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Pharmacological characterisation of the adenosine receptor mediating increased ion transport in the mouse isolated trachea and the effect of allergen challenge

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1 The effect of adenosine on transepithelial ion transport was investigated in isolated preparations of murine trachea mounted in Ussing chambers. The possible regulation of adenosine receptors in an established model of allergic airway inflammation was also investigated.

2 Mucosally applied adenosine caused increases in short-circuit current (I_{SC}) that corresponded to approximately 50% of the response to the most efficacious secretogogue, ATP (ΔI_{SC} $69.5 \pm 6.7\,\mu A\,cm^2$). In contrast, submucosally applied adenosine caused only small (<20%) increases in I_{SC}, which were not investigated further.

3 The A_1-selective (N^6-cyclopentyladenosine, CPA, 1 nM–10 μM), A_{2A}-selective (2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxoamido adenosine; CGS 21680; 0.1–100 μM) and A_3-selective (1-deoxy-1-[6-[[(3-iodophenyl)-methyl]amino]-9H-purin-9-yl]-*N*-methyl-β-D-ribofuranuronamide; IB-MECA; 30 nM–100 μM) adenosine receptor agonists were either equipotent or less potent than adenosine, suggesting that these receptors do not mediate the response to adenosine.

4 The A_1 receptor selective antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 10 nM–1 μM) caused a rightward shift of the adenosine concentration–effect curve only at 1 μM. The mixed A_{2A}/A_{2B} receptor antagonist 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM 241385) also caused rightward shift of the adenosine concentration–effect curve, again only at micromolar concentrations, suggestive of the involvement of A_{2B} receptors.

5 In preparations from animals sensitised to ovalbumin and challenged over 3 days with aerosol ovalbumin, a decrease in baseline I_{SC} was observed and responses to ATP were diminished. Similarly, the amplitude of responses to adenosine were attenuated although there was no change in potency.

6 These results suggest that the A_{2B} receptor mediates the I_{SC} response to adenosine in the mouse trachea. This receptor does not appear to be regulated in a standard asthma model.
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Abbreviations: CPA, N^6-cyclopentyladenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; B-MECA, 1-deoxy-1-[6-[[(3-iodophenyl)-methyl]amino]-9H-purin-9-yl]-*N*-methyl-β-D-ribofuranuronamide; NECA, 5′-*N*-ethyl-carboxamidoadenosine

Introduction

Extracellular purine nucleotides and nucleosides signal *via* two families of receptors, adenosine (P1) receptors and P2 receptors. The two families can be distinguished pharmacologically by the purines that activate them (Ralevic & Burnstock, 1998; Bucheimer & Linden, 2004). Thus, adenosine acts primarily at adenosine (A_1, A_{2A}, A_{2B}, A_3) receptors, whereas other purines such as ATP, ADP and UDP potently activate P2 receptors. P2 receptors are further divided into G-protein-coupled P2Y receptors and P2X receptors, which are ligand-gated ion channels (Ralevic & Burnstock, 1998; Bucheimer & Linden, 2004). Purinoceptors have been identified at most epithelial surfaces and modulate epithelial ion transport in the gastrointestinal system, kidney and lung (Bucheimer & Linden, 2004). ATP has been demonstrated to increase chloride conductance by the airway epithelium *via* $P2Y_2$ receptors (Cressman *et al*., 1999; Kellerman *et al*., 2002; Bucheimer & Linden, 2004). Release of ATP by airway epithelial cells has also been demonstrated, suggesting that ATP may act in an autocrine or paracrine manner (Homolya *et al*., 2000).

Adenosine is the breakdown product of ATP *via* endogenous ecto-ATPases and is also present at cell surfaces in cultured airway epithelial cells (Lazarowski *et al*., 2004). However, the pharmacology of the effect of adenosine on ion transport by the epithelium of the lower airways has rarely been investigated. Adenosine has been demonstrated to increase chloride secretion in human primary cultures of nasal epithelium as well as in transformed and other epithelium-like cell lines (Lazarowski *et al*., 1992; Cobb *et al*., 2002). Although the effect of adenosine on cultured human lower airway epithelial cells has been demonstrated previously (Lazarowski *et al*., 2004), a thorough pharmacological analysis has not been

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performed. Furthermore, data from freshly isolated preparations are completely lacking. Thus, the primary aim of the present study was to determine which receptor(s) mediates ion transport changes in the mouse isolated trachea, a species now widely used in respiratory research.

Adenosine is also an interesting mediator as it is a provocative challenge that selectively causes bronchoconstriction in asthmatics only (Barnes *et al*., 1998). There has been interest in adenosine and regulation of adenosine receptors in asthmatics and animal models of the disease (El-Hashim *et al*., 1999; Fozard *et al*., 2002), including those in mice (Fan *et al*., 2003). Therefore, a second aim of the present study was to establish if the effect of adenosine on ion transport was affected by allergic sensitisation and challenge.

Methods

Animals and experimental procedure

All experiments were carried out under The Animals (Scientific Procedures) Act, 1986. Female BALB/c mice (Harlan, Bichester, Oxon., U.K.), 6–8 weeks old were killed with an overdose of urethane (20 g kg^{-1} i.p.) and the entire trachea was removed and opened longitudinally along the dorsal surface to form a flat sheet. This sheet was then mounted between two perspex Ussing chambers (with a surface area of 0.06 cm^2), which contained a modified Krebs solution (composition (mM): NaCl 118; KCl 5.4; $MgSO_4$ 0.57; glucose 11; KH_2PO_4 1.2; $NaHCO_3$ 25; $CaCl_2$ 2.5), which was maintained at 37°C and continuously bubbled with 95% O_2/5% CO_2. Current and voltage electrodes (containing 4% agar in 2 M KCl; World Precision Instruments; Stevenage, Herts., U.K.) attached to the chambers were connected to an amplifier (DVC 1000, World Precision Instruments), which was then used to voltage clamp the tissue at 0 mV. Changes in short circuit current (I_{SC}) were used as a measure of the effect of adenosine and other drugs on the preparation.

After a 30 min equilibration period, the maximum secretory response of each preparation was determined by adding ATP (100 μM; lumenal side), which from preliminary experiments was found to be the most efficacious secretogogue in this preparation. The peak response to maximum concentrations of ATP (100 μM) in preliminary experiments was 69.5 ± 6.7 $\mu A\,cm^{-2}$, greater than substance P (1 μM; 45.7 ± 4.3), bradykinin (1 μM; 13.2 ± 2.2) and adenosine (100 μM; 38.8 ± 5.2; all luminal, $n = 7$ for each). All responses to adenosine and other agonists are expressed as a percentage of this maximal ATP response. After washout of ATP, the preparations were allowed to equilibrate for a further 30 min before the addition of agonists. Preliminary experiments established that adenosine caused monophasic increases in I_{SC}, which reached a plateau that was steady for >30 s, allowing cumulative concentration–effect curves to be established. The symmetry of these curves did not indicate that receptor desensitisation was occurring during agonist addition. However, in some preparations a second curve, repeated up to an hour after the initial curve was depressed or slightly right-shifted. Therefore, for the purposes of this study only one concentration–effect curve was performed on each tissue. Where the effects of antagonists were examined, these were added after the washout of ATP.

Allergic sensitisation and challenge

On day 1, mice were sensitised with an i.p. injection of 10 μg chicken egg ovalbumin (Sigma, Poole, Dorset, U.K.) per mouse in 0.1 M $Al_2(OH)_3$ adjuvant in saline and boosted in the same way 7 days later. Control mice were sham sensitised and boosted with alum/saline only. On days 14–17, the animals were exposed to a 30 min aerosol challenge of ovalbumin (1 mg ml^{-1}). This protocol consistently produces lung eosinophilia and airway hyper-responsiveness in this laboratory (Riffo-Vasquez *et al*., 2000; Pitchford *et al*., 2004). Tissues were isolated for study on day 18 as described above.

Statistics

Concentration–effect curves were fitted by nonlinear regression using the computer programme Graphpad Prism (Version 2.01; Graphpad Software, San Diego, CA, U.S.A.) to determine pEC_{50} values. Statistical differences between these values were assessed using one-way analysis of variance with Newman–Keuls post-test. Student's *t*-tests were used to compare differences between control and immunised animals. A *P*-value of less than 0.05 was considered significant.

Drugs

Adenosine hemisulphate, ATP, N^6-cyclopentyladenosine (CPA) and 5′-*N*-ethyl-carboxamidoadenosine (NECA) were purchased from Sigma. 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxoamido adenosine hydrochloride (CGS 21680), 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM 241385), 1-deoxy-1-[6-[[(3-iodophenyl)-methyl]amino]-9H-purin-9-yl]-*N*-methyl-β-D-ribofuranuronamide (IB-MECA) and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) were purchased from Tocris Cookson (Avonmouth, Bristol, U.K.).

Adenosine and NECA were prepared as 100 mM solutions in distilled water. All other drugs were prepared as 100 mM stocks in DMSO and subsequently diluted in water, with the exception of IB-MECA, which was diluted in 50% DMSO to 1 mM and subsequently in distilled water. The volumes of DMSO added to the bath did not alone cause significant changes in I_{SC}.

Results

Effect of adenosine on I_{SC}

In four preparations, the effect of adenosine on I_{SC} when applied to the adventitial side of the preparation was examined. Three preparations responded with small (range 13–18% of ATP) increases in I_{SC} with a pEC_{50} of 4.68 ± 0.22 (Figure 1a). These small responses were not investigated further.

Adenosine caused increases in I_{SC} reaching a maximum of approximately 50% of ATP in all preparations studied when added to the lumenal surface of the preparation (Figure 1b). With the exception of CGS 21680, all adenosine analogues tested caused I_{SC} changes with similar efficacy, although potencies varied (Figure 1b; Table 1). The nonselective adenosine receptor agonist NECA was the most potent, the

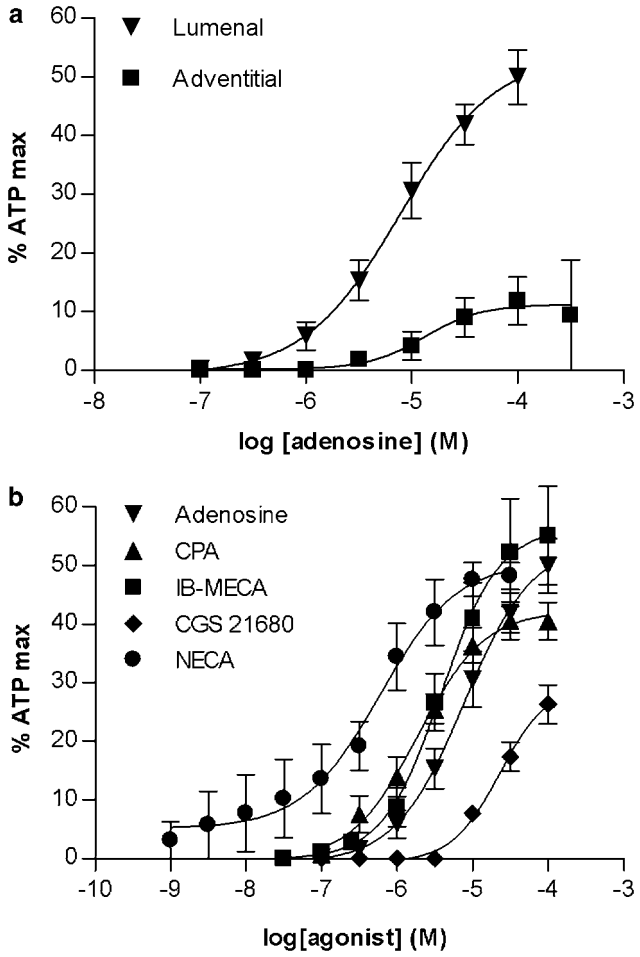

Figure 1 (a) Comparison of the effect of cumulatively added adenosine on I_{SC} when applied to the lumenal or adventitial surface of the isolated mouse trachea ($n=4$–5). Responses have been normalised to the response of the tissue to ATP, the most efficacious secretogogue in this preparation. (b) Comparison of several mucosally applied adenosine analogues in this assay ($n=4$–6). The concentration–effect curve for adenosine is reproduced from panel (a) in panel (b).

Table 1 pEC$_{50}$ and maximum response values for adenosine and adenosine analogues in the Ussing chamber assay

Compound	p*EC$_{50}$*	*% ATP max*	n
Adenosine	5.11±0.2	53±7	6
NECA	6.24±0.3	51±7	4[a]
CPA	5.73±0.1	47±3	4
IB-MECA	5.42±0.2	56±6	4
CGS 21680	4.63±0.2	30±5	4[a]

[a]Significantly different to the potency of adenosine, $P<0.05$ (one-way analysis of variance with Newman–Kuels post-test)

A_1 selective agonist CGS 21680 the least potent, while IB-MECA and CPA had similar potencies to adenosine. Thus, the rank order of potencies for adenosine and adenosine analogues in this preparation is NECA > adenosine = CPA = IB-MECA > CGS 21680.

Effects of adenosine receptor antagonists

The A_1-selective antagonist DPCPX (10 nM–1 μM) caused a rightward shift of the adenosine concentration–effect curve only at the highest concentration tested (Figure 2a). The A_{2A}-preferring, mixed A_{2A}/A_{2B} antagonist ZM 241385 (0.3–10 μM) shifted the adenosine concentration–effect curve to the right with an apparent pA_2 of 7.2 (Figure 2b and c). Neither DPCPX nor ZM 241385 altered baseline I_{SC} at concentrations that affected the adenosine concentration–effect relationship.

Effect of allergen challenge on responses to adenosine

Preparations from allergic animals had a significantly lower baseline I_{SC} (Figure 3a) and produced smaller responses to ATP (Figure 3b), suggesting that secretion was impaired. There was no difference in the potency of adenosine between sensitised ($pEC_{50}=5.10\pm0.26$) and sham-sensitised ($pEC_{50}=5.26\pm0.15$) mice, and when normalised to the response to ATP, there was no difference in efficacy (Figure 3c).

Discussion and conclusions

The present study confirms that, like ATP, adenosine is a secretogogue in the normal, freshly isolated airway epithelium. This response appears to be mediated by adenosine receptors of the A_{2B} subtype, based on the potencies both of agonists and antagonists for these receptors. However, there does not appear to be any significant regulation of this receptor in an acute model of allergic airway disease.

Adenosine can act at four distinct receptor subtypes: A_1, A_{2A}, A_{2B} and A_3. Agonists with known increased potency relative to adenosine (Klotz, 2000) at A_1 (CPA), A_{2A} (CGS 21680) and A_3 (IB-MECA) were either equipotent or less potent than adenosine, suggesting that none of these receptors mediate the action of adenosine in this preparation. Indeed the rank order of agonists (NECA > Adenosine = CPA = IB-MECA > CGS 21680) is similar to that reported for A_{2B} receptors in other tissues such as human coronary arteries (Kemp & Cocks, 1999) and rat mesenteric artery (Prentice *et al*., 1997), as well as in receptor expression systems (Patel *et al*., 2003). The results of studies using relatively selective receptor antagonists provide further evidence that A_{2B} receptors mediate the response to adenosine. Thus, the A_1 selective antagonist DPCPX, which antagonises A_1 receptors in the nanomolar range (e.g. pA_2 9.3–9.7 in the rat duodenum; Nicholls & Hourani, 1997; see also Klotz, 2000), caused rightward shifts of the adenosine concentration–effect curve only at micromolar concentrations, in agreement with studies of A_{2B} receptors in other systems (Fozard *et al*., 2003). Similarly, the A_{2A}-selective antagonist ZM 241385 caused rightward shift of the adenosine concentration–effect curve only at micomolar concentrations with a pA_2 of 7.2, consistent with the known potency of this compound at A_{2B} receptors in several species (Poucher *et al*., 1995; Ongini *et al*., 1999; Fozard *et al*., 2003). Therefore, both agonist and antagonist studies support a role for A_{2B} in the mouse tracheal epithelium. However, the effect of ZM 241385 at high concentrations, where increased concentrations of this antagonist appeared to fail to further right-shift the adenosine concentration–effect

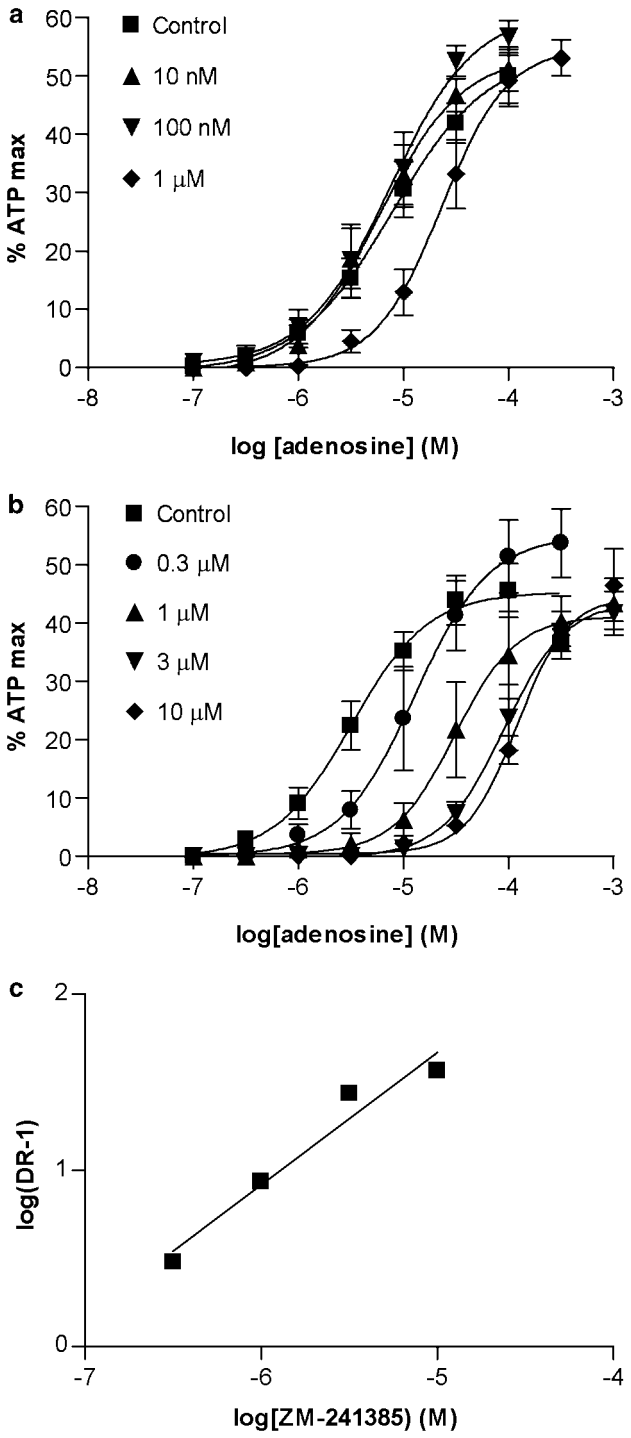

Figure 2 Effect of adenosine receptor antagonists on cumulative concentration–effect curves for adenosine in the mouse trachea. (a) The A_1-selective antagonist DPCPX had no effect on the concentration–effect relationship at concentrations less than 1 μM ($n=3$–6). (b) Dose-dependent rightward shift of the adenosine concentration-effect curve by the A_{2A}/A_{2B} antagonist ZM 241385 ($n=4$–5). (c) Schild analysis of the effect of ZM 241385 on the adenosine concentration–effect curve, demonstrating a slope close to unity. The pA_2 derived from this analysis was 7.2.

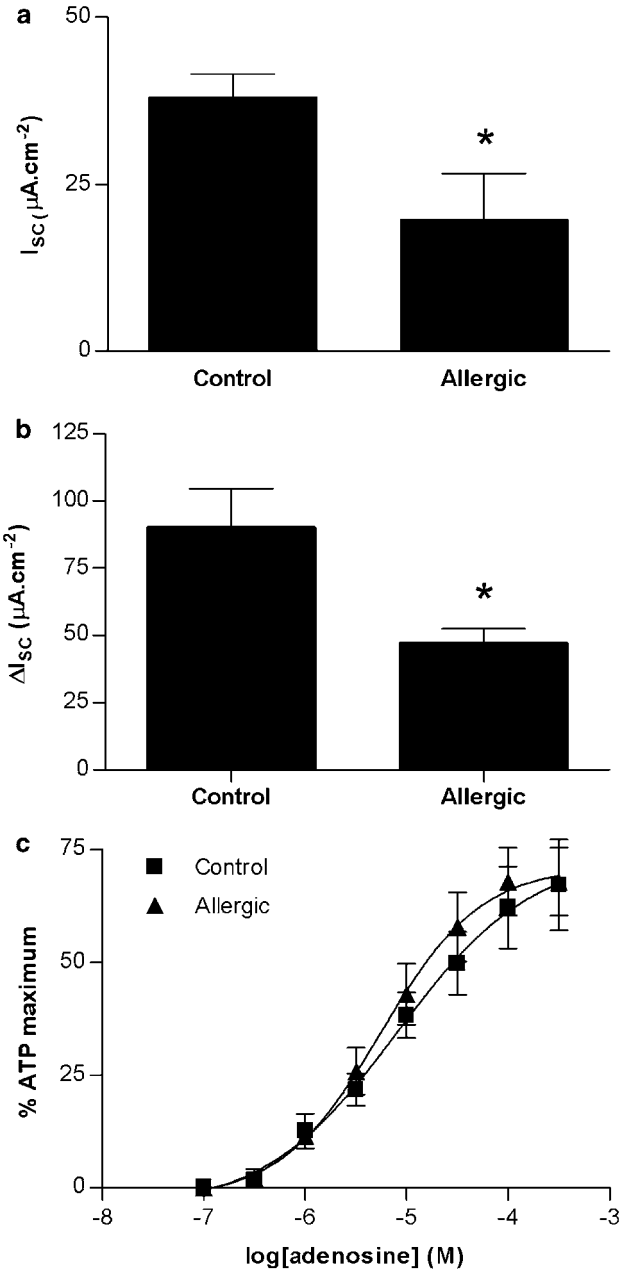

Figure 3 Effect of allergic sensitisation and 3 days of aerosol challenge on the I_{SC} response of the murine trachea to adenosine. (a) Unstimulated I_{SC} was significantly lower in the allergic compared with sham-sensitised controls ($n=5$ each). (b) The increase in I_{SC} following ATP addition (100 μM) was also significantly attenuated in the allergic group ($n=5$ each). (c) Normalised to the response to ATP, there was no difference in the potency or efficacy of the response to adenosine between the two groups ($n=5$ each). *Indicates a significant difference ($P<0.05$) between the sham and allergic groups (unpaired t-test).

curve, may indicate the presence of a low affinity, non-A_{2B}-binding site. Nevertheless, the predominant adenosine receptor in this preparation appears to be a A_{2B}, and as cultured human bronchial epithelial cells have been suggested to express A_{2B} receptors coupled to ion secretion (Lazarowski *et al*., 2004), mice may be a useful model for *in vivo* experimentation in this area.

Inhaled adenosine selectively causes bronchoconstriction only in asthmatics and adenosine receptors have been implicated in asthma and are a potential target for future therapies (Barnes *et al*., 1998; Fozard, 2003). Airways from

asthmatics have increased numbers of mucus-secreting cells, and in fatal asthma mucus is frequently found to be plugging the airways (Jeffery, 2003; Cohn *et al*., 2004). Since ion transport by the epithelium maintains the sol layer upon which mucus is moved by ciliated epithelial cells, any dysfunction in ion transport would impact on mucus clearance (Boucher, 2003). In this and a previous study (Cloutier *et al*., 2004), aerosol challenge of sensitised mice with allergen resulted in a decreased baseline I_{SC} and blunted responses to secretogogues. Whether this is a feature of the human disease does not appear to have been investigated, despite the important role of ion transport in the functioning of the mucocilliary escalator that must cope with an increased mucous burden in asthma. In a recent study, nasal epithelium was used as a surrogate for bronchial epithelial secretory responsiveness, and no difference between control and mild asthmatics was observed (Chung *et al*., 2003). Further studies with cells isolated from bronchial biopsies might reveal more detail about possible dysfunction of ion transport in asthmatics. In culture, two important cytokines that are involved in the allergic response, IL-4 (Galietta *et al*., 2002) and IL-13 (Danahay *et al*., 2002), cause a hypersecretory phenotype in human airway epithelial cells and both of these cytokines have been repeatedly shown to be present and important in conventional acute murine allergic models (Elias *et al*., 2003) where we observed a hyposecretory effect of allergen challenge. Furthermore, in the intestine during an immune response to nematode infection in mice, in addition to hypersecretion due to reduced glucose transport, responses to secretory agonists are impaired (Madden *et al*., 2004), suggesting that alterations in ion transport may be a common feature of mucosal surfaces following antigen challenge. It will be important to determine whether the opposite effects of these cytokines on ion transport between murine and human cells is due to a species difference or the experimental conditions (*in vitro* vs *in vivo*).

Of concern to the present studies is how to interpret the effect of the allergic response on the response to adenosine when baseline and stimulated I_{SC} appears to be downregulated in the allergic animals. As raw I_{SC} measurements, the response to adenosine is significantly blunted, yet if the data are normalised to the response to ATP – as we have presented them – then there is no difference between the maxima of the curves. Irrespective of whether the data are normalised or not, the potency of adenosine remained unchanged. Thus, while whole lung A_{2B} receptor mRNA expression increases in murine asthma models, and A_{2B} receptors may contribute to bronchomotor responses to adenosine in allergic mice (Fan *et al*., 2003), no change in A_{2B} receptor function is observed in the airway epithelium. These findings suggest that the regulation of A_{2B} receptor expression is cell-specific during the acute inflammatory response to allergen.

In unstimulated cultured human airway epithelial cells, endogenous adenosine has recently been shown to activate A_{2B} receptors tonically (Lazarowski *et al*., 2004). In the present study, however, A_{2B} antagonists had no effect on baseline I_{SC}. This difference is probably due to the huge difference in the volumes bathing the preparations in each study. Thus, in the present study each side of the preparation was bathed by 5 ml of physiological saline and the surface area of the preparation was 0.06 cm^2. By contrast, very small volumes of media – mimicking the airway surface liquid layer – were used in the cell culture model. It would be of interest to determine whether A_{2B} antagonists have any effect on baseline transepithelial ion fluxes *in vivo*.

In conclusion, mucosally applied adenosine causes an increase in I_{SC} in the mouse isolated trachea that is mediated by adenosine A_{2B} receptors. However, in a well-characterised allergic murine model, there was no change in the potency of adenosine in producing this effect, although the baseline and stimulated I_{SC} was impaired in allergic animals.

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