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Characterization of adenosine receptors involved in adenosine-induced bronchoconstriction in allergic rabbits

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1 Recent work has suggested that adenosine may be involved in asthma via the activation of A_1 receptors. However, the role of the recently cloned A_3 receptor in airways is largely unknown. In the present study, we have investigated the role of the A_3 receptor in adenosine-induced bronchoconstriction in allergic rabbits.

2 Aerosol challenge of antigen (Ag) immunized rabbits with the adenosine precursor, adenosine 5'-monophosphate (AMP), resulted in a dose-dependent fall in dynamic compliance (C_{dyn}). The maximum fall in C_{dyn} in these rabbits was significantly greater than that in litter matched, sham immunized animals ($P<0.05$). However, there was no significant difference in the maximum increase in airways resistance (R_L) between Ag and sham immunized rabbits ($P>0.05$).

3 Aerosol challenge of Ag immunized rabbits with cyclopentyl-adenosine (CPA) (A_1-receptor agonist) elicited a dose-dependent fall in C_{dyn} in Ag immunized rabbits and the maximum fall in C_{dyn} in these rabbits was significantly greater than that observed in sham immunized rabbits ($P<0.05$). Similarly, CPA induced dose-dependent increases in R_L in Ag immunized rabbits whereas sham immunized rabbits failed to respond to CPA within the same dose range. The maximum increase in R_L in Ag immunized rabbits was significantly greater than that of sham immunized rabbits ($P<0.05$).

4 Aerosol challenge of either Ag or sham immunized rabbits with the A_3 agonist aminophenylethyladenosine (APNEA) did not elicit dose-dependent changes in either R_L or C_{dyn}. Moreover, there was no significant difference in the maximum response, measured by either parameter, between the two animal groups ($P>0.05$).

5 These data provide further evidence for a role of the A_1 receptor in the airways, but do not support a role for the A_3 receptor in adenosine-induced bronchoconstriction in the allergic rabbit.

Keywords: Adenosine; airway hyperresponsiveness; immunized; rabbits

Introduction

Asthma is a disease characterized by several features, including (1) airway obstruction, (2) airway inflammation and (3) airway hyperresponsiveness (AHR) to a variety of stimuli (National Asthma Education Program, 1991). Traditionally, AHR has been clinically and experimentally defined as increased airway responsiveness to inhaled histamine or methacholine and asthmatics have been found to be relatively non-selectively hyperresponsive (Woolcock *et al.*, 1984; Sterk *et al.*, 1985). However, this concept is increasingly being challenged as it is now recognized that asthmatics are particularly sensitive to certain agents including bradykinin (Fuller *et al.*, 1987) and adenosine (Cushley *et al.*, 1983). It is therefore of interest to understand the mechanism underlying adenosine-induced effects in the airways.

The evidence for the involvement of the purine nucleoside, adenosine, in asthma is growing. In man, adenosine has been shown to induce bronchoconstriction when inhaled by allergic asthmatics (Cushley *et al.*, 1983). Although allergic non-asthmatics bronchoconstrict when exposed to adenosine, they do so to a lesser degree than atopic asthmatics, and normal subjects show little or no response (Cushley *et al.*, 1983). Dipyridamole, an inhibitor of adenosine uptake, enhances adenosine-induced bronchospasm in asthmatics when administered intravenously or by inhalation (Crimi *et al.*, 1988), an effect that can be inhibited by theophylline, an adenosine receptor antagonist (Cushley *et al.*, 1984). This suggests that there may be increased expression of adenosine receptor(s) in asthmatic airways. In fact, three different classes of adenosine receptor have been characterized so far based on biochemical (Jacobson *et al.*, 1992), functional (Van Calker *et al.*, 1979) and receptor-cloning studies (Zhou *et al.*, 1992). These include the A_1, A_{2A}, A_{2B} and A_3 receptor subtypes. Adenosine interacts with these surface receptors which either inhibit (A_1) adenylyl cyclase (Londos *et al.*, 1980), stimulate (A_{2A} and A_{2B}) adenylyl cyclase (Collis & Hourani, 1993; Gilfillan & Rooney, 1987) or activate (A_3) phospholipase C (Ali *et al.*, 1990). However, the particular subtype responsible for the adenosine-induced bronchoconstriction seen in asthmatics is not yet known.

In an allergic rabbit model, having many similarities with allergic asthma (Herd & Page, 1996), rabbits immunized from birth with antigen have been shown to develop AHR to adenosine (Ali *et al.*, 1994a). Furthermore, it has been found that bronchial smooth muscle strips from these allergic rabbits, unlike those from normals, contract when exposed to adenosine (Ali *et al.*, 1994a). Interestingly, however, sensitized rabbits bronchoconstrict to a greater degree following aerosol challenge with the A_1 receptor agonist cyclopentyladenosine (CPA) than with adenosine suggesting that this subtype of the adenosine receptor may mediate the adenosine-induced bronchoconstriction, or that this receptor subtype is upregulated in allergic rabbits (Ali *et al.*, 1994b). However, the exact mechanisms underlying adenosine-induced bronchoconstriction in asthmatics are unknown. There is evidence to implicate mast cell derived mediators (Meade, 1995) and activation of airway nerves (Crimi *et al.*, 1992; Polosa *et al.*, 1992). However, the role of the recently cloned A_3 receptor has not been considered in the context of the lung, and in the present study we have investigated the role of the A_3 receptor in adenosine-induced bronchoconstriction in allergic rabbits.

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Methods

Animals

New Zealand White (NZW) rabbits of either sex weighing 2.5–4.5 kg at 3 months of age were obtained from Froxfield Farms, Petersfield, Hants and used throughout the study.

Immunisation protocols

Immunisation of neonatal NZW rabbits was performed by modifying (Coyle *et al.*, 1989) a previously described method (Shampain *et al.*, 1982). Allergen – immunised rabbits were injected intraperitoneally (0.5 ml) within 24 h of birth with *Alternaria tenuis* extract (40,000 pnu ml^{-1}) in aluminium hydroxide ($Al(OH)_3$) moist gel (10 mg) and 0.9% sterile saline in the ratio 2:1:1. Concurrently, sham-immunised littermate controls were injected intraperitoneally with $Al(OH)_3$ and saline in the ratio of 1:3. The intraperitoneal administration of allergen and/or adjuvant was repeated weekly for the first month and then biweekly for the following two months.

Pulmonary function measurement

Rabbits were prepared for pulmonary function testing at 3 months of age, 4–7 days after their last intraperitoneal injection of antigen. Premedication of the rabbits with an i.p. injection of diazepam (5 mg kg^{-1}) was followed 15 min later by an i.m. injection of Hypnorm (0.4 mg kg^{-1}, a mixture of fentanyl citrate, 0.315 mg ml^{-1} and fluanisone, 10 mg ml^{-1}). This regime produces a neuroleptic analgesic state and is recommended for recovery procedures in laboratory rabbits (Flecknell, 1987). The animals were placed in a supine position on a padded animal board and intubated with a 3.0 mm i.d. cuffed endotracheal tube. The cuff was inflated and the external end of the tube attached to a heated Fleisch pneumotachograph (size 00).

Flow values were measured by using a Validyne differential pressure transducer and inspiration was taken, by convention, to register a positive flow. The flow was continuously integrated to give an indication of tidal volume. Pleural pressure was estimated by placing a polythene catheter with an attached latex balloon in the lower third of the oesophagus. An index of transpulmonary pressure, the difference between thoracic and pleural pressure, was recorded by another differential pressure transducer connected between the outflow of the oesophageal balloon and the external atmosphere. The position of the balloon was adjusted to obtain the maximum index of transpulmonary pressure, where it remained throughout the experiment. Measurements were made as described previously (Minshall *et al.*, 1993). Total lung resistance (R_L; $cmH_2O\ l^{-1}\ s^{-1}$) and dynamic compliance (C_{dyn}; ml cmH_2O^{-1}) values were calculated by an on-line pulmonary monitoring system (P.M.S. version 5.1; Mumed Ltd., London) according to the method of Von Neergaard and Wirtz (Von Neergaard & Wirtz, 1927). C_{dyn} was obtained by dividing the change in tidal volume by the change in the transpulmonary pressure between the points of zero flow. The contribution made to the total lung resistance (R_L) by the endotracheal tube was negligible at flow rates monitored between 0 and 60 ml min^{-1}.

Experimental protocol

Airway responsiveness to inhaled adenosine analogues Airway responsiveness to adenosine 5'-monophosphate (AMP) was determined by performing a cumulative dose-response curve to inhaled AMP (1.87–120 mg ml^{-1}; aerosol was generated with a Devilbiss ultrasonic nebuliser, particle size 0.5–5 μm). Following each 2 min aerosol of AMP, 10 breaths were recorded and the mean values of R_L and C_{dyn} were calculated.

Airway responsiveness to inhaled CPA Airway responsiveness to CPA was determined by performing a cumulative dose-response curve to inhaled CPA (0.078–10 mg ml^{-1}; aerosol was generated with a jet nebulizer, turbo turret, particle size 0.3–3 μm). Again following each 2 min aerosol of CPA, 10 breaths were recorded and the mean values of R_L and C_{dyn} were calculated.

Airway responsiveness to inhaled APNEA Airway responsiveness to aminophenylethyladenosine (APNEA) was determined by performing a cumulative dose-response curve to inhaled APNEA (0.126–4 mg ml^{-1}, aerosol was generated with a jet nebulizer, turbo turret particle size 0.3–3 μm). Again following each 2 min aerosol of APNEA, 10 breaths were recorded and the mean values of R_L and C_{dyn} were calculated.

DPCPX challenge

Ten minutes before challenge with the A_3 agonist APNEA, the relatively selective A_1 antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (10^{-4} M in 100% dimethylsulphoxide (DMSO)) was aerosolized for 2 min with the turbo turret nebulizer in order to eliminate A_1 receptor involvement. Pulmonary function was recorded before and after pretreatment with DPCPX to determine its effect on basal airway tone.

Two different nebulizers were used for AMP and the other adenosine agonists because different solvents were used to dissolve these compounds (i.e. saline was used to dissolve AMP whereas dimethylsulphoxide (DMSO) and ethanol were used to dissolve the adenosine agonists) which led to differences in the viscosity in the resulting solutions.

Drugs and chemicals

All reagents were of analytical grade. Drugs and chemicals used were: AMP (Sigma chemical Co., Poole, Dorset); DPCPX, APNEA and CPA (Semat Technical [UK] Ltd, St. Albans, Hertfordshire); diazepam (Roche Products Ltd, Welwyn Garden City, Hertfordshire); Hypnorm (a mixture of fentanyl citrate, 0.315 mg ml^{-1}, and fluanisone, 10 mg ml^{-1}; Janssen Pharmaceutical Ltd., Grove, Oxfordshire); sterile pyrogen-free 0.9% sodium chloride solution (saline; Baxter Healthcare Ltd., Thetford, Norfolk). Stock solutions of AMP were made up in 0.9% saline, those of APNEA and CPA were made up in 50% DMSO in water and 50% ethanol, respectively. With the exception of DPCPX, which was dissolved in 100% DMSO, all subsequent dilutions of drugs were made in 0.9% sterile saline.

Data expression and analysis

Airway responsiveness to inhaled AMP and related compounds have been expressed as the percentage change in R_L and C_{dyn} from baseline values in response to increasing doses of the inhaled compounds. The maximum percentage increase in R_L and decrease in C_{dyn} within the studied dose range was recorded and used as an index of airway responsiveness. Unpaired Student's t test was used throughout to test the significance of our observations.

Results

Effect of AMP on lung function

Aerosol challenge of both Ag and sham immunized rabbits with AMP did not elicit significant changes in R_L (Figure 1a) although aerosol challenge with AMP elicited a dose-dependent decrease in C_{dyn} in Ag and sham immunized rabbits (Figure 1b).

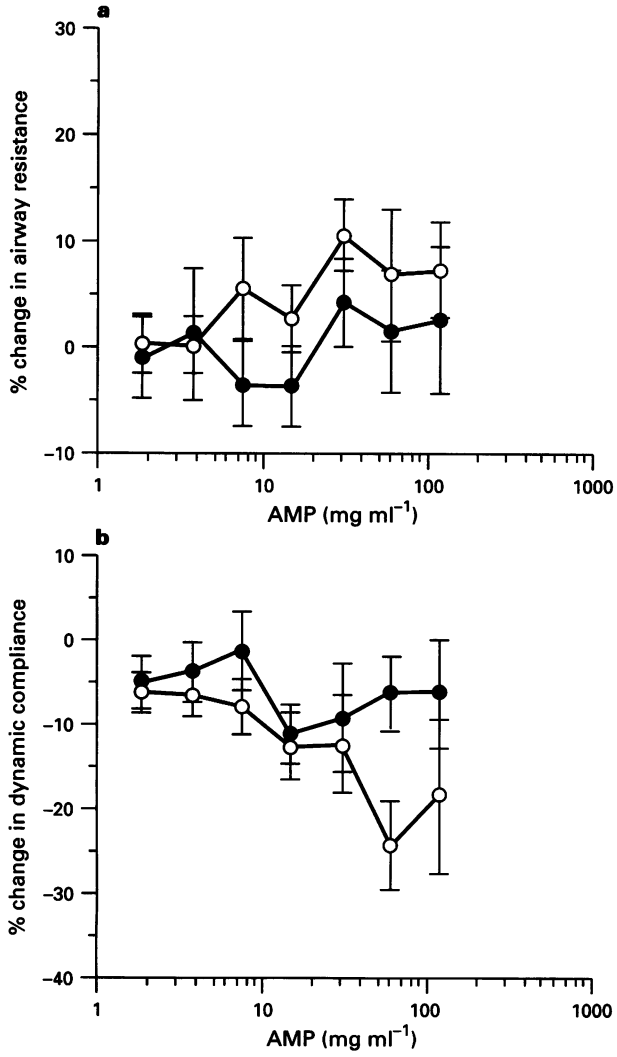

Figure 1 Changes in (a) airway resistance and (b) dynamic compliance from baseline values were measured following inhalation of increasing concentrations of AMP (1.87–120 mg ml^{-1}). Responses were measured in Ag immunized (○, $n=8$) and sham immunized rabbits (●, $n=8$) and have been plotted as % change against concentration of AMP (log scale); vertical lines show s.e.mean.

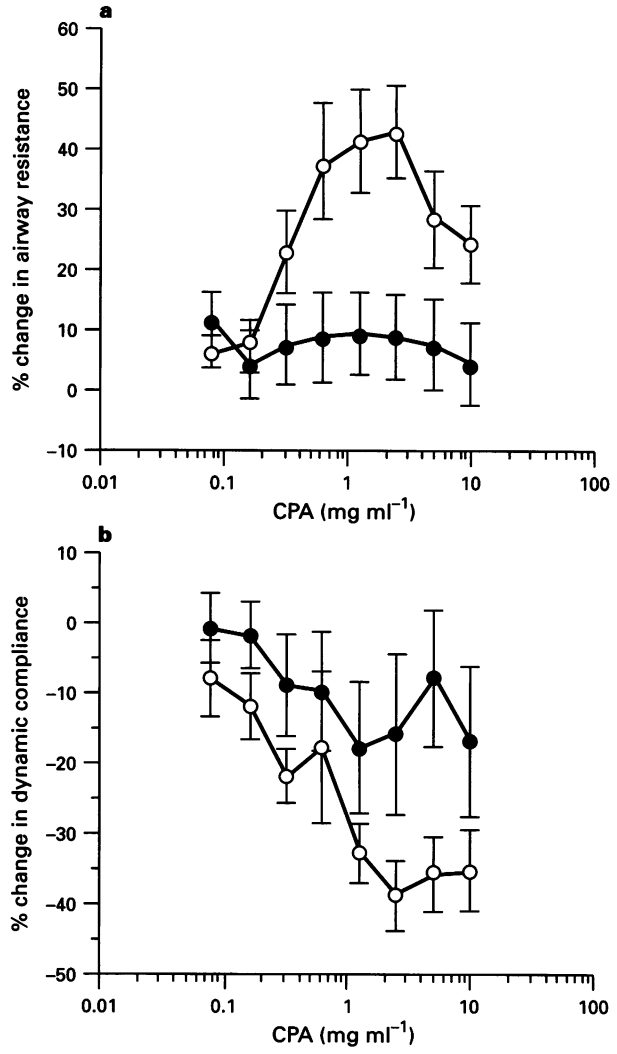

Figure 2 Change in (a) airway resistance and (b) dynamic compliance from baseline values were measured following inhalation of increasing concentration of cyclopentyl-adenosine (CPA, 0.078–10 mg ml^{-1}). Responses were measured in Ag immunized (○, $n=8$) and sham immunized rabbits (●, $n=8$) and have been plotted as % change against concentration of CPA (log scale); vertical lines show s.e.mean.

Effect of CPA on lung function

Aerosol challenge with CPA elicited a dose-dependent increase in R_L in Ag immunized rabbits. However, sham immunized rabbits did not respond to CPA at the same doses (Figure 2a). Aerosol challenge with CPA elicited a dose-dependent fall in C_{dyn} in both Ag and sham immunized rabbits (Figure 2b).

Effect of APNEA on lung function

Aerosol challenge with APNEA did not elicit a dose-dependent increase in R_L in either group (Figure 3a). Similarly, aerosol challenge with APNEA did not elicit a dose-dependent fall in C_{dyn} in either animal group (Figure 3b).

Maximum changes in R_L induced by AMP, CPA and APNEA

Aerosol challenge with AMP did not elicit maximum changes in R_L in either Ag or sham immunized rabbits that were significantly different (Figure 4a). CPA induced a maximum increase in R_L in Ag immunized rabbits that was significantly greater than that induced in sham immunized rabbits ($P<0.05$) and was significantly greater than changes induced by either AMP ($P<0.05$) or APNEA ($P<0.05$) in both animal groups. Furthermore, APNEA did not elicit maximum changes in R_L in either Ag or sham immunized rabbits that were significantly different (Figure 4a).

Maximum changes in C_{dyn} induced by AMP, CPA and APNEA

Aerosol challenge of Ag immunized rabbits with AMP induced a maximum fall in C_{dyn} that was significantly greater than that induced in sham immunized rabbits ($P<0.05$) (Figure 4b). Furthermore, the CPA induced maximum fall in C_{dyn} was significantly greater than that induced in sham immunized rabbits ($P<0.05$) and was also significantly greater than that induced by APNEA ($P<0.05$) but not AMP ($P>0.05$). Challenge with APNEA did not result in maximum changes in C_{dyn} in the two groups that were significantly different.

There was no significant difference in baseline values of both R_L and C_{dyn} between the different rabbit batches.

Discussion

Our results show that the non-selective adenosine receptor agonist AMP induced bronchoconstriction in Ag immunized but not in sham immunized rabbits. This finding is consistent

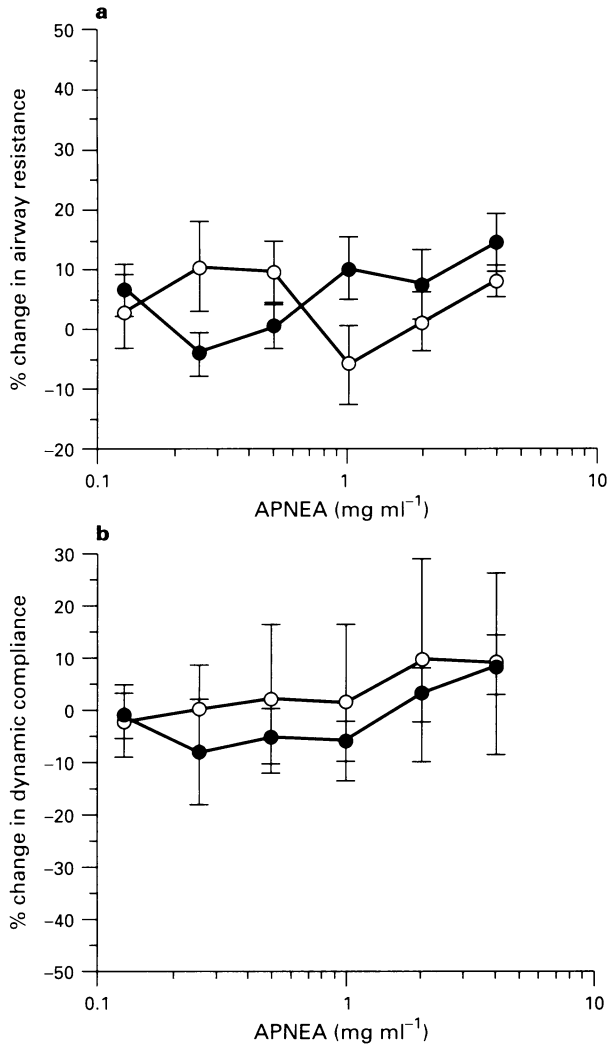

Figure 3 Change in (a) airway resistance and (b) dynamic compliance from baseline values were measured following inhalation of increasing concentration of aminophenylethyladenosine (APNEA, 0.126–4 mg ml^{-1}). Responses were measured in Ag immunized (○, $n=6$) and sham immunized rabbits (●, $n=6$) and have been plotted as % change against concentration of APNEA (log scale); vertical lines show s.e.mean.

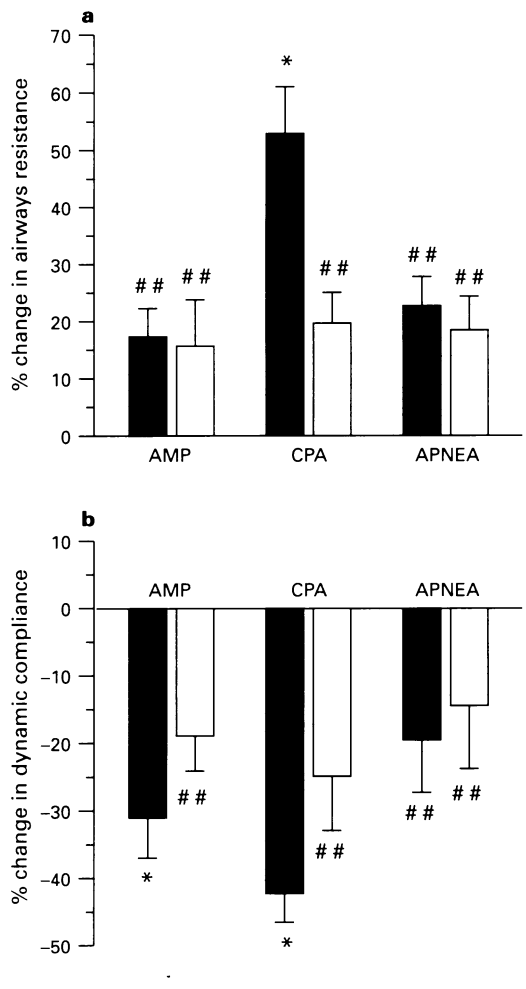

Figure 4 Maximum changes in (a) airway resistance and (b) dynamic compliance induced by AMP, cyclopentyl adenosine (CPA) and aminophenylethyladenosine (APNEA) in Ag (■)) and sham (□) immunized rabbits were calculated. Mean ± s.e.mean values are shown ($n=6-8$). *$P<0.05$ compared with corresponding sham immunized group; ##$P<0.05$ compared with CPA Ag immunized group.

with previous studies undertaken in our laboratory (Minshall *et al.*, 1996). Furthermore, the A_1 agonist CPA but not the A_3 agonist APNEA induced bronchoconstriction in Ag immunized rabbits. These results suggest that A_1 and not A_3 receptors are involved in adenosine-induced bronchoconstriction in allergic rabbits.

The recent surge in interest concerning the role of adenosine in asthma has stemmed from the observation that the inhalation of adenosine or its more soluble precursor, AMP, causes a significant degree of bronchoconstriction in asthmatics, whereas non-asthmatics consistently fail to bronchoconstrict (Cushley *et al.*, 1983). This all or nothing response is in contrast to observations with the majority of other substances commonly used to assess airway responsiveness in asthmatics (e.g. methacholine or histamine), where both asthmatics and normals respond, although to differing degrees (Cockcroft *et al.*, 1977; Sterk & Bel, 1991).

Our data show that AMP induced a concentration-dependent fall in dynamic compliance in Ag immunized rabbits. Moreover, the maximum % fall in dynamic compliance in Ag immunized rabbits was significantly greater than that of sham immunized rabbits. However, there was no significant difference in terms of changes in R_L. This may be due to the fact that AMP being unselective is not potent enough to significantly alter flow in the larger airways whilst having the ability to alter flow in smaller airways, reflected by changes in R_L and C_{dyn}, respectively. Moreover, the A_1 receptor agonist (CPA) elicited dose-dependent increases in airway resistance and parallel decreases in dynamic compliance. Furthermore, airway responsiveness to CPA in Ag immunized rabbits was significantly greater than that observed in sham immunized rabbits.

The mechanisms whereby adenosine induces bronchoconstriction are not completely understood. Both direct and indirect modes of action have been suggested. One possible mechanism of action for adenosine-induced bronchoconstriction may be through direct effects on airway smooth muscle. Evidence shows that guinea-pig tracheal smooth muscle preparations *in vitro* demonstrate a transient contraction to low doses of adenosine (Lunblad & Persson, 1988), which is followed by a more powerful and sustained relaxation of the precontracted tissue at higher doses of adenosine (Brown & Collis, 1982). Furthermore, preparations of guinea-pig whole isolated perfused lung have been shown to contract inconsistently in response to adenosine. However, these contractions are greatly enhanced by prior sensitization of the animal to ovalbumin (Thorne & Broadley, 1988). It is possible, therefore, that failure of adenosine to induce a clear contraction in these tissues is due to the fact that normal tissue had been used i.e. tissue

lacking the allergic state. This seems likely, as the situation is somewhat similar in human airway tissue where adenosine induces weak contraction of bronchi from normal humans (Finney *et al.*, 1985). However, in bronchi obtained from asthmatic patients, adenosine induces a dose-dependent contraction of the muscle (Bjorck *et al.*, 1992). Moreover, a recent study has shown that tracheal strips from rabbits immunized with house dust mite are more responsive to adenosine than tracheal strips isolated from naive rabbits (Ali *et al.*, 1994a). In addition, the peripheral airways from these rabbits exhibit greater sensitivity to CPA than central (tracheal) airways which corroborates our *in vivo* data where changes in C_{dyn} in immunized rabbits challenged with AMP and CPA were more marked than changes in R_L.

Adenosine may mediate bronchoconstriction indirectly, secondary to activation of mast cells. Adenosine has been shown to induce a marked histamine release from peritoneal mast cells (Church *et al.*, 1986). In human lung mast cells and peripheral blood basophils, AMP can enhance histamine release provided that the nucleoside is added to the cell preparation following application of the secretory stimulus (Church *et al.*, 1983; Hughes *et al.*, 1984). There is also evidence to suggest that the A_{2b} receptor may be involved in adenosine induced histamine from human mast cells and, interestingly, this mechanism seems to be sensitive to enprofylline (Feoktistov & Biaggioni, 1995). More direct evidence for the involvement of histamine has been provided by studies demonstrating an increase in histamine plasma levels concomittant with the onset of bronchoconstriction following the inhalation of AMP in atopic subjects (Phillips *et al.*, 1990). Recently, it has also been shown that local instillation of AMP into asthmatic airways results in the release of histamine (Polosa *et al.*, 1995). Moreover, so-called mast cell stabilizers, such as sodium cromoglycate and nedocromil sodium, are capable of suppressing mast cell mediator release, protecting atopic and non-atopic asthmatic subjects against bronchoconstriction induced by inhaled AMP and adenosine (Polosa *et al.*, 1989). However, this cannot be taken as proof of mast cell involvement because these drugs possess a wide variety of other pharmacological actions including inhibitory activities on neutrophils and eosinophils as well as suppression of C-fibre neural function in the lung (Norris, 1995).

Another possible explanation for the mechanism of action of adenosine is that it may, at least in part, provoke a reflex bronchoconstriction. The modulator effects of adenine nucleotides and nucleosides on synaptic transmission are well established (Ginsborg & Hirst, 1972). However, it has been found that, in airway tissues, adenosine has no effect on cholinergic-induced contractile responses of fresh, *post mortem*, specimens of human tracheal muscle (Bai *et al*, 1989) or guinea-pig trachea (Grundstrom *et al.*, 1981) to electrical field stimulation. In contrast, in rabbit bronchial smooth muscle, it has been demonstrated that adenosine enhances the constrictor response to transmural nerve stimulation (Gustafsson *et al.*, 1986). Moreover, data from *in vivo* studies are also inconclusive. Some have shown that muscarinic blockade with ipratropium bromide failed to affect greatly the airway responses to inhaled adenosine (Mann *et al.*, 1985). However, other groups (Okayama *et al.*, 1986; Crimi *et al.*, 1992) have obtained a significant attenuation of adenosine-induced bronchoconstriction in asthmatics following administration of atropine or ipratropium bromide, respectively. Furthermore, it has been suggested that the mechanism of action of AMP - induced bronchoconstriction may be through the activation of sensory C (non-myelinated) fibres (Polosa *et al.*, 1992).

Recent studies have suggested that the bronchoconstrictor effects of non-selective adenosine analogues in the allergic rabbit model are mediated primarily by the A_1 receptor (Ali *et al.*, 1994a). However, the role of the A_3 adenosine receptor in adenosine-induced bronchoconstriction has not been studied formally. This receptor was first described in a cultured mast line cell line (RBL-2H3) as a novel adenosine receptor with features distinct from those of the classical A_1 and A_2 receptors (Ribeiro & Sebastiao, 1994). The receptor does not mediate the activation of adenylyl cyclase, but instead activates phospholipase C via a G protein, inducing transient production of inositol phosphates and transient elevation of the concentration of cytosolic calcium (Ca^{2+}) (Ali *et al.*, 1990).

In the present experiments, we found that aerosol challenge with APNEA did not result in airway responses in either sham or neonatally immunized rabbits. In contrast, others have shown that i.v. administration of APNEA to spontaneously breathing BDE-type rats increased R_L (Meade, 1995). Furthermore, in the rat this effect seems to be mediated through the mast cell as agents that either deplete or stabilize mast cells significantly reduce APNEA-induced increases in R_L (Meade, 1995), supporting the findings of others that incubation with APNEA facilitates release of allergic mediators from mast cells (Ramkumar *et al.*, 1993). Furthermore, an NK_2 receptor antagonist (L-659877) was shown to inhibit APNEA-induced bronchospasm suggesting a link between A_3 receptors and sensory nerves in this model (Meade, 1995). Interestingly, in confirmation of our findings, it has also been shown that APNEA lacked any effects on either R_L or C_{dyn} in rats, although APNEA did elicit hypotensive effects mediated through the activation of A_3 receptors on mast cells (Hannon *et al.*, 1995; Fozard *et al.*, 1996). The inability of APNEA to elicit a response in our allergic rabbit model in terms of airway changes may suggest that this receptor subtype is either not expressed in rabbit airways or, if expressed, does not mediate bronchoconstriction.

Our results show that challenge of immunized rabbits with CPA elicited dose-dependent increases in airway resistance and parallel decreases in dynamic compliance. Furthermore, airway responsiveness to CPA in Ag immunized rabbits was significantly greater than that observed in sham immunized rabbits. These results confirm a previous finding where immunized and non-immunized rabbits were shown to differ significantly in terms of their responsiveness to CPA, suggesting perhaps that immunization may upregulate the A_1 receptor (Ali *et al.*, 1994b). Our data also suggest that CPA may be a useful marker of airway responsiveness.

In conclusion, our data do not support a role for A_3 receptors in adenosine-induced bronchoconstriction but emphasize the role of A_1 receptors in this regard.

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