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Evidence for an atypical receptor mediating the augmented bronchoconstrictor response to adenosine induced by allergen challenge in actively sensitized Brown Norway rats

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1 The bronchoconstrictor response to adenosine is markedly and selectively increased following ovalbumin (OA) challenge in actively sensitized, Brown Norway rats. We present a pharmacological analysis of the receptor mediating this response.

2 Like adenosine, the broad-spectrum adenosine receptor agonist, NECA, induced dose-related bronchoconstriction in actively sensitized, OA-challenged animals. In contrast, CPA, CGS 21680 and 2-Cl-IB-MECA, agonists selective for A_1 A_{2A} and A_3 receptors, respectively, induced no, or minimal, bronchoconstriction.

3 Neither the selective A_1 receptor antagonist, DPCPX, nor the selective A_{2A} receptor antagonist, ZM 241385, blocked the bronchoconstrictor response to adenosine.

4 MRS 1754, which has similar affinity for rat A_{2B} and A_1 receptors, failed to block the bronchoconstrictor response to adenosine despite blockade of the A_1 receptor-mediated bradycardia induced by NECA.

5 8-SPT and CGS 15943, antagonists at A_1, A_{2A}, and A_{2B} but not A_3 receptors, inhibited the bronchoconstrictor response to adenosine. However, the degree of blockade (approximately 3 fold) did not reflect the plasma concentrations, which were 139 and 21 times greater than the K_B value at the rat A_{2B} receptor, respectively.

6 Adenosine and NECA, but not CPA, CGS 21680 or 2-Cl-IB-MECA, induced contraction of parenchymal strip preparations from actively sensitized OA-challenged animals. Responses to adenosine could not be antagonized by 8-SPT or MRS 1754 at concentrations >50 times their affinities at the rat A_{2B} receptor.

7 The receptor mediating the bronchoconstrictor response to adenosine augmented following allergen challenge in actively sensitized BN rats cannot be categorized as one of the four recognized adenosine receptor subtypes.

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Abbreviations: AHR, airway hyperresponsiveness; BN, Brown Norway; CGS 21680, 2-[*p*-(2-carboxylethyl) phenethylamino]-5′-N-ethylcarboxamidoadenosine; 2-Cl-IB-MECA, 2-chloro-N^6-(3-iodobenzyl) 5′-N-methylcarboxamidoadenosine; CGS 15943, 9-chloro-2,2-(furanyl)[1,2,4]triazolo[1,5-c]quinazolin-5-amine; CPA, N^6-cyclopentyladenosine; DMSO, dimethylsulphoxide; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; HR, heart rate; 5-HT, 5-hydroxytryptamine; i.t., intratracheal; LC-MS/MS, Liquid chromatography-mass spectrometry/mass spectrometry; MABP, mean arterial blood pressure; MRS 1754, N-(4-cyano-phenyl)-2-[4-(2,6-dioxo-1,3-dipropyl-2,3,4,5,6,7-hexahydro-1H-purin-8-yl)-phenoxy]-acetamide; NECA, 5′-N-ethylcarboxamido adenosine; OA, ovalbumin; 8-SPT, 8-(*p*-sulphophenyl) theophylline; ZM 241385, 4-(2-[7-amino-2-(2-furyl) [1,2,4] triazolo [2,3-a][1,3,5] triazin-5-yl amino]ethyl) phenol

Introduction

We have recently described a marked and selective augmentation of the bronchoconstrictor response to adenosine following allergen challenge in actively sensitized, Brown Norway (BN) rats (Fozard & Hannon, 2000; Hannon *et al*., 2001). The response occurs against a background of mild pulmonary inflammation (Palser *et al*., 2000), exhibits tachyphylaxis (Fozard & Hannon, 2000) and is primarily, if not exclusively, a consequence of mast cell activation (Hannon *et al*., 2001). In all these respects it bears similarity to the bronchoconstrictor response to inhaled adenosine seen in asthmatic patients (Phillips & Holgate, 1995; Jacobson & Bai, 1997; Marquardt, 1997; Polosa & Holgate, 1997; Feoktistov *et al*., 1998; Forsythe & Ennis, 1999; Meade *et al*., 2001).

The receptor mediating the bronchoconstrictor response to adenosine in asthmatics has not been established, although evidence is available implicating the A_{2B} receptor (Feoktistov *et al*., 1998; Fozard & Hannon, 1999). The aim of the present work was to characterize the receptor(s) mediating the

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bronchoconstrictor response to adenosine, augmented following allergen challenge, in actively sensitized BN rats. To this end, the effect of allergen challenge on the sensitivity of the airways to a range of subtype selective adenosine receptor agonists was defined both *in vivo* and *in vitro*. In addition, the effects of a series of adenosine receptor antagonists, some of them subtype selective, on the augmented bronchoconstrictor response to adenosine were investigated.

A part of these results has been presented to the British Pharmacological Society (Hannon *et al*., 1999a, b; Fozard *et al*., 2001).

Methods

Animals

Male BN rats weighing 200–300 g were supplied by Biological Research Laboratories (Füllinsdorf, Switzerland). They were kept at an ambient temperature of 22°C under a 12 h normal phase light-dark cycle and fed on NAFAG® pellets supplied by Nahr und Futtermittel AG, Gossau, Switzerland. Drinking water was freely available. All experiments were carried out with the approval of the Veterinary Authority of the City of Basel (Kantonales Veterinaeramt, Basel-Stadt).

Sensitization and challenge with allergen

The procedure is based on that described by Tarayre *et al*. (1992). Ovalbumin (OA; 20 μg ml^{-1}) was mixed (30 min on ice) in a blender (Polytron, Kinematica Ltd.) with aluminium hydroxide (20 mg ml^{-1}) and injected (0.5 ml per animal s.c.) coincidentally with *Acullulare pertussis* adsorbat vaccine (0.2 ml per animal i.p.; diluted 1 : 4 with saline 0.9% w v^{-1}). Injection of OA, together with adjuvant, was repeated 14 and 21 days later. Sensitized animals were used in experiments between days 28 and 35. For challenge with OA, animals were briefly anaesthetized (4% isofluran) in an anaesthetic chamber. OA or vehicle (saline, 0.2 ml per animal) was administered intratracheally (i.t.) and the animals allowed to recover.

Measurement of lung function

Animals were anaesthetized with sodium pentothal (70 mg kg^{-1} i.p.) and a tracheotomy performed. Heparinized polyethylene catheters were inserted into the left carotid artery for recording blood pressure and into the left jugular vein for drug administration. To suppress spontaneous respiration animals were given an intramuscular injection of vecuronium bromide (12 mg kg^{-1}). No experiment lasted longer than 90 min, during which time surgical anaesthesia was maintained without the need for supplementary anaesthesia. Body temperature was maintained at 37°C with a heated pad controlled by a rectal thermistor.

Animals were ventilated (7 ml kg^{-1}, 1 Hz) *via* the tracheal cannula with a mixture of air and oxygen (50 : 50) in order to maintain the pO_2, pCO_2 and pH of the blood within the physiological range. Ventilation was monitored at the trachea by a pneumotachograph (Fleisch 0000, Zabona, Switzerland) in line with the respiratory pump and connected to a differential pressure transducer (MP 4514871, Validyne, U.S.A.). Coincident pressure changes within the thorax were measured *via* an intrathoracic cannula, using a differential pressure transducer (MP 4524, Validyne, U.S.A). From measurements of airflow and transpulmonary pressure, airway resistance (R_L, cm H_2O l^{-1} s^{-1}) was calculated after each respiratory cycle by use of a digital electronic pulmonary monitoring system (PMS, Mumed, London, U.K.). Mean arterial blood pressure (MABP) and heart rate (HR; by derivation) was recorded from the carotid artery by means of a pressure transducer (P23Dd, Gould, U.S.A).

In vivo *studies: experimental protocols*

Agonist analyses Responses to the different adenosine receptor agonists were determined 3 h following challenge of actively sensitized animals with either vehicle (saline, 0.2 ml i.t.) or OA (0.3 mg kg^{-1} i.t.). This dose of OA and the pretreatment time of 3 h give a marked and consistent increase in the bronchoconstrictor response to adenosine (Hannon *et al*., 2001). Agonists were given by bolus i.v. injection, and to avoid tachyphylaxis only one response was generated per animal. The agonists used were 5′-N-ethylcarboxamido adenosine (NECA), a broad spectrum adenosine receptor ligand (Bruns *et al*., 1986), N^6-cyclopentyladenosine (CPA), a selective A_1 receptor ligand (Londos *et al*., 1980), 2-[*p*-(2-carboxylethyl) phenethylamino]-5′-N-ethylcarboxamidoadenosine (CGS 21680), a selective A_{2A} receptor ligand (Jarvis *et al*., 1989; Wan *et al*., 1990), and 2-chloro-N^6-(3-iodobenzyl) 5′-N-methylcarboxamidoadenosine (2-Cl-IB-MECA), an agonist with high affinity and selectivity for the A_3 receptor (Kim *et al*., 1994). The doses used were derived from the literature where they have been shown to give cardiovascular effects in rats consistent with the subtype selectivity of the agonist in question (Fozard & Carruthers, 1993; van Schaick *et al*., 1996).

Antagonist analyses Three hours following challenge with OA (0.3 mg kg^{-1} i.t.), animals were given a bolus i.v. injection of one of the following antagonists; the broad spectrum adenosine A_1, A_{2A}, A_{2B} receptor antagonists, 8-(*p*-sulphophenyl) theophylline (8-SPT; Jacobson *et al*., 1999) and 9-chloro-2,2-(furanyl)[1,2,4]triazolo [1,5-c]quinazolin-5-amine (CGS 15943; Kim *et al*., 1998), the A_1 receptor selective antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX; Bruns *et al*., 1987; Jacobson *et al*., 1999), the A_{2A} receptor selective antagonist, 4-(2-[7-amino-2-(2-furyl) [1,2,4] triazolo [2,3-a][1,3,5] triazin-5-yl amino]ethyl) phenol (ZM 241385; Palmer *et al*., 1995; Poucher *et al*., 1995; Kim *et al*., 1998) the A_{2B} receptor selective antagonist, N-(4-cyano-phenyl)-2-[4-(2,6-dioxo-1,3-dipropyl-2,3,4,5,6,7-hexahydro-1H-purin-8-yl)-phenoxy]-acetamide (MRS 1754; Kim *et al*., 2000), or their respective vehicles. Five min later animals were given adenosine (1 mg kg^{-1}) by bolus i.v. injection and 15 min later a dose-response curve to 5-HT (3–30 μg kg^{-1} i.v.) was performed; the interval between doses was 2 min. After a further 5 min a dose-response curve to an adenosine receptor agonist corresponding to the selectivity and/or dose of the antagonist utilized was established. Thus, CPA was used in conjunction with DPCPX, CGS 21680 with ZM 241385 and NECA with 8-SPT, CGS 15943 and MRS 1754.

Adenosine receptor antagonist concentrations in plasma

Animals were anaesthetized with sodium pentobarbitone (60 mg kg^{-1} i.p.) and one femoral vein and carotid artery cannulated. Compounds were given i.v. and blood samples were taken after 2, 5, 10, 20 and 30 min. Plasma was prepared by centrifugation and the drug concentrations measured by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS).

Measurement of histamine and 5-HT in plasma

Actively sensitized animals were challenged with OA (0.3 mg kg^{-1}, i.t.) and 2.5 h later anaesthetized with sodium pentothal (70 mg kg^{-1} i.p.). Polyethylene catheters were placed in both the left carotid artery (for blood collection) and right jugular vein (for drug administration). After set-up, the animals were left for a stabilization period of at least 20 min. Three hours after OA challenge animals were given a bolus injection of either 8-SPT (40 mg kg^{-1}) or vehicle (saline, 1 ml kg^{-1}). One min later a blood sample was taken from the carotid artery to give the baseline value. Five min later, animals were given adenosine (1 mg kg^{-1}) by bolus i.v. injection and 1 min later a second blood sample was taken. Blood samples (approximately 1 ml) were taken into 1.5 ml ethylenediaminetetraacetate-coated plastic collection tubes and chilled on ice. Samples were immediately centrifuged ($1700 \times g$, 30 min, 4°C; Omnifuge 2.0, Heraeus Sepratech, CH) and the overlying plasma aspirated and stored at −30°C prior to assay. The concentrations of histamine and 5-HT in the plasma were measured by colorimetric assay using commercial kits (Immunotech) as previously described (Hannon *et al*., 2001).

In vitro *studies*

Lung parenchymal strip The method is described in detail in Hannon *et al*. (2001). In brief, Brown Norway rats, sensitized to OA as described above, were challenged with OA (0.3 mg kg^{-1}) or vehicle (saline) 3 h prior to death by exposure to carbon dioxide. The lungs were perfused *in situ* with modified Krebs' solution of the following composition (mM): NaCl 118; KCl 4.8; $MgSO_4$, 1.2; $CaCl_2$ 2.5; KH_2PO_4, 1.2; $NaHCO_3$, 25; glucose 11, prior to slices being prepared and set up for recording isotonic tension. After a stabilization period of 1 h, during which time the tissues were repeatedly washed, a supramaximal concentration of bethanechol (0.1 mM) was added. After repeated washing during 1–1.5 h, a single concentration of adenosine or an adenosine receptor agonist was added to the bath, followed 1 h later, after repeated washing, by establishment of a dose-response curve to 5-HT (100 nM – 0.1 mM) and, a further 1 h later, a curve to bethanechol (100 nM – 0.1 mM). Tension changes were expressed relative to the maximal response to bethanechol. In the antagonist studies, compounds were included in the Krebs' solution from 15 min prior to establishment of a response to adenosine (1 mM) for the duration of the experiment.

Longitudinal muscle from colon The distal colon was rapidly removed from BN rats killed by exposure to carbon dioxide and placed in modified Krebs' solution of the composition described above. The longitudinal muscle and the muscularis mucosa were separated as described by Bailey & Hourani (1992). Segments of longitudinal muscle (10 mm long, 3 mm wide) were set up for recording isotonic tension in 10 ml organ baths containing modified Krebs' solution at 37°C bubbled with 95% O_2/5% CO_2. Resting tension was maintained at 0.5 g. After a stabilization period of 1 h, during which time the tissues were repeatedly washed, a supramaximal concentration of bethanechol (0.1 mM) was added to the bath. After repeated washing during 1–1.5 h, bethanechol (0.1 mM) was added to the bath followed again by washout. The tissue was then exposed a third time to bethanechol (0.1 mM) followed 20 min later by establishment of a relaxant concentration-response curve to NECA (10 nM – 0.1 mM) in the presence of the bethanechol. After repeated washing, a second concentration-response curve to NECA in the presence of bethanechol was established. Antagonists or vehicle (dimethylsulphoxide (DMSO), final bath concentration 0.2%) were included in the Krebs' solution 30 min prior to establishment of the second concentration-response to NECA. Relaxant effects were expressed relative to the maximal response to bethanechol. The dissociation constant of the antagonist-receptor complex, K_B, was calculated from the equation: $\frac{[A*]}{[A]} - 1 = \frac{[B]}{K_B}$; where $\frac{[A*]}{[A]}$ is the ratio of concentrations of agonist giving an equal response in the presence and in the absence of a given concentration of antagonist, B (Furchgott, 1972).

Materials

Aluminium hydroxide was from Merck, Germany. *Acullulare pertussis* adsorbat vaccine was from the Vaccinal and Serotherapic Institute of Bern, Switzerland. Pentothal (thiopentalum natricum) and Forene (isofluran, 100%) were obtained from Abbott, Switzerland. Norcuron (vecuronium bromide) was from Organon Teknika, Holland. Ovalbumin was obtained from Fluka, Switzerland. Bethanechol (carbamyl-β-methyl-choline chloride), 5-hydroxytryptamine creatinine sulphate and adenosine hemisulphate were obtained from Sigma, Switzerland. 9-Chloro-2,2-(furanyl)[1,2,4]triazolo[1,5-c]quinazolin-5-amine (CGS 15943), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), 8-*p*-(sulphophenyl) theophylline (8-SPT), 5′-N-ethylcarboxamidoadenosine (NECA), 2-[p-(2-carboxyethyl)phenylamino]-5′-N-ethylcarboxamidoadenosine (CGS 21680), N^6-cyclopentyladenosine (CPA) and 2-chloro-N^6-(3-iodobenzyl)adenosine-5′-N-methyl-carboxamide (2-Cl-IB-MECA) were obtained from Research Biochemicals International, U.S.A. 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM 241385) was a gift from Astra Zeneca Pharmaceuticals, U.K. N-(4-cyano-phenyl)-2-[4-(2,6-dioxo-1,3-dipropyl-2,3,4,5,6,7-hexahydro-1H-purin-8-yl)-phenoxy]-acetamide (MRS 1754) was synthesized at Novartis Horsham Research Centre U.K. 8-SPT was made up in saline, (NaCl 0.9% w v^{-1}) solution. The adenosine receptor agonists, DPCPX and ZM 243185 were dissolved at 1 mg ml^{-1} in 50% DMSO in distilled water and diluted immediately before use in saline. CGS 19543 and MRS 1754 (at the 1 mg kg^{-1} dose) were dissolved in a solution of DMSO, Sandimmun® placebo solution (Novartis Pharma AG, Switzerland) and distilled water (1 : 4 : 5). For the 3 mg kg^{-1} dose of MRS1754, the vehicle was polyethyleneglycol 200 containing 2% DMSO.

Data analysis

All data are presented as means ± s.e.mean. Statistical analysis was performed on raw data by means of Student's *t*-test for paired data or analysis of variance with *post hoc* pairwise multiple comparison procedures, using SigmaStat for Windows, version 2.03. A P value <0.05 was considered significant.

Results

Effect of subtype selective adenosine receptor agonists in actively sensitized BN rats challenged with OA

In actively sensitized BN rats challenged with vehicle (saline, 0.2 ml i.t.) 3 h previously, the broad-spectrum adenosine receptor agonist, NECA (3–100 μg kg^{-1} i.v.) induced dose-dependent bronchoconstrictor responses. In animals challenged with OA (0.3 mg kg^{-1} i.t.), responses to NECA were markedly and significantly augmented (Figure 1). NECA also induced falls in both MABP and HR, reflecting its broad range of adenosine receptor activities. Unlike the effects on the airways, the cardiovascular effects were poorly dose-related, a consequence of even the lowest dose of NECA inducing close to a maximum change in both MABP and HR (Table 1).

The selective A_1 receptor agonist, CPA (10 and 30 μg kg^{-1} i.v.), induced profound bradycardia, and hypotension which was maximal and similar in both vehicle- and OA-challenged animals (Table 1). Thus, the adenosine A_1 receptor is activated at these doses. CPA produced only weak bronchoconstrictor responses in animals challenged with vehicle. Similarly, in the OA-challenged animals bronchoconstrictor responses were small, although at the higher dose (30 μg kg^{-1}) a significant increase relative to the vehicle-treated animals was observed (Figure 1).

The selective A_{2A} receptor agonist, CGS 21680 (30 μg kg^{-1} i.v.), induced a prominent hypotensive response indicating that the dose was adequate to activate the A_{2A} receptors of the systemic circulation to induce vasodilatation (Table 1). Despite this, CGS 21680 did not induce bronchoconstrictor effects following either vehicle- or OA-challenge (Figure 1).

The selective A_3 receptor agonist, 2-Cl-IB-MECA (40–1000 μg kg^{-1} i.v.), induced similar pronounced, dose-related hypotensive responses in animals challenged with vehicle or OA (Table 1), consistent with activation of A_3 receptors. In animals challenged with vehicle, 2-Cl-IB-MECA induced little or no effect on airway resistance. In contrast, in animals challenged with OA, 2-Cl-IB-MECA induced small bronchoconstrictor responses at the two highest doses. Although significant relative to the effects in vehicle-challenged animals, the bronchoconstrictor responses were markedly less than those seen with either adenosine or NECA (Figure 1).

Effect of adenosine receptor antagonists on the augmented response to adenosine induced by allergen challenge in actively sensitized BN rats

Broad spectrum adenosine receptor antagonists 8-SPT (20 and 40 mg kg^{-1} i.v.), induced dose-dependent inhibition of the augmented bronchoconstrictor response to adenosine in

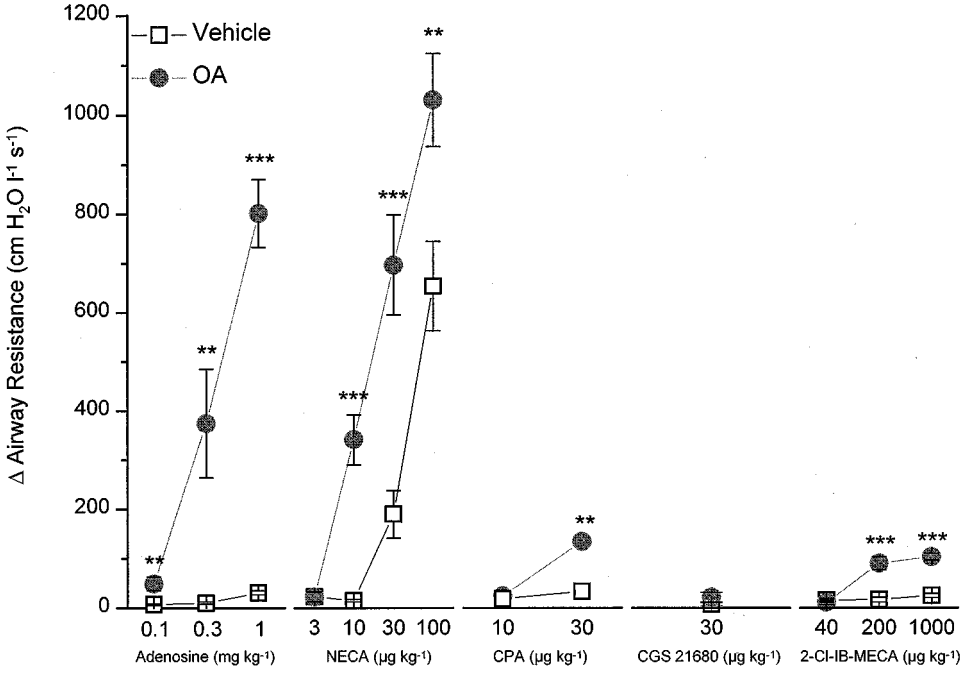

Figure 1 The bronchoconstrictor effects of adenosine, NECA, CPA, CGS 21680 and 2-Cl-IB-MECA in actively sensitized, Brown Norway rats 3 h post intratracheal instillation of vehicle (saline, 0.2 ml) or ovalbumin (OA, 0.3 mg kg^{-1}). The agonists were given i.v. and to avoid tachyphylaxis, only one response was generated per animal. Results are expressed as means ± s.e. means ($n=4-5$). $^{**}P<0.01$, $^{***}P<0.001$ that the value is significantly different from the equivalent value in the vehicle-challenged group. For corresponding cardiovascular data, see Table 1.

actively sensitized, OA-challenged animals, by 55 and 82%, respectively. By reference to the dose response relationship in Figure 1, such inhibition represents an approximate 3 and 8 fold blockade of the response to adenosine, respectively. Bronchoconstrictor responses to 5-HT were similar in both 8-SPT and vehicle-treated animals. Testifying to the non-selective antagonist properties of 8-SPT, both the bradycardia and hypotension induced by adenosine were blocked dose-dependently by 8-SPT as were the cardiovascular effects of subsequent administration of NECA (Figure 2). CGS 15943 (1 mg kg^{-1} i.v.) induced a significant, 62%, inhibition (corresponding to an approximate 3 fold blockade) of the bronchoconstrictor response to adenosine, with no blockade of the bronchoconstrictor response to 5-HT. Both the

Table 1 Effects of adenosine agonists on mean arterial blood pressure (MABP) and heart rate (HR) in actively sensitized Brown Norway rats, challenged intratracheally 3 h previously with either vehicle (saline, 0.2 ml) or ovalbumin (OA, 0.3 mg kg^{-1})

		Vehicle-Challenged					*OA-Challenged*				
	Dose (μg kg^{-1})	*MABP** (mmHg)	*MABP†* %	*HR** (b.p.m.)	*HR†* %		*MABP** (mmHg)	*MABP†* %	*HR** (b.p.m.)	*HR†* %	
Agonist	*i.v*	*Baseline*	*Change*	*Baseline*	*Change*	n	*Baseline*	*Change*	*Baseline*	*Change*	n
Adenosine	100	145±4	−23±2	367±11	−18±3	5	145±10	−29±4	365±7	−22±3	5
	300	136±1	−47±2	346±8	−43±3	5	132±4	−56±3	344±11	−41±4	5
	1000	137±2	−71±1	350±4	−67±4	5	133±5	−73±1	353±4	−73±3	5
NECA	3	125±6	−69±2	395±7	−61±3	5	119±9	−67±1	377±15	−59±4	5
	10	122±3	−76±1	383±12	−79±2	5	116±5	−75±1	376±2	−78±1	5
	30	123±6	−73±2	392±8	−80±2	5	117±4	−80±1	376±5	−79±2	5
	100	116±7	−77±1	374±7	−80±1	5	123±8	−75±2	359±19	−83±1	5
CPA	10	119±8	−53±7	360±14	−63±5	5	114±10	−56±7	373±9	−66±8	5
	30	125±1	−76±1	382±10	−72±3	5	119±5	−64±3	375±12	−63±9	5
CGS 21680	30	124±3	−57±3	360±14	−4.8±2.3	5	119±6	−69±9	359±17	−10±6	5
2-CI-IB-MECA	40	123±4	−26±7	357±11	−3.5±1.2	4	125±1	−31±1	368±4	−1.7±0.8	4
	200	129±3	−41±2	345±27	−2.4±0.7	5	123±5	−50±3	351±17	−3.7±1.5	5
	1000	126±4	−50±6	358±7	−5.8±2.2	5	122±6	−59±2	362±11	−7.6±3.2	5

Values represent means±s.e.means of *n* observations. *Value immediately before injection of agonist; †maximum change from baseline.

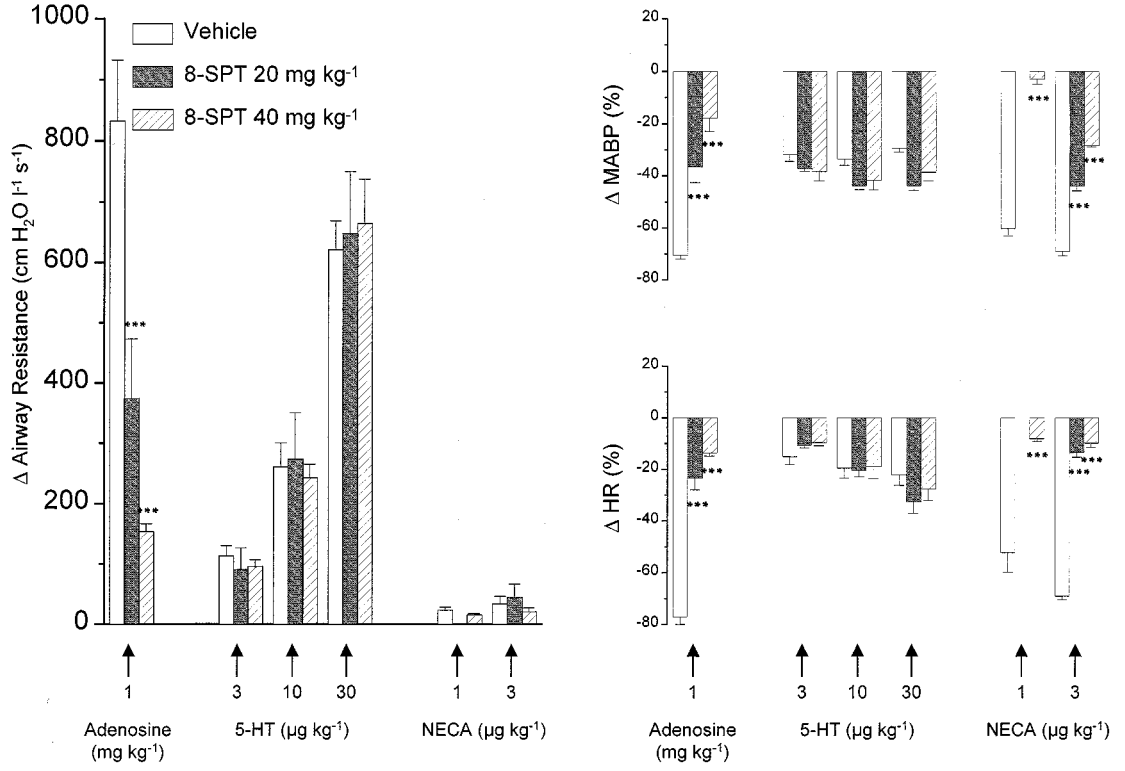

Figure 2 Effect of 8-SPT (20–40 mg kg^{-1}, given i.v. 5 min prior to adenosine), on responses to i.v. administration of adenosine, 5-HT and NECA, with respect to airway resistance, mean arterial blood pressure (MABP) and heart rate (HR) in actively sensitized Brown Norway rats 3 h post intratracheal instillation of ovalbumin (0.3 mg kg^{-1}). Means±s.e.means are presented (n=4–8). ***P<0.001 that the value is significantly different from the equivalent value in the vehicle-treated group.

hypotension and bradycardia in response to adenosine and NECA were markedly and significantly reduced by CGS 15943 (Figure 3).

Selective adenosine receptor antagonists The selective A_1 receptor antagonist, DPCPX (100 μg kg^{-1} i.v.), had no effect on the augmented bronchoconstrictor response to adenosine, nor did it affect the bronchoconstrictor responses to 5-HT (Figure 4). Clear evidence that the dose of DPCPX was sufficient to block the A_1 receptor comes from the observation that the bradycardia induced both by adenosine and by the selective A_1 receptor agonist, CPA, was markedly and significantly suppressed in these animals (Figure 4). The selective A_{2A} receptor antagonist, ZM 241385 (30 μg kg^{-1} i.v.), had no effect on the bronchoconstrictor response to adenosine augmented following OA-challenge. Responses to the two highest doses of 5-HT were significantly reduced following ZM 241385 (Figure 5). That the dose of ZM 241385 was sufficient to establish adenosine A_{2A} receptor blockade in these animals was demonstrated by the marked inhibition of the hypotensive response induced by the selective A_{2A} receptor agonist, CGS 21680 (Figure 5).

The selective A_{2B} receptor antagonist, MRS 1754, given at a dose of 1 mg kg^{-1} i.v. had no significant effects on the airway or cardiovascular responses to adenosine, 5-HT or NECA (Figure 6A). Similarly, at the 3 mg kg^{-1} dose, MRS 1754 had no significant effects on the bronchoconstrictor effects of adenosine although the responses to the lower doses of 5-HT were suppressed (Figure 6B). The vehicle used to deliver the 3 mg kg^{-1} dose caused some loss of sensitivity to the cardiovascular effects of NECA (see Figure 6). Despite this, both the fall in blood pressure, and in particular the bradycardia, induced by adenosine and NECA were significantly inhibited (Figure 6).

Histamine and 5-HT concentrations following adenosine administration: effects of 8-SPT

In agreement with previous results (Tigani *et al*., 2000; Hannon *et al*., 2001), adenosine (1 mg kg^{-1}) administered i.v. to actively sensitized animals challenged 3 h previously with OA, increased the plasma concentrations of histamine and 5-HT in the 5 min period following injection. The increases in histamine and 5-HT were significantly inhibited by pretreatment with 8-SPT (40 mg kg^{-1} i.v., 5 min prior to adenosine) by 51 and 69%, respectively (Figure 7).

Plasma concentrations of antagonists: relationship to affinity for rat A_1, A_{2B} and A_3 receptors

The data are shown in Table 2. The plasma concentrations of 8-SPT, CGS 15943 and MRS 1754 measured 5 min after i.v. administration of 20, 1 and 1 mg kg^{-1} respectively (corresponding to the time when adenosine was administered in the functional studies) were high, exceeding the K_B values at the A_{2B} receptor present in rat colon longitudinal muscle strip by 139, 21 and 184 fold, respectively. Similarly, the plasma concentrations of 8-SPT, CGS 15943 and MRS 1754 at the time of starting the NECA dose response curve exceeded the

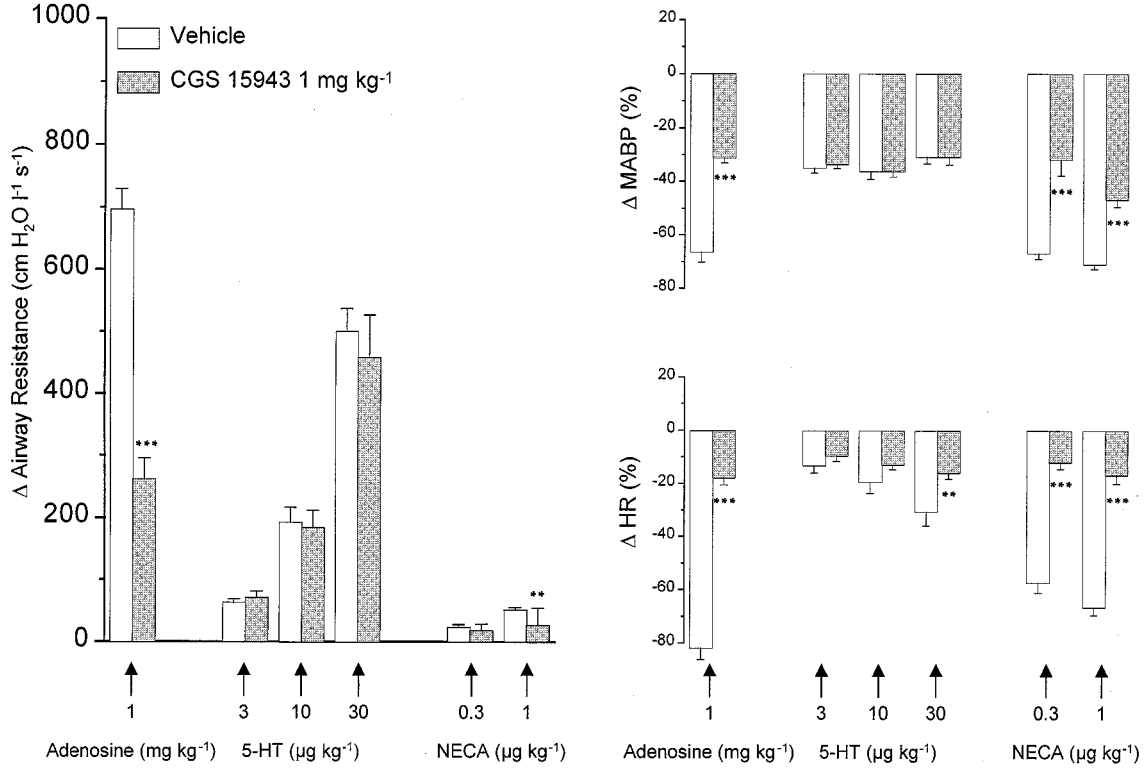

Figure 3 Effect of CGS 15943 (1 mg kg^{-1}, given i.v. 5 min prior to adenosine) on responses to adenosine, 5-HT and NECA), with respect to airway resistance, mean arterial blood pressure (MABP) and heart rate (HR) in actively sensitized Brown Norway rats 3 h post intratracheal instillation of ovalbumin (0.3 mg kg^{-1}). Means$\pm$s.e.means are presented ($n=5$). $^{**}P<0.01$, $^{***}P<0.001$ that the value is significantly different from the equivalent value in the vehicle-treated group.

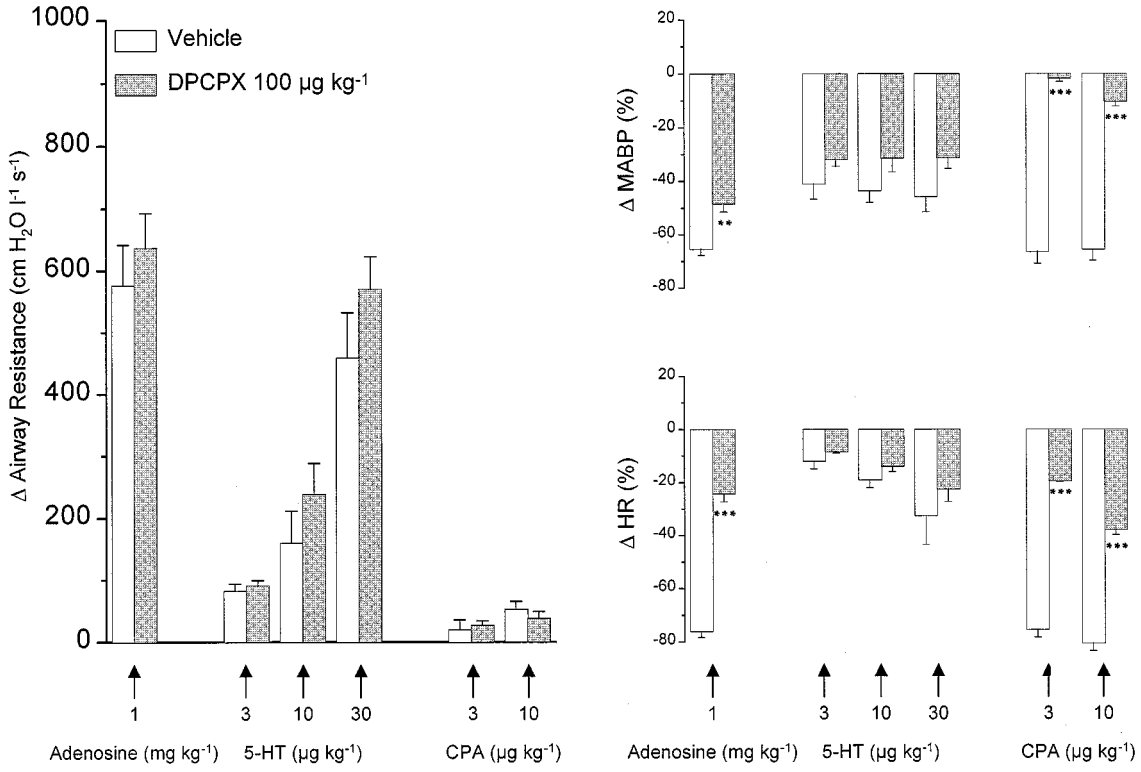

Figure 4 Effect of DPCPX (100 μg kg^{-1}, given i.v. 5 min prior to adenosine) on responses to adenosine, 5-HT and CPA), with respect to airway resistance, mean arterial blood pressure (MABP) and heart rate (HR) in actively sensitized Brown Norway rats 3 h post intratracheal instillation of ovalbumin (0.3 mg kg^{-1}). Means$\pm$s.e. means are presented ($n=5$). $^{**}P<0.01$, $^{***}P<0.001$ that the value is significantly different from the equivalent value in the vehicle-treated group.

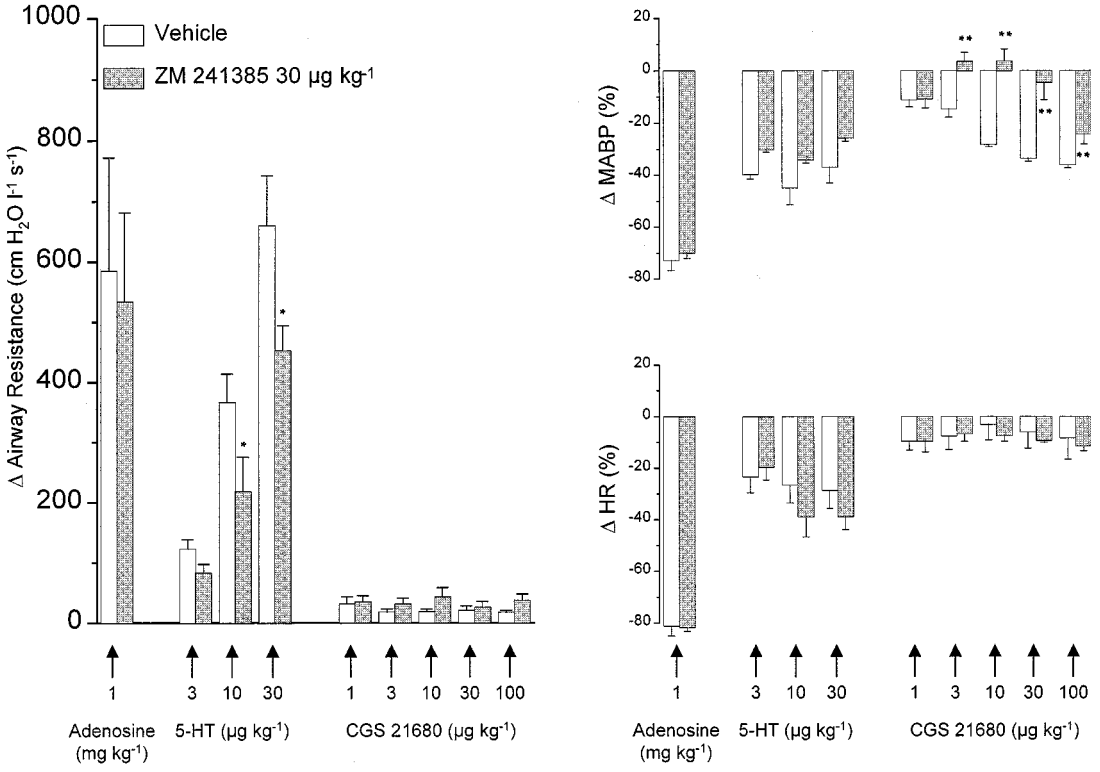

Figure 5 Effect of ZM 241385 (30 μg kg^{-1}, given i.v. 5 min prior to adenosine) on responses to adenosine, 5-HT and CGS 21680, with respect to airway resistance, mean arterial blood pressure (MABP) and heart rate (HR) in actively sensitized Brown Norway rats 3 h post intratracheal instillation of ovalbumin (0.3 mg kg^{-1}). Means$\pm$s.e. means are presented ($n=5$). $^{*}P<0.05$, $^{**}P<0.01$ that the value is significantly different from the equivalent value in the vehicle-treated group.

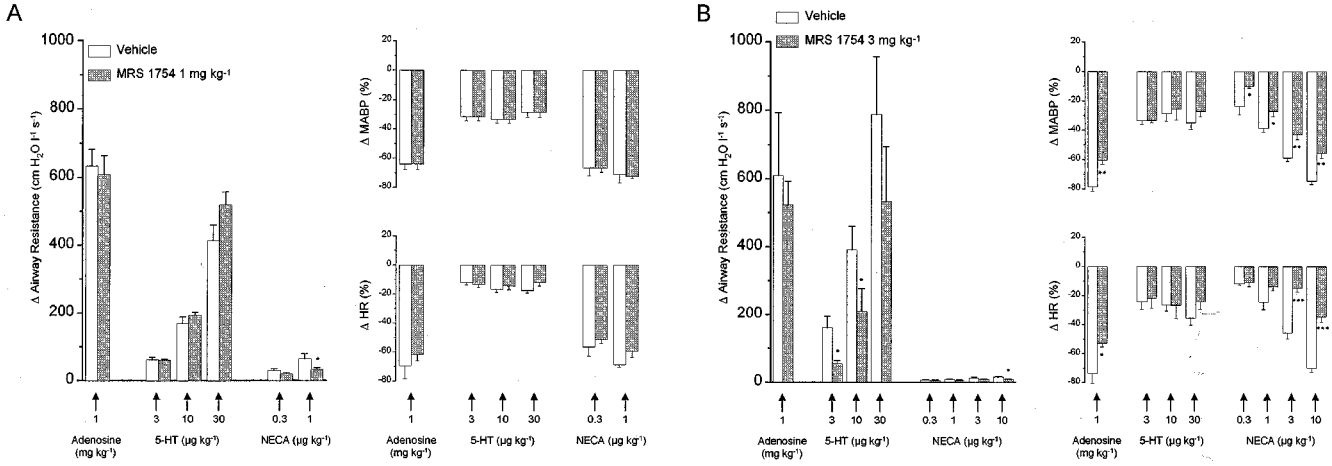

Figure 6 Effect of MRS 1754 (1 and 3 mg kg^{-1}, given i.v. 5 min prior to adenosine, represented in panels A, B, respectively) on responses to adenosine, 5-HT and NECA, with respect to airway resistance, mean arterial blood pressure (MABP) and heart rate (HR) in actively sensitized Brown Norway rats 3 h post intratracheal instillation of ovalbumin (0.3 mg kg^{-1}). Means$\pm$s.e. means are presented ($n=4-5$). *$P<0.05$, **$P<0.01$, ***$P<0.001$ that the value is significantly different from the equivalent value in the vehicle-treated group.

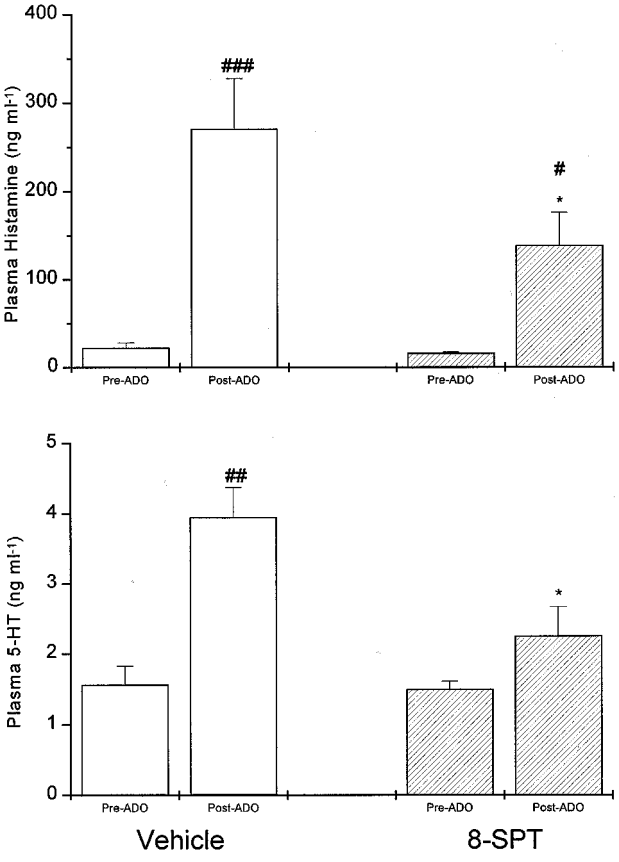

Figure 7 Changes in histamine (upper panel) or 5-HT (lower panel) plasma concentrations induced by adenosine (ADO, 1 mg kg^{-1} i.v.) in actively sensitized Brown Norway rats challenged 3 h previously with ovalbumin: Effects of pretreatment with 8-SPT (40 mg kg^{-1} i.v.), or vehicle (saline, 1 ml kg^{-1} i.v.), given 5 min prior to adenosine. Each column represents the mean$\pm$s.e.mean of four animals. #$P<0.05$, ##$P<0.01$, ###$P<0.001$ indicates significant difference between pre- and post-adenosine values in the same animals. *$P<0.05$ indicates significant difference between post adenosine values in vehicle- and 8-SPT-treated animals.

K_B values at the A_1 receptor (literature values) by 7, 16 and 27 fold, respectively.

In vitro *studies on lung parenchymal strips*

Lung parenchymal strips taken from sensitized animals challenged with OA manifest concentration-dependent constrictor responses to adenosine which reflect the *in vivo* findings, in that they are augmented relative to the responses obtained on strips from vehicle-challenged animals and that the response is mast cell mediated (Hannon *et al*., 2001). NECA also induced contractions of strips taken from animals challenged with OA, which were significantly greater than the responses seen in strips from vehicle-challenged animals. In contrast, neither CPA, CGS 21580 nor 2-Cl-IB-MECA, applied at concentrations 100 times their K_i values for the rat A_1, A_{2A} and A_3 receptors, respectively (Jacobson *et al*., 1995), induced contraction of the strips (Figure 8). When applied to the tissue, 15 min prior to challenge with adenosine (1 mM), neither 8-SPT (100 μM) nor MRS 1754 (1 μM) had any inhibitory effect on the response (Figure 9). In contrast, the response to adenosine was blocked significantly by CGS 15943 (1 μM). Reference to the dose-response curve to adenosine in Figure 9, indicates that the degree of inhibition was 3 fold from which a K_B of 506 nM can be calculated.

Discussion

We have used a range of adenosine receptor agonists and antagonists in order to define the adenosine receptor subtype which mediates the augmented bronchoconstrictor response to adenosine in actively sensitized, OA-challenged animals. With respect to the agonists, these were NECA, the archetypal broad-spectrum adenosine receptor agonist (Bruns *et al*., 1986), CPA, an A_1-selective agonist (Londos *et al*., 1980), CGS 21680, an A_{2A}-selective agonist (Jarvis *et al*.,

Table 2 The plasma concentrations of adenosine receptor antagonists, following i.v. administration to anaesthetized Brown Norway rats. Relationship to their affinities for rat adenosine receptor subtypes.

Ligand	*Dose* (mg kg^{-1})	*Plasma concentration* (μM)		*Receptor affinities* (μM)					*5 min* [plasma] K_BrA_{2B}	*30 min* [plasma] K_BrA_1
	i.v.	5 min*	30 min*	*n*	rA_{2B} (K_B)	*n*	rA_1 (K_i)	rA_3 (K_i)		
8-SPT	20	226.9±20.1	27.6±2.5	4	1.63±0.23[e]	3	4.20[a]	>100[b]	139	6.6
CGS 15943	1	1.50±0.12	0.33±0.06	5	0.07±0.012[e]	5	0.021[c]	>100[c]	21	16
MRS 1754	1	3.50±0.76	0.46±0.07	4	0.019±0.06[e]	3	0.017[d]	n.d.	184	27

*Time after antagonist administration; [a]Jacobson *et al*., 1999; [b]Gao *et al*., 2001; [c]van Galen *et al*., 1994; [d]Kim *et al*., 2000; [e]present studies; n.d. not determined.

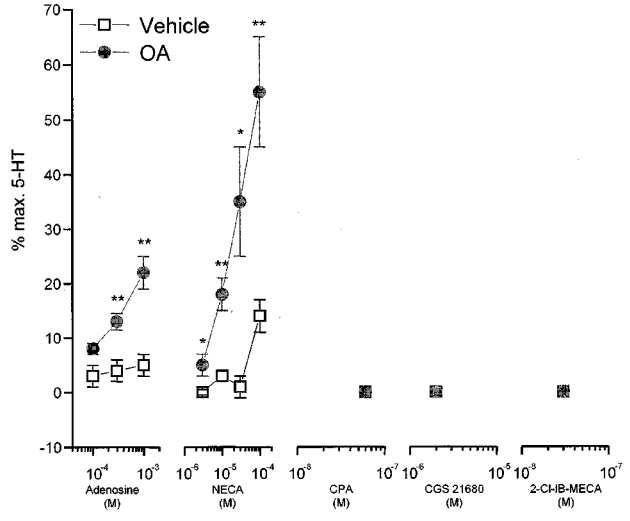

Figure 8 Effects of adenosine, NECA, CPA, CGS 21680 and 2-Cl-IB-MECA on parenchymal strips prepared from lungs removed from Brown Norway rats actively sensitized to ovalbumin (OA) and challenged 3 h previously with vehicle (saline, 0.2 ml, i.t.) or OA (0.3 mg kg^{-1} i.t.). Responses are expressed relative to the response to 5-HT (10^{-4} M). Results are expressed as means±s.e.means of between three and six individual experiments. *$P<0.05$, **$P<0.01$ that the value is significantly different from the equivalent value in the vehicle-challenged group.

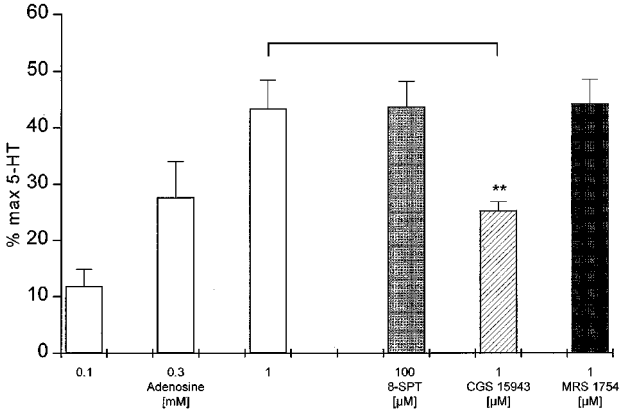

Figure 9 The effect of the adenosine receptor antagonists 8-SPT (100 μM), CGS 15943 (1 μM) and MRS 1754 (1 μM) on responses to adenosine (1 mM) on parenchymal strips prepared from lungs removed from Brown Norway rats actively sensitized to OA and challenged 3 h previously with OA (0.3 mg kg^{-1} i.t.). Responses are expressed relative to the maximal response to 5-HT (10^{-4} M). The results represent the means±s.e.means of 4–9 individual experiments. **$P<0.01$ that the value differs significantly from the response to adenosine (1 mM) in the absence of CGS 15943.

1989; Wan *et al*., 1990), and 2-Cl-IB-MECA, an agonist with high affinity and selectivity for A_3 adenosine receptors (Kim *et al*., 1994). With respect to the antagonists we used DPCPX, which is A_1-selective (Bruns *et al*., 1987; Jacobson *et al*., 1999), ZM 241385, which is A_{2A}-selective (Palmer *et al*., 1995; Poucher *et al*., 1995; Kim *et al*., 1998), MRS 1754, which is A_{2B} -selective (Kim *et al*., 2000) and 8-SPT and CGS 15943, which are broad-spectrum adenosine receptor antagonists showing potency at A_1, A_{2A}, A_{2B} receptors but negligible affinity for A_3 receptors (Ji *et al*., 1994; Fozard & Hannon, 1999; see also Table 2). The data provide a pharmacological definition of the receptor subtype involved in the augmented bronchoconstrictor response to adenosine in actively sensitized, OA-challenged animals.

First, the ability of NECA, a stable analogue of adenosine with negligible affinity for the adenosine transporter (Balwierczak *et al*., 1989; Jimenez *et al*., 2000), to mimic the response to adenosine implies the involvement of cell surface adenosine receptors. Moreover, cross-desensitization readily occurs between adenosine and NECA (Fozard & Hannon, 2000) suggesting that the two agonists share a common mechanism of action. However, since NECA, like adenosine has affinity for each of the recognized adenosine receptor subtypes (Klotz *et al*., 1998; Ralevic & Burnstock, 1998), the observation provides no help in identifying which receptor site is involved. The analysis was therefore extended to include agonists selective for the adenosine A_1, A_{2A} and A_3 receptors (CPA, CGS 21680 and 2-Cl-IB-MECA, respectively). The doses of CPA and CGS 21680 used in this study stem from the observations of Fozard & Carruthers (1993) who demonstrated, in pithed Sprague-Dawley rats with blood pressures supported by angiotensin II, pronounced bradycardic or hypotensive responses *via* the activation of A_1 or A_{2A} adenosine receptors, respectively. The doses of 2-Cl-IB-MECA were selected based on the observations of van Schaick *et al*. (1996) who showed that intravenous injection of 200 μg kg^{-1} in the conscious Wistar rat induced hypotension and a marked increase in the plasma histamine concentration, a consequence of A_3 receptor activation. The cardiovascular effects of CPA, CGS 21680 and 2-Cl-IB-MECA were confirmed in the present studies (Table 1), thus establishing that the doses used were sufficient for subtype-selective activation of the respective adenosine receptors.

The fact that at the 10 μg kg^{-1} dose of CPA and following 30 μg kg^{-1} CGS 21680, there was no bronchoconstriction despite clear cardiovascular evidence of A_1 and A_{2A} receptor

activation, respectively, effectively rules out a role for these receptor subtypes in the bronchoconstrictor response to adenosine. However, small but significant bronchoconstrictor effects were seen following CPA (30 μg kg^{-1} i.v.) and the highest doses of 2-Cl-IB-MECA. Since such doses when administered to Sprague-Dawley or Wistar rats stimulate A_3 receptors leading to mast cell degranulation (Fozard & Carruthers, 1993; Hannon *et al*., 1995, Fozard *et al*., 1996; van Schaick *et al*., 1996), it is possible that the bronchoconstrictor effects of the higher doses of CPA and 2-Cl-IB-MECA in the present study reflect A_3 receptor activation. It is important to emphasize however that the bronchoconstrictor responses to CPA and 2-Cl-IB-MECA are small ($\leqslant$100% increase over baseline) relative to those produced by adenosine or NECA (increases $>$850 % of basal values).

Thus, from the analysis of the effects of the adenosine receptor agonists, neither A_1 nor A_{2A} receptors appear to be involved in the bronchoconstrictor response to adenosine and the consequences of A_3 receptor activation are minimal. The fact that the broad-spectrum adenosine receptor agonists, adenosine and NECA are effective suggests the A_{2B} receptor as the major contributor to the bronchoconstrictor response to adenosine augmented following allergen challenge in the actively sensitized BN rat. We carried out an antagonist analysis to seek support for this conclusion.

DPCPX did not block the bronchoconstrictor response to adenosine despite strongly inhibiting the bradycardia induced by CPA. Similarly, ZM 241385 induced no blockade of the response to adenosine, despite markedly inhibiting the hypotensive response to CGS 21680. Bronchoconstrictor responses to 5-HT, used as a control for the sensitivity of the bronchial smooth muscle to the mediator involved in the response to adenosine (Hannon *et al*., 2001), were unaltered (DPCPX) or slightly reduced (ZM 241385). Together with the observations that neither CPA nor CGS 21680 induced bronchoconstriction, these data rule out unequivocally the involvement of A_1 or A_{2A} receptors in the bronchoconstrictor response to adenosine, augmented following OA-challenge in actively sensitized BN rats.

8-SPT induced dose-dependent blockade of the bronchoconstrictor response to adenosine with no effects on those to 5-HT. 8-SPT also inhibited the adenosine-induced increase in plasma histamine and 5-HT concentrations at the same dose at which the bronchoconstrictor effects are inhibited. These observations are consistent with the anti-bronchoconstrictor effect of 8-SPT being a consequence of blockade of the receptor present on airway mast cells through which adenosine induces mediator release and bronchoconstriction (Hannon *et al*., 2001). 8-SPT has broadly similar antagonist potencies at A_1, A_{2A} and A_{2B} receptors but has only weak effects at rat A_3 receptors (Ji *et al*., 1994; van Galen *et al*., 1994; see also Table 2). Indeed, our earlier studies established that the higher dose of 8-SPT used in the present study (40 mg kg^{-1}) did not block A_3 receptor mediated hypotension in the rat (Fozard & Carruthers, 1993). Given that A_1 and A_{2A} receptors are not involved in the response to adenosine, the most plausible explanation for the marked and dose-dependent inhibition seen in animals treated with 8-SPT would be blockade of adenosine A_{2B} receptors. CGS 15943, like 8-SPT, has affinity for rat A_1, A_{2A} and A2B receptors (although it is significantly more potent) and has negligible affinity for rat A_3 receptors (Kim *et al*., 1998; Gao *et al*., 2001). Blockade of the response to adenosine by relatively low doses of CGS 15943 is again ostensibly in support of a role for A_{2B} receptors.

Further support for this conclusion was sought by using MRS 1754, a xanthine derivative with high affinity for the human A_{2B} receptor (K_i 2 nM) and selectivity ($>$200 fold) *vis à vis* the other human adenosine receptor subtypes (Kim *et al*., 2000). Although our data indicate that the K_B for the rat A_{2B} receptor is somewhat lower (16 nM) than that at the human receptor and similar to the K_i at the rat A_1 receptor (17 nM; Kim *et al*., 2000), MRS 1754 provides a useful tool to implicate A_{2B} receptors in the response. Surprisingly, MRS 1754 failed to block the bronchoconstrictor response to adenosine despite, at the higher dose of 3 mg kg^{-1}, producing blockade of the A_1 receptor-mediated bradycardia, induced by NECA. Given that MRS 1754 has approximately equal affinity for the rat A_1 and A_{2B} receptors and that the plasma concentration at the time of administration of adenosine is appreciably greater than that at the time of starting the NECA sequence (7 fold in the case of the 1 mg kg^{-1} dose level), the observation is difficult to reconcile with the involvement of A_{2B} receptors in the bronchoconstrictor response to adenosine.

Consideration of the pharmacokinetic profile of 8-SPT after intravenous administration under identical conditions to those of the functional studies further questions the role of the A_{2B} receptor in the bronchoconstrictor response to adenosine augmented after allergen challenge. Thus, following a dose of 20 mg kg^{-1} of 8-SPT, which induced an approximate 3 fold blockade of the bronchoconstrictor response to adenosine, the concentration of 8-SPT present in the plasma at the time of adenosine challenge was 139 fold higher than its K_B at the rat A_{2B} receptor. This should be contrasted with the substantially greater than 3 fold blockade of the A_1 receptor-mediated bradycardia induced by NECA between 30 and 40 min after drug administration when the plasma concentration exceeded the K_B at the rat A_1 receptor by only 6.6 fold. Similar reasoning can be applied to CGS 15943, where the plasma concentrations at the times of testing adenosine and NECA exceed the K_B values at the A_{2B} and A_1 receptors by 21 and 16 fold, respectively, and the degree of blockade of the A_1 receptor-mediated bradycardia is clearly greater than that of the bronchoconstrictor response to adenosine. Although a possible confounding factor in relating plasma drug concentrations to pharmacodynamic responses is the degree of protein binding, this parameter is independent of drug concentration and would not detract from the reasoning set out above.

Finally, data from our *in vitro* studies lend strong support to the conclusion that the augmented response to adenosine following allergen challenge is not mediated by A_{2B} receptors. The augmented contractile response of lung parenchymal strips removed from actively sensitized OA-challenged animals to adenosine mirrors closely the *in vivo* bronchoconstrictor response with respect to agonist pharmacology (Figures 1 and 8) and the involvement of mast cells (Hannon *et al*., 2001). Nevertheless, the response to adenosine is resistant to blockade by 8-SPT, and MRS 1754 at concentrations $>$50 times their respective K_B values for blockade of the rat A_{2B} receptor. Moreover, although CGS 15943 is able to antagonize the response to adenosine, its

affinity in this respect (K_B, 506 nM) is appreciably less than its affinity for the A_{2B} receptor of the longitudinal muscle of the rat colon (K_B 70 nM) defined under similar experimental conditions.

Thus, we are left with the surprising conclusion that the receptor mediating the bronchoconstrictor response to adenosine augmented following allergen challenge in the actively sensitized BN rat cannot be categorized as one of the four recognized adenosine receptor subtypes. It remains a challenge for the future to define this receptor further and to establish whether the receptor mediating the response to adenosine of the human asthmatic airways is the same.

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