptor-mediated inhibition of glutamate release (Poli *et al.*, 1991; Yang *et al.*, 2007; Nascimento *et al.*, 2010; Rau *et al.*, 2014).

Our data also showed that D-AP5 completely reverses the DPCPX-enhanced citric acid-induced airway obstruction. This further highlights the important role for brainstem glutamate/GluN1 receptors in mediating signals from

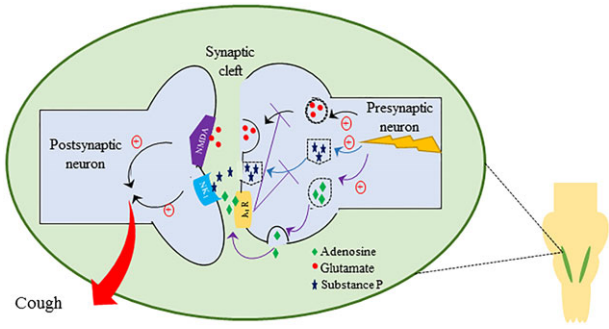

Figure 7

Summary of our findings and a proposed mechanism for the interaction between the adenosine/A_1 receptor and glutamate/GluN1 (NMDA) and the substance P/NK_1 receptor signalling pathways in controlling reflexes such as cough and airway obstruction. Blockade of the A_1 receptor results in an increased cough response and airway obstruction that is reversed following antagonism of the GluN1 and NK_1 receptors. This suggests that stimulation of airway nerves results in an increased central adenosine release which, *via* the A_1 receptor, limits the cough response possibly by reducing central glutamate and substance P release. We therefore propose that in conditions such as CHS, this cough inhibitory adenosine pathway may be dysfunctional.

sensory afferents to the brainstem and also underscores the inhibitory role of adenosine A_1 receptors on this pathway. These findings are in line with studies showing that glutamate release in the nTS is increased following airway sensory nerve stimulation, and this also correlated with airway smooth muscle contraction (Haxhiu *et al*., 2000). Moreover, denervation of the airways inhibits glutamate release in the nTS (Haxhiu *et al*., 2005). It is thus of interest to note that glutamate-mediated EPSPs in the AVPN, in the rostral nucleus ambiguous, results in increased ACh-induced bronchoconstriction in the airways (Haxhiu *et al*., 2005; Haxhiu *et al*., 2000). In this regard, both GluN1 and AMPA receptors have been reported to be involved in transmitting the bronchoconstrictive inputs from the airways to the nTS to the AVPN.

In this study, we also investigated whether the substance P/NK_1 receptor, another well-established pathway in cough, is also involved in the DPCPX-enhanced cough and airway obstruction. Our data show that there was a dose-dependent reduction in the DPCPX-enhanced cough following blockade of the substance P/NK_1 receptor pathway. This effect was not due to interference with the baseline cough since blockade of the NK_1 receptor with FK-888 alone had no significant effect. This is in contrast to previous studies that have reported central substance P/NK_1 receptor signalling is involved in the mediation of cough. The lack of effect of FK-888 on baseline cough may be due to the already low baseline cough. Nevertheless, it is clear that treatment with FK-888 significantly inhibited the DPCPX-enhanced cough. This implies that activation of the adenosine A_1 receptor results in reduced excitatory tachykininergic transmission and points to an interaction between the adenosine A_1 receptor and the central substance P/NK_1 receptor signalling pathway. There is a paucity of studies looking at this interaction in the CNS, and only few studies have looked at this interaction in the peripheral nervous system. For example, endogenous adenosine has been reported to inhibit evoked substance P release from perifused networks of myenteric ganglia (Moneta *et al*., 1997). Adenosine A_1 receptors have also been demonstrated to inhibit tachykinin release from perifused enteric nerve endings (Christofi *et al*., 1990; Broad *et al*., 1992). Taken together, these studies indicate that adenosine receptor(s), at least on the myenteric nerve endings, are coupled negatively to tachykinin release. Thus, it is possible that activation of A_1 receptors, on tachykininergic nerves in the nTs, or other cough controlling brain regions, decreases the release of excitatory substance P (see Figure 7). Furthermore, treatment with FK888 also resulted in dose-dependent reduction in the DPCPX-enhanced airway obstruction as evidenced by the reduction in Penh. This suggests that central substance P/NK_1 receptor signalling affects the airway obstruction response. This is in agreement with reports that i.c.v. administration of substance P induces a significant degree of bronchospasm and that bradykinin-induced hyperresponsiveness to histamine is reversed by centrally administered neurokinin receptor antagonists (Mazzone and Canning, 2002a; Mazzone and Canning, 2002b).

Since activation of the A_1 receptor inhibited both the citric acid-induced cough and airway obstruction, and its blockade potentiates both, we investigated whether blockade of the GABAergic and opioidergic pathways would also result in the enhancement of cough or airway obstruction. If this was the case, it would imply that they are also tonically activated in response to tussigenic stimulation. Our data show that blockade of the $GABA_B$ or the μ receptors, at doses shown to have effects *in vivo* (Takahama and Shirasaki, 2007), had no significant effect on cough or airway obstruction. These findings suggest that the role of the opioidergic and the GABAergic systems, in the cough reflex, is different from that of the adenosine A_1 receptor-mediated purinergic pathway and that the latter may play a distinct role in controlling cough.

In summary, our data show that activation of airway and central adenosine A_1 receptors suppress citric acid-induced cough and airway obstruction whilst blockade of central adenosine A_1 receptors has an enhancing effect on both these responses. This is likely to be secondary to modulation of central excitatory glutamatergic and tachykininergic activity in the nTS or other brain regions that regulate cough (Figure 7). Thus, adenosine A_1 receptors act as a 'braking system' to limit excessive airway responses. Our findings may have relevance to patients with chronic cough where their demonstrated CHS may not be due to enhanced activity of the central excitatory pathways but rather to decreased activity of the inhibitory pathways such as the A_1 receptor-mediated purinergic pathway.

Acknowledgements

We are greatly indebted to our friend and mentor the late Dr Dom Spina, a great respiratory pharmacologist, for his insightful feedback on the work and constructive criticism of this manuscript. We are also grateful to Professors Samuel Kombian and Yunus Luqmani for their help with the preparation of the manuscript and to Ala Abdelali for preparing the schematic diagram. We also acknowledge the support of Ms Ola Zahran and Mr Sunny Ojoko from the Animal Resources Center of the Health Sciences Centre, Kuwait University.

Author contributions

A.Z.E.-H. devised the experiments and wrote the manuscript. S.M. and F.A.-S. performed the experiments and the statistical analyses.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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