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Mechanism of adenosine-induced airways obstruction in allergic guinea pigs

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1 Inhaled adenosine induces airway obstruction in asthmatic but not healthy subjects, a phenomenon that is also observed in various animal species when they are immunised to a relevant antigen, but which does not occur in naïve animals. The purpose of this study was to investigate the mechanisms of airway responsiveness to adenosine receptor agonists in anaesthetised allergic guinea pigs.

2 Inhaled adenosine 5′-monophosphate (AMP), the A_1-selective adenosine receptor agonist N^6-cyclopentyladenosine (CPA) and ovalbumin all caused airway obstruction in allergic guinea pigs, but not naïve animals, as assessed by changes in total lung resistance. In contrast, the A_{2a}-selective (CGS 21680; 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxoamido adenosine) and A_3-selective (IB-MECA; 1-deoxy-1-[6-[[3-iodophenyl)-methyl]amino]-9*H*-purin-9-yl]-*N*-methyl-*β*-D-ribofuranuronamide) adenosine receptor agonists failed to elicit airway obstruction in passively sensitised guinea pigs.

3 Airway obstruction induced by AMP or CPA was not inhibited by the H_1 receptor antagonist, mepyramine (1 mg kg^{-1}) in passively sensitised guinea-pigs. In contrast, airway obstruction to ovalbumin was significantly inhibited by this antagonist.

4 Airway obstruction induced by AMP and CPA was significantly inhibited in sensitised animals chronically treated with capsaicin. In contrast, airway obstruction to ovalbumin was not inhibited by this treatment.

5 Airway obstruction induced by AMP, CPA and ovalbumin was significantly inhibited following bilateral vagotomy or pharmacological treatment with atropine (2 mg kg^{-1}).

6 Airway obstruction to CPA was inhibited by the adenosine A_1 receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX: 0.1–1 mg kg^{-1}). In contrast, airway obstruction to ovalbumin was not inhibited by this treatment.

7 These observations provide evidence indicating that AMP and CPA may induce airway obstruction in sensitised guinea pigs by a mechanism unrelated to histamine release from mast cells, but is mediated *via* an adenosine A_1-receptor-dependent mechanism. The inhibition of AMP- and CPA-induced airway obstruction by atropine, capsaicin and bilateral vagotomy suggests a neuronal-dependent mechanism with the particular involvement of capsaicin-sensitive nerves.
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Abbreviations: AMP, adenosine-5′-monophosphate; CGS 21680, 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxoamido adenosine; CPA, N^6-cyclopentyladenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; IB-MECA, 1-deoxy-1-[6-[[3-iodophenyl)-methyl]amino]-9*H*-purin-9-yl]-*N*-methyl-*β*-D-ribofuranuronamide

Introduction

Airway hyper-responsiveness (AHR) represents an increase in the sensitivity and reactivity of the airways in response to a number of bronchoconstrictor stimuli and is a characteristic feature of asthma (Woolcock *et al*., 1984). AHR is most commonly assessed in response to inhalation challenge of methacholine or histamine, both of which are likely to cause bronchoconstriction following the direct activation of receptors on airway smooth muscle (Van Schoor *et al*., 2000). Twenty years ago, Cushley *et al*. (1983) first reported that the inhalation of adenosine causes bronchoconstriction in asthmatic but not normal subjects. There is now increasing evidence that evaluating airway responsiveness through adenosine-induced bronchoconstriction may be valuable in monitoring disease progression and the effects of anti-inflammatory therapy in asthma (Polosa *et al*., 2000; Van Schoor *et al*., 2000). Recent studies have reported that adenosine responsiveness is more closely related with allergic airway inflammation in subjects with asthma and allergic rhinitis than using methacholine responsiveness (Polosa *et al*., 2000). Furthermore, isolated bronchi obtained from asthmatic subjects, exhibit increased contraction in response to adeno-

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sine compared with nonasthmatic bronchi *via* an adenosine A_1 receptor mechanism (Bjorck *et al*., 1992), and more recently, mucin induction in asthmatic airway tissue has been demonstrated to occur *via* an A_1-receptor-dependent mechanism (McNamara *et al*., 2004). Moreover, we have recently shown an upregulation of adenosine A_1 receptors on airway structures in biopsies obtained from patients with mild asthma (Brown *et al*., 2005).

Various animal species including rabbits (El-Hashim *et al*., 1996), Brown Norway rats (Hannon *et al*., 2001), mice (Fan & Mustafa, 2002; Wyss *et al*., 2005) and guinea pigs (Thorne & Broadley, 1994) undergo airways obstruction in response to adenosine following sensitisation. There are clearly species differences with regard to the mechanism of action and adenosine receptor subtypes implicated in the bronchoconstrictor response (Fozard & Hannon, 2000; Meade *et al*., 2001). We have previously reported that rabbits sensitised to *Alternaria tenuis* bronchoconstrict to adenosine *via* an adenosine A_1-receptor-dependent mechanism in the absence of allergen challenge (El-Hashim *et al*., 1996) supporting observations made in bronchi from asthmatic subjects (Bjorck *et al*., 1992). Moreover, asthmatics are uniquely sensitive to adenosine in the absence of exacerbation of their disease process (Meade *et al*., 2001), and therefore modelling this process may enable us to better understand the mechanism underlying this phenotypic change in asthma.

The role of mast cells and the subsequent release of preformed and newly formed mediators have received considerable attention as a contributory mechanism to adenosine-induced airway obstruction (Rafferty *et al*., 1987; Polosa *et al*., 1995). However, adenosine can activate many other cell types in the airways, including pulmonary C-fibres (Hong *et al*., 1998; Chuaychoo *et al*., 2005) and induces dysponea in healthy volunteers possibly by the activation of these afferent nerves (Burki *et al*., 2005). Hence, the possibility that adenosine might elicit airways obstruction *via* the activation of neuronal pathways is plausible, but remains to be established. Thus, the aims of the present study were to investigate the mechanisms of adenosine-induced airway obstruction in anaesthetised, sensitised guinea pigs *in vivo* and in particular to evaluate the contribution of neuronal pathways to this phenomenon.

Methods

Animals

Male, Dunkin–Hartley guinea pigs (250–450 g; Harlan) were used throughout this study. Guinea pigs were housed on-site for at least 24 h prior to experimentation and given free access to food and water. All studies were carried out under the Animals (Scientific Procedures) Act of 1986.

Sensitisation protocol

Ovalbumin was prepared in saline (25 mg ml^{-1}) and mixed with aluminium hydroxide (v v^{-1}: 1 : 10). This solution was injected intraperitoneally (i.p.) into recipients (5 mg kg^{-1}), and after 10 days, this procedure was repeated. After a further 7 days, blood was collected *via* a cannula inserted into the carotid artery under i.p. anaesthesia with urethane (1.75 g kg^{-1}) and added to heparin (0.2 ml; 100 U ml^{-1}). Blood was centrifuged ($2000 \times g$ for 15 min) and the plasma was removed and stored (-20°C). For passive sensitisation, this anti-ovalbumin guinea pig plasma was injected intravenously *via* the saphenous vein of recipient naïve conscious guinea pigs (1 ml animal^{-1}). In some cases, recipient animals received serum from sham-immunised guinea pigs. Lung function was recorded 7–10 days later.

Measurement of pulmonary function

Guinea pigs were anaesthetised by an i.p. injection of urethane (1.75 mg kg^{-1}). Lung function was assessed 30 min later. Guinea pigs were ventilated (8 ml kg^{-1}; 60 breaths min^{-1}) through a tracheal cannula connected to a pneumotachograph and a pressure transducer ($\pm$2 cm H_2O: model MP-45-14-871; Validyne Engineering, Northridge, CA, U.S.A.). Changes in airflow were measured using an automated lung function recording system (Pulmonary Monitoring System, version 5.0; Mumed, London, U.K.) and displayed in real time on a personal computer. The flow signal was integrated to give a measure of tidal volume. An intrathoracic cannula was inserted between the third and fifth intercostal space and connected to the negative side of the pressure transducer ($\pm$20 cm H_2O; Validyne). The positive side of the transducer was connected to the side of the pneumotachograph proximal to the animal. The difference between mouth and thoracic pressure was used as a measure of transpulmonary pressure (TPP). Total lung resistance (R_L; cm H_2O s l^{-1}) was calculated from flow, tidal volume and TPP by integration. The jugular vein was cannulated for the administration of various compounds, with blood pressure and heart rate being recorded *via* a pressure transducer attached through an arterial cannula inserted into the carotid artery.

Airway obstruction (peak response) was measured following administration of aerosolised AMP (10 mg ml^{-1}; 10 s), the A_1-selective (CPA; 10 mg ml^{-1}; 10 s); A_{2a}-selective (CGS21680; 10 mg ml^{-1}; 10 s), A_3-selective (IB-MECA; 10 mg ml^{-1}; 10 s) receptor agonist and ovalbumin (5 mg ml^{-1}; 10 s). In some cases, airway obstruction was recorded in response to histamine (0.1–1 mg ml^{-1}; 10 s). These agonists were aerosolised and delivered to the airways *via* a DeVilbiss nebuliser at 4 ml breath^{-1} and 60 breaths min^{-1}.

Pharmacological treatment

Guinea pigs were randomised into different treatment groups that received either mepyramine (1 mg kg^{-1}) or DPCPX (0.1–1 mg kg^{-1}). In other experiments, passively immunised animals were treated with atropine (2 mg kg^{-1}) or underwent bilateral vagotomy and were then assessed on the same occasion in blocks with two animals randomised to each treatment group alongside two untreated animals per block. In further experiments, airway obstruction was recorded in passively immunised guinea pigs in response to intravenously administered methacholine (1–3 μg kg^{-1}) or histamine (2–4 μg kg^{-1}) in the absence or presence of atropine and mepyramine (1 mg kg^{-1}), respectively. Antagonists were administered 15 min prior to administration of the agonist.

Capsaicin treatment

One group of guinea pigs was chronically treated with capsaicin as previously described prior to passive sensitisation (Keir *et al*., 2002). Briefly, guinea pigs were anaesthetised with an intramuscular injection of ketamine (35 mg kg^{-1}) and xylazine (5 mg kg^{-1}), followed by an i.p. injection of mepyramine (10 mg kg^{-1}) and salbutamol (1 mg kg^{-1}) to protect animals from the acute adverse effects of capsaicin. Animals were injected 15 min later with capsaicin (subcutaneously) prepared in ethanol, Tween 80 and 0.9% saline (v v^{-1}; 1 : 1 : 8) in the dorsal region of the neck. Animals were treated twice a day (6 h apart) on 3 consecutive days, receiving a total dose of 80 mg kg^{-1} capsaicin. Control animals were treated in a similar manner, with the exception that they received vehicle only (ethanol: Tween 80 : 0.9% saline; v v^{-1}; 1 : 1 : 8). In both cases, animals were passively immunised a day after the last vehicle or capsaicin injection and were anaesthetised 7–10 days later for the measurement of lung mechanics.

Bronchoalveolar lavage

BAL was performed immediately after the completion of the measurement of lung function. Animals were terminated with an overdose of anaesthetic followed by instilling 5 ml sterile saline into the lungs *via* the tracheal cannula and the fluid was immediately aspirated. The same fluid was then reinjected and the procedure repeated three times. This resulted in 40–60% recovery of BAL fluid from the lungs of each guinea pig. Total cell counts were performed using an improved Neubauer haemocytometer. Cytospin preparations were prepared using the Shandon cytospin 2 centrifuge. BAL fluid (100 μl) was added to the each cytospin cup and the samples were centrifuged for 1 min at 1300 r.p.m. Slides were fixed and stained using Diff-Quick®. The cell types were classified as eosinophils, neutrophils and mononuclear cells according to standard morphological criteria. Cells were counted in a blinded manner. The results are expressed as total number of cells per ml BAL fluid and eosinophils as a percentage of the total number of cells counted.

Drugs

The following reagents were used: ovalbumin (grade V), urethane, atropine, CPA, AMP, histamine, Tween 80, mepyramine, DPCPX, capsaicin, IB-MECA (Sigma, Poole, Dorset, U.K.), aluminium hydroxide solution (Sanofi, Rio de Janeiro, Brazil) and CGS 21680 (Tocris, U.K.).

Data analysis

Airway obstruction was calculated as a percentage increase in total lung resistance (R_L) above baseline (the individual animal's own baseline value). Data are expressed as arithmetic means ± standard error of the mean (s.e.m), with the exception of the provocative concentration that gave rise to a 50% increase in baseline total lung resistance (PC_{50} R_L) in response to histamine, which was expressed as geometric mean together with 95% confidence interval. The effect of drug treatment was analysed by unpaired Student's *t*-test, and when appropriate, a Bonferroni correction was made in the case of multiple comparisons between means and considered statistically significant if the *P*-value was less than 0.05.

Results

Baseline total airway resistance

There was no significant difference in baseline R_L (cm H_2O s l^{-1}) between naïve (195.3 ± 15.6, $n = 24$) and passively immunised guinea pigs (164.3 ± 12.3, $n = 30$). Furthermore, baseline pulmonary function was unaffected by any of the treatment protocols used (data not shown). The total number of cells recovered from BAL was not significantly different between naïve and passively immunised animals (17.7 ± 0.7 vs 22.7 ± 2.3 × 10^4 cells ml^{-1}). In contrast, the percentage of eosinophils recovered in BAL was significantly greater in passively sensitised guinea pigs (22.6 ± 2.7 vs 42.2 ± 4.7, respectively, $P < 0.05$).

Airway obstruction to adenosine agonists

Aerosolised administration of AMP or CPA (10 mg ml^{-1}; 10 s) to naïve animals that had received serum from nonimmunised animals did not bronchoconstrict to these agonists (% increase in R_L; 2.8 ± 0.8 and 4.4 ± 1.3, respectively, $n = 4$).

The aerosolised administration of AMP (10 mg ml^{-1}; 10 s) or CPA (10 mg ml^{-1}; 10 s) caused airway obstruction in allergic guinea pigs compared with littermate naïve guinea pigs ($P < 0.01$; $n = 5$ per treatment group; Figure 1a). In contrast, aerosol administration of the A_{2a}-selective (CGS21680, 10 mg ml^{-1}; 10 s) or A_3-selective (IB-MECA; 10 mg ml^{-1}; 10 s) receptor agonist failed to elicit airway obstruction in either allergic or naïve guinea pigs ($P > 0.05$; $n = 5$ per treatment group; Figure 1a). CPA induced a dose-dependent increase in total lung resistance (Figure 1b) in allergic guinea pigs and higher concentrations could not be tested because of poor solubility. The aerosolised administration of AMP and CPA produced a fall (% decrease) in blood pressure (AMP: 53.2 ± 5.4%; CPA: 56.1 ± 4.2%), which recovered to baseline after 15 min.

The intravenous administration of the A_1 receptor antagonist DPCPX (100–300 μg kg^{-1}) 15 min prior to measuring lung function significantly inhibited airway obstruction induced by CPA (DPCPX 300 μg kg^{-1} vs control, $P < 0.05$; $n = 3$–4 per group; Figure 1c).

In a further series of experiments, the effect of sensitisation on airways responsiveness to histamine was compared. There was no significant difference in the PC_{50} R_L value for aerosolised histamine (mg ml^{-1}) between littermate naïve and passively immunised guinea pigs (geometric mean, 95% confidence interval; 3.07 (1.9–4.8), $n = 5$ vs 2.4 (1.8–3.4), $n = 6$, respectively, $P > 0.05$).

The time (s) to peak airways obstruction in response to ovalbumin (5 mg ml^{-1}) and CPA (10 mg ml^{-1}) was 114 ± 35 s ($n = 7$) and 89 ± 10 s ($n = 5$), respectively. The time for airway obstruction to reverse by 50% in response to ovalbumin was 61 ± 24 s, while for CPA, airway obstruction was maintained over a 10 min period.

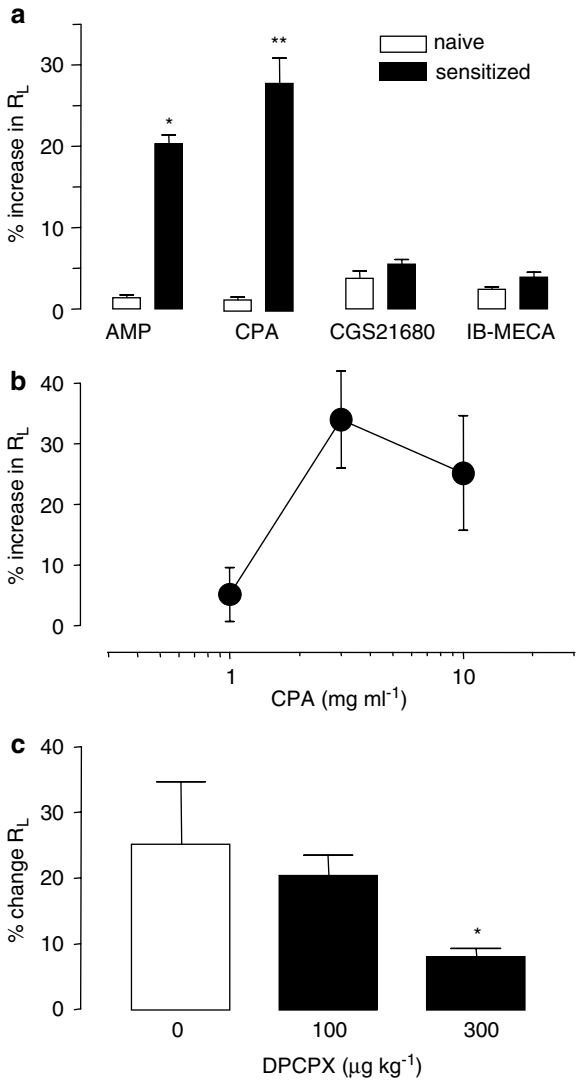

Figure 1 (a) Airway obstruction (% increase in baseline R_L) to AMP, CPA, CGS21680 and IB-MECA (10 mg ml^{-1}; 10 s) in naïve (open columns) and allergic animals (closed columns). $N=5$ per group. (b) Dose-dependent increase in airway obstruction (% increase in baseline R_L) to CPA. $N=3$ per group. (c) Airway obstruction (% increase in baseline R_L) to CPA in the absence or following intravenous treatment with DPCPX. $N=3$–6 per group. Results expressed as mean and standard error of the mean. $^*P<0.05$, $^{**}P<0.01$ compared with response obtained in naïve animals (a) or absence of DPCPX (c).

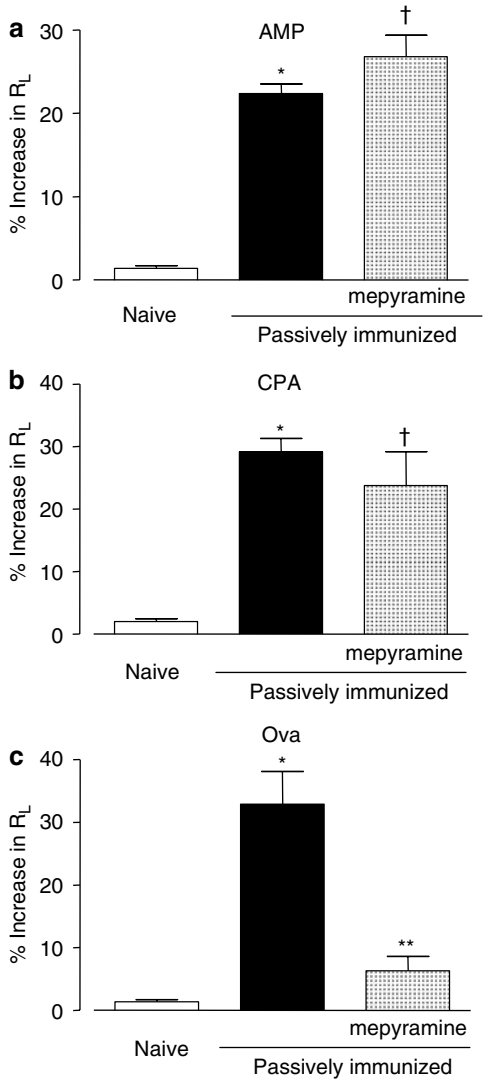

Figure 2 Airway obstruction (% increase in baseline R_L) to (a) AMP (10 mg ml^{-1}; 10 s), (b) CPA (10 mg ml^{-1}; 10 s) and (c) ovalbumin (Ova; 5 mg ml^{-1}; 10 s) in naïve littermate controls (open columns) and passively sensitised guinea pigs (closed columns, stippled columns). Passively immunised animals were either untreated (closed columns) or treated with mepyramine (1 mg kg^{-1}; stippled columns). Results expressed as mean and standard error of the mean of five observations per group. $^*P<0.05$ cf. naïve group, $^{**}P<0.05$ cf. untreated sensitised group; $^{\dagger}P>0.05$ cf. untreated sensitised group.

Pharmacological studies with mepyramine

There was no significant difference in the increase in total R_L in response to aerosolised administration of AMP or CPA (10 mg ml^{-1}) between animals treated with mepyramine (1 mg kg^{-1}) 15 min prior to recording lung function, and vehicle-treated animals (Figure 2a and b; $P>0.05$, $n=5$–6 per group). In contrast, mepyramine significantly inhibited the increase in baseline R_L in response to ovalbumin (5 mg ml^{-1}; Figure 2c; $P<0.05$).

In control experiments, airways obstruction (% increase in R_L) in response to intravenously administered histamine (2 μg kg^{-1}; 53.6±21.2; 4 μg kg^{-1}; 169.7±40.3, $n=3$) was significantly inhibited (2.0±0.2 and 7.3±3.0, respectively, $n=3$; $P<0.05$) by mepyramine (1 mg kg^{-1}).

Pharmacological studies with capsaicin

Chronic pretreatment with capsaicin significantly inhibited airway obstruction induced by AMP ($P<0.05$; $n=5$ per group; Figure 3a) and CPA ($P<0.05$; $n=5$ per group; Figure 3b) compared with vehicle-treated passively immunised animals. Consistent with previous findings, animals chronically treated with capsaicin failed to respond to the intravenous administration of capsaicin (100 μg kg^{-1}) compared with vehicle-treated animals, confirming the success of this treatment

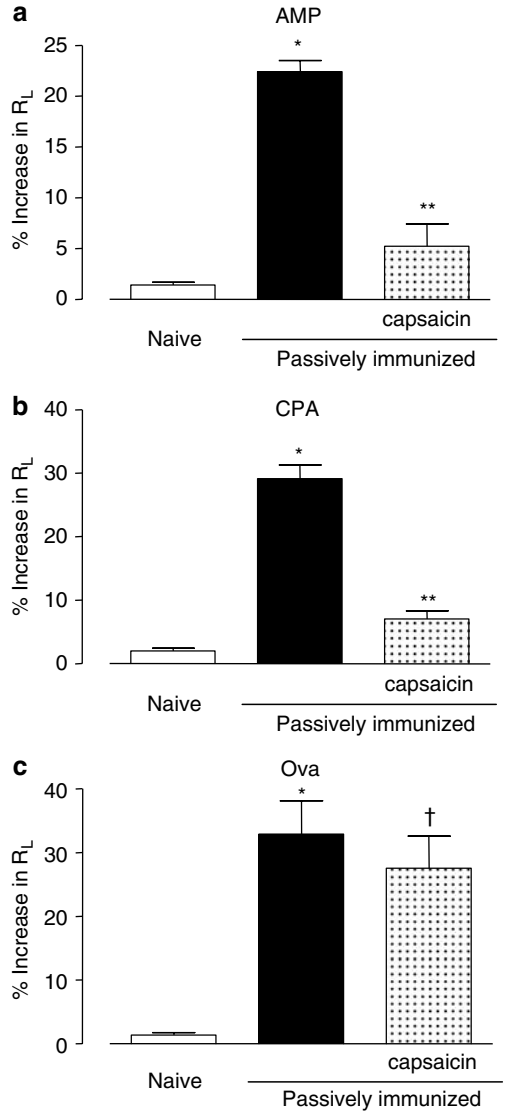

Figure 3 Airway obstruction (% increase in baseline R_L) to (a) AMP (10 mg ml^{-1}; 10 s), (b) CPA (10 mg ml^{-1}; 10 s) and (c) ovalbumin (Ova; 5 mg ml^{-1}; 10 s) in naïve littermate controls (open columns) and passively sensitised guinea pigs (closed columns, stippled columns). Passively immunised animals were vehicle treated (closed columns) or had been chronically treated with capsaicin (80 mg kg^{-1}; stippled columns). Results expressed as mean and standard error of the mean of five observations per group. *$P<0.05$ cf. naïve group, **$P<0.05$ cf. untreated sensitised group; $^{\dagger}P>0.05$ cf. untreated sensitised group.

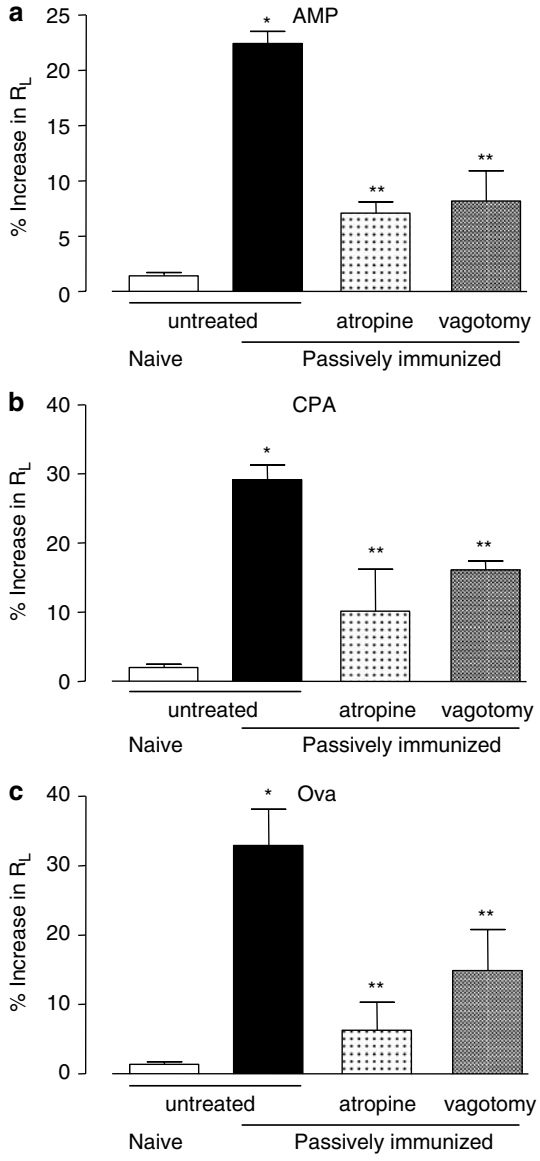

Figure 4 Airway obstruction (% increase in baseline R_L) to (a) AMP (10 mg ml^{-1}; 10 s), (b) CPA (10 mg ml^{-1}; 10 s) and (c) ovalbumin (Ova; 5 mg ml^{-1}; 10 s) in naïve littermate controls (open columns) and passively sensitised guinea pigs (closed columns, stippled columns, dark stippled columns). Passively immunised animals were either untreated (closed columns) or treated with atropine (2 mg kg^{-1}; stippled columns). In other groups, sensitised animals underwent bilateral vagotomy (dark stippled columns). Results expressed as mean and standard error of the mean of five observations per group. *$P<0.05$ cf. naïve group, **$P<0.05$ cf. untreated sensitised group.

(data not shown). In contrast, airway obstruction to ovalbumin was not significantly altered in passively immunised guinea pigs chronically treated with capsaicin ($P>0.05$; Figure 3c).

Pharmacological treatment with atropine or bilateral vagotomy

The intravenous administration of atropine (2 mg kg^{-1}) or bilateral vagotomy, 15 min prior to measuring lung function, significantly inhibited airway obstruction induced by AMP ($P<0.05$; $n=5$ per group; Figure 4a) and CPA ($P<0.05$; $n=5$ per group; Figure 4b). Similarly, bilateral treatment with atropine or bilateral vagotomy significantly inhibited airway obstruction induced by ovalbumin ($P<0.05$; $n=5$ per group; Figure 4c). In control experiments, the percentage increase in baseline R_L in response to methacholine (1 μg kg^{-1}; 9.8$\pm$2.1; 3 μg kg^{-1}; 62.6$\pm$7.8, $n=3$) was significantly inhibited (4.9$\pm$4.3 and 3.8$\pm$1.6, respectively, $n=3$; $P<0.05$) in the presence of atropine.

Pharmacological treatment with DPCPX

Intravenous administration of the A_1 receptor antagonist DPCPX (1 mg kg^{-1}) 15 min prior to measuring lung function

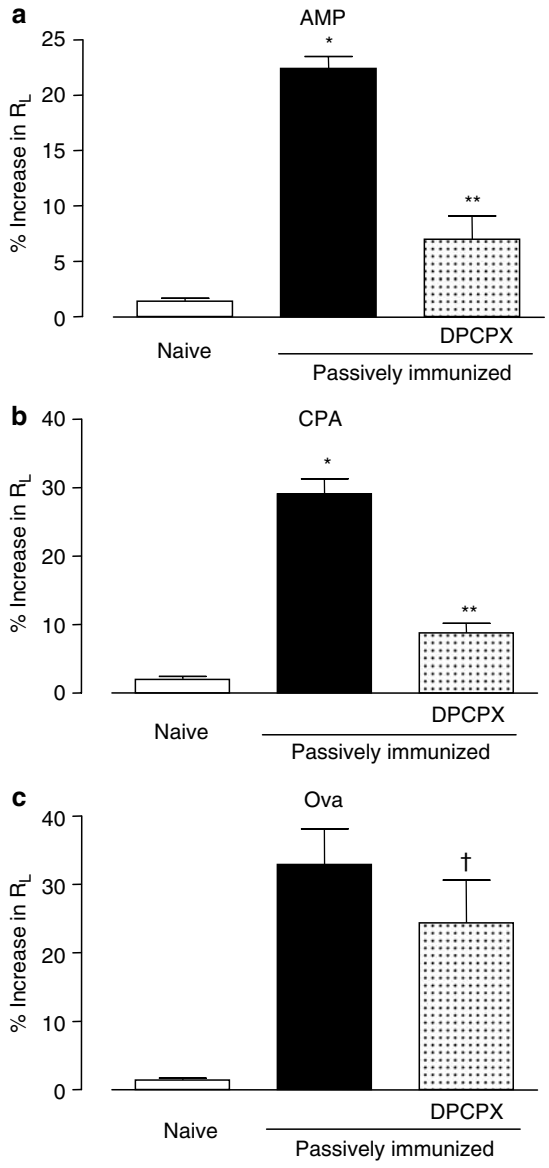

Figure 5 Airway obstruction (% increase in baseline R_L) to (a) AMP (10 mg ml^{-1}; 10 s), (b) CPA (10 mg ml^{-1}; 10 s) and (c) ovalbumin (Ova; 5 mg ml^{-1}; 10 s) in naïve littermate controls (open columns) and passively sensitised guinea pigs (closed columns, stippled columns). Passively immunised animals were either untreated (closed columns) or treated with DPCPX (1 mg kg^{-1}; stippled columns). Results expressed as mean and standard error of the mean of five observations per group. *$P<0.05$ cf. naïve group, **$P<0.05$ cf. untreated sensitised group; $^{\dagger}P>0.05$ cf untreated sensitised group.

significantly inhibited airway obstruction induced by AMP ($P<0.05$; $n=5$ per group; Figure 5a) and CPA ($P<0.05$; $n=5$ per group; Figure 5b) compared with vehicle-treated animals. In contrast, there was no significant difference in the increase in R_L in response to the aerosolised administration of ovalbumin in animals treated with the A_1 receptor antagonist DPCPX ($P>0.05$; $n=5$ per group; Figure 5c).

Discussion

We have demonstrated that AMP causes airway obstruction in allergic guinea pigs, consistent with previous findings in conscious animals (Thorne & Broadley, 1994). This finding is not unique to the guinea pig and is a feature of sensitised rabbits (El-Hashim *et al*., 1996), Brown Norway rats (Hannon *et al*., 2001) and mice (Fan & Mustafa, 2002; Wyss *et al*., 2005). These observations support the clinical observation that asthmatic, but not healthy subjects, bronchoconstrict in response to inhaled adenosine (Cushley *et al*., 1983). Allergic guinea pigs have long been used as an animal model of certain features of asthma. Aerosol challenge with antigen in suitably sensitised guinea pigs robustly induces airway obstruction and inflammation similar to that seen in asthma (Kallos & Kallos, 1984), indices that were reproduced in this study. We have also shown that the response to adenosine in these allergic guinea pigs to adenosine is A_1 receptor dependent, supporting earlier work in allergic rabbits (El-Hashim *et al*., 1996) and in human bronchus obtained from subjects with asthma (Bjorck *et al*., 1992).

Numerous studies have previously investigated the potential mechanisms of adenosine-induced airway obstruction in the guinea pig. A number of *in vitro* experiments have shown that adenosine causes substantial contraction of sensitised guinea-pig airways that appeared to be adenosine A_3 receptor dependent (Thorne & Broadley, 1992; Martin & Broadley, 2002). Moreover, other studies have revealed that inhalation of AMP caused a rapid migration of eosinophils and macrophages into the airways in sensitised guinea pigs, which could be blocked by the A_3 antagonist MRS-1220 (Thorne *et al*., 1996; Spruntulis & Broadley, 2001). However, our results presented here suggest that the bronchoconstrictor response induced by AMP is reproduced by the adenosine A_1 receptor agonist, CPA, suggesting that this effect is primarily an A_1-receptor-dependent mechanism. This adenosine receptor A_1-mediated response is further confirmed by the fact that the adenosine A_{2A}-receptor-selective agonist CGS21680 and the adenosine A_3-receptor-selective agonist IB-MECA failed to elicit any bronchoconstrictor response in allergic guinea pigs. Furthermore, the adenosine A_1 receptor antagonist, DPCPX, inhibited airway obstruction to both AMP and CPA. It is noteworthy that bronchoconstriction to adenosine agonists in this model was not dependent upon allergen challenge, but only on sensitisation. This is similar to other allergic models (El-Hashim *et al*., 1996; Wyss *et al*., 2005) and human asthma (Cushley *et al*., 1983). The mechanism by which sensitisation leads to increased responsiveness to adenosine does not appear to be dependent upon altered airway smooth muscle contractility. Airway sensitivity to histamine was not increased in our model, which is consistent with the studies cited earlier, which show that allergen challenge is required before AHR to histamine can be documented. This heterogeneity of AHR is well established and is characteristic of asthma (O'Connor *et al*., 1999) and animal models of hyper-responsiveness (Hoshiko & Morley, 1993).

Earlier studies in allergic rabbits have also supported the hypothesis that airway obstruction induced by adenosine is mediated *via* the adenosine A_1 receptor (El-Hashim *et al*., 1996). Moreover, others still have suggested an involvement of adenosine A_{2B} receptors on mast cells to explain AMP-induced airways obstruction in allergic subjects (Feoktistov & Biaggioni, 1995). However, this hypothesis is not consistent with our data since airway obstruction induced by CPA was not blocked by the histamine H_1 receptor antagonist mepyramine. In contrast, the bronchoconstrictor response to ovalbumin was substantially inhibited by mepyramine, highlighting the

important role histamine plays in mediating bronchoconstriction in response to ovalbumin in this model. Indeed, it is well established that histamine is the major autacoid-mediating acute bronchoconstriction to antigen in the allergic guinea pig (Daffonchio *et al*., 1987). Therefore, if mast cell-derived products are responsible for mediating bronchoconstriction to CPA either directly or indirectly *via* activation of sensory nerves, then this is not a major pathway in this model.

These two findings rule out a role for mast cell-derived histamine in the response to AMP in this model and suggest that airway obstruction induced by antigen and adenosine are mediated by different mechanisms. These observations differ from the proposed major role for the mast cell in adenosine-induced airways obstruction (Holgate, 2005), since adenosine might activate mast cell adenosine A_{2B} receptors and thereby lead to the release of various spasmogens, including histamine, 5-HT, leukotrienes and prostaglandins, resulting in bronchoconstriction (Peachell *et al*., 1989; Polosa *et al*., 1995; Van Schoor *et al*., 2000). Studies in man have also highlighted a discrepancy in the role of histamine as a mediator of the bronchoconstrictor response to adenosine. Certainly in asthmatics, adenosine can elicit bronchoconstriction that is partly reduced by an H_1-receptor antagonist (Rafferty *et al*., 1987) and adenosine induces a rise in levels of histamine in bronchial lavage (Polosa *et al*., 1995), although this is not always a consistent observation (Crummy *et al*., 2004). Furthermore, a number of clinical studies also do not support an important role for adenosine A_{2B} receptors in mediating bronchoconstriction to adenosine. Firstly, adenosine does not mediate a late-phase response in asthma, clearly indicating that activation of the mast cell differs between this mediator and allergens (Holgate, 2005). Secondly, enprophylline, a weak adenosine A_{2B} receptor antagonist ($K_i = 4.7\,\mu$M) (Kim *et al*., 2002), appeared to be less effective than theophylline in functionally antagonising bronchoconstriction to AMP (Clarke *et al*., 1989). Moreover, acute administration of theophylline or enprophylline had modest effects against the early-phase response that is most likely attributed to bronchodilation and not inhibition of mast cell degranulation *via* adenosine receptor antagonism (Pauwels *et al*., 1985; Cockcroft *et al*., 1989) and theophylline did not inhibit the release of mast cell-derived histamine into bronchoalveolar lavage fluid (Jaffar *et al*., 1996). The observation that adenosine A_1 receptors mediate contraction of isolated bronchial tissue from asthmatic subjects in response to adenosine highlights the importance of other receptor subtypes in this response (Bjorck *et al*., 1992).

Our findings showing a cholinergic component of the response to allergen is also consistent with other studies in the guinea pig. Thus, ovalbumin can induce activation of parasympathetic ganglia, and acetylcholine release from parasympathetic neurones in tissues from sensitised animals. This effect is likely to be secondary to the release of histamine from tissue mast cells (Myers & Undem, 1995). Hence, histamine released from mast cells could stimulate parasympathetic ganglia either directly (Myers & Undem, 1995) or indirectly, *via* activation of afferent nerves (Mills & Widdicombe, 1970). While a number of studies have also reported a cholinergic component mediating bronchospasm to allergen challenge in primates (Richards *et al*., 1983), guinea pigs (Takahashi *et al*., 1997) and in asthmatic subjects (Clarke *et al*., 1982), it is clearly not the major mechanism underlying acute bronchoconstriction to antigen in asthma (Howarth *et al*., 1985), and it is likely that differences in the sensitivity of asthmatic subjects or antigen dose alters the proportion of the total bronchoconstrictor response mediated by parasympathetic stimulation.

We also show clearly that bilateral vagotomy and treatment with atropine inhibited AMP- and CPA-induced airway obstruction, suggesting that adenosine causes airway obstruction in the guinea pig *via* pathways involving cholinergic reflexes and vagal nerve activation. This is in contrast to the findings in allergic rats, where atropine and bilateral vagotomy were ineffective against the increase in airways responsiveness to adenosine following antigen challenge (Hannon *et al*., 2001). The role of cholinergic nerves in exacerbation of airway responsiveness to adenosine was not the primary focus of our study. However, airway obstruction induced by AMP and CPA was also inhibited by capsaicin, highlighting a role for capsaicin-sensitive sensory nerves in this response. This is consistent with electrophysiological data in the rat (Hong *et al*., 1998) and guinea pig (Chuaychoo *et al*., 2005), showing that adenosine can activate C-fibres and supports other recent work from our laboratory that CPA can also induce cholinergic reflex-mediated contraction of tracheal smooth muscle in allergic guinea pigs *in situ* (Reynolds *et al*., 2004). Furthermore, there is now considerable evidence showing that AHR to a variety of nonallergic and allergic stimuli can be inhibited following chemical ablation of sensory nerves by capsaicin (Spina & Page, 2002). The mechanism by which sensitisation *per se* leads to increased airway responsiveness to adenosine remains to be established. It is unclear whether this is related to increased expression of adenosine receptors on sensory nerves or increased neuronal activity. In either case, activation of afferent C-fibres by adenosine results in reflex bronchoconstriction in this guinea-pig model.

Interestingly, capsaicin treatment failed to abolish airway obstruction in response to ovalbumin and highlights an important difference in the response induced by adenosine and antigen in allergic guinea pigs. Ovalbumin may activate parasympathetic ganglia (Riccio *et al*., 1996), thereby inducing an atropine-sensitive bronchoconstrictor response independently of the activation of C-fibres and our results support other studies showing that ovalbumin-induced airway obstruction is not dependent upon C-fibre activation (Ingenito *et al*., 1991; Keir *et al*., 2002).

In conclusion, we have provided evidence indicating that AMP and CPA induce airway obstruction in sensitised guinea pigs by an adenosine A_1-receptor-dependent mechanism. The inhibition of this response by atropine, capsaicin and bilateral vagotomy suggests the involvement of C-fibres and parasympathetic reflexes in this phenomenon. The role of mast cell-derived histamine in the response of sensitised animals to AMP or CPA is questioned by the fact that mepyramine does not block airways obstruction induced by either AMP or CPA. Furthermore, ovalbumin-induced airways obstruction was not affected by chronic pretreatment with capsaicin but was sensitive to mepyramine, highlighting the different pathways involved in airway obstruction induced by adenosine and antigen in allergic guinea pigs.

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