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The receptor mechanism mediating the contractile response to adenosine on lung parenchymal strips from actively sensitised, allergen-challenged Brown Norway rats

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Abstract Parenchymal strips prepared from lungs removed from actively sensitised Brown Norway rats challenged with allergen show hyperresponsiveness to adenosine. The response is mast cell mediated and a preliminary pharmacological analysis suggested the involvement of a receptor (or receptors) that could not be classified as any of the known adenosine receptor subtypes. We present a further analysis of the response. Male Brown Norway (BN) rats, actively sensitised to ovalbumin (OA), were challenged intratracheally with OA and killed 3 h later to provide parenchymal strip preparations. The augmented contractile responses to adenosine were partially blocked by the 5-HT receptor antagonist, methysergide, or the A_1 receptor antagonist, DPCPX, and abolished in the presence of both antagonists. Responses to high concentrations of the A_1 receptor agonist, CPA were, like those to adenosine, augmented on tissues from allergen-challenged animals and blocked by a combination of methysergide and DPCPX. The A_3 receptor agonist, Cl-IB-MECA, did not contract the tissue, but partially blocked the response to adenosine. A combination of Cl-IB-MECA and methysergide induced a similar degree of blockade to that seen with either drug given alone. Combination of Cl-IB-MECA and/or methysergide with DPCPX abolished the response to adenosine. The effects of the A_3 receptor agonist, inosine, were augmented on tissues from allergen-challenged animals and markedly inhibited by disodium cromoglycate, methysergide or Cl-IB-MECA. Responses to adenosine were abolished when

parenchymal strips were taken from rats pretreated 48 h previously with pertussis toxin. 8-SPT, CGS 15943, XAC, MRS 1754, DPCPX and theophylline, at concentrations which inhibit the A_1 , A_{2A} and/or A_{2B} receptors but have negligible affinity for the rat A_3 receptor, inhibited responses to adenosine, but high concentrations were required and blockade was incomplete. MRS 1523 and MRS 1191, which are antagonists at the rat A_3 receptor, had no effect on the response to adenosine. The present results support and clarify our earlier conclusion that an atypical receptor mechanism mediates contraction of the parenchymal strip prepared from the lungs of actively sensitised BN rats challenged with allergen to adenosine. The response arises from a combined effect of adenosine on the A_1 receptor and a receptor with similarities to the A_3 receptor, but where Cl-IB-MECA behaves as an antagonist and MRS 1523 and MRS 1191 are inactive at concentrations that substantially exceed their affinities for the rat A_3 receptor.

Keywords Adenosine A_1 receptors · Adenosine A_3 receptors · Inosine · Airway hyperresponsiveness · Allergen challenge · Brown Norway rat · Lung parenchymal strip · Pertussis toxin

Abbreviations BN: Brown Norway · CGS 15943: 9-Chloro-2,2-(furanyl)[1,2,4]triazolo[1,5-c]quinazolin-5-amine · DMSO: Dimethylsulphoxide · DPCPX: 1,3-Dipropyl-8-cyclopentylxanthine · HR: Heart rate · 5-HT: 5-Hydroxytryptamine · i.t.: Intratracheal · MABP: Mean arterial blood pressure · MRS 1523: 3-Propyl-6-ethyl-5-[(ethyl-thio)carbonyl]-2-phenyl-4-propyl-3-pyridine carboxylate · MRS 1191: 3-Ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate · MRS 1754: *N*-(4-cyano-phenyl)-2-[4-(2,6-dioxo-1,3-dipropyl-2,3,4,5,6,7-hexahydro-1*H*-purin-8-yl)-phenoxy]-acetamide · NECA: 5'-*N*-ethylcarboxamido adenosine · OA: Ovalbumin · 8-SPT: 8-(*p*-sulphophenyl) theophylline · XAC: 8-[-4-[[[(2-aminoethyl)-amino] carbonyl]ethyl]oxy]phenyl]-1,3-dipropylxanthine

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Introduction

We have recently described a marked and selective augmentation of the bronchoconstrictor response to adenosine following antigen challenge in actively sensitised Brown Norway (BN) rats (Fozard and Hannon 2000; Hannon et al. 2001; Fozard et al. 2003b). Parenchymal strips prepared from lungs removed from actively sensitised BN rats challenged with allergen also show hyperresponsiveness to adenosine (Hannon et al. 2001). Both the bronchoconstrictor response in vivo and the contractile response on the parenchymal strip ex vivo are mast cell mediated and a preliminary pharmacological analysis suggested the involvement of a receptor (or receptors) which could not be classified as any of the known adenosine receptor subtypes (Hannon et al. 2001, 2002; Fozard et al. 2003b).

A key factor in excluding a role for three out of the four recognised adenosine receptor subtypes was that the subtype selective agonists, CPA (A_1), CGS 21680 (A_{2A}) and Cl-IB-MECA (A_3), unlike the non-selective agonists, adenosine and NECA, did not induce contraction of the strips from sensitised, challenged animals despite the use of concentrations 100-fold their K_i values at the receptor sites for which they are selective (Hannon et al. 2002). It was noteworthy, however, that the concentrations of adenosine and NECA required to contract the strip were high. For example, we routinely used a concentration of adenosine of 1 mM which exceeds by 14,000-fold the K_i value at the rat A_1 receptor and by 150 times the K_i value at the rat A_3 receptor (Mueller 2003). In the case of NECA, the concentrations ranged between 3 and 100 μ M which are 10–300 times the K_i value at the rat A_3 receptor and 400–14,000 times the K_i value at the A_1 receptor, respectively (Fredholm et al. 2001a). On this basis, we decided to re-evaluate the effects of CPA and Cl-IB-MECA at higher concentrations than those used previously. In the case of Cl-IB-MECA, we also explored its effect as an antagonist of the response to adenosine. We have also evaluated the response to inosine, a relatively weak, though selective, A_3 receptor agonist (Jin et al. 1997; Fredholm et al. 2001b). We used pertussis toxin to identify whether the signalling pathway triggered by the receptor(s) involves a GTP-binding protein of the G_i/G_o type. Finally, the effects of adenosine receptor antagonists with different subtype selectivities on the response to adenosine have been measured. Our results show that the atypical aspect of the receptor mechanism involved in the contraction of the parenchymal strip to adenosine stems from a combined effect of adenosine on the A_1 receptor and a site with similar pharmacology to, but not identical with, that of the A_3 receptor.

Materials and methods

Animals Male BN rats weighing 200–300 g were supplied by Biological Research Laboratories (Fuellinsdorf, Switzerland). They were kept at an ambient temperature of $22 \pm 2^\circ\text{C}$ 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 Veterinäramt, Basel-Stadt).

Sensitisation and challenge with allergen The procedure is based on that described by Tarayre et al. (1992). Ovalbumin (OA; $20 \mu\text{g}/\text{ml}^{-1}$) was mixed (30 min on ice) in a blender (Polytron, Kinematica, Lucerne, Switzerland) with aluminium hydroxide ($20 \text{ mg}/\text{ml}^{-1}$) and injected (0.5 ml per animal subcutaneously [s.c.]). Injection of OA, together with adjuvant, was repeated 14 and 21 days later. Sensitised animals were used in experiments between days 28 and 35. For challenge with OA, animals were briefly anaesthetised (4% isoflurane) in an anaesthetic chamber. OA ($0.3 \text{ mg}/\text{kg}^{-1}$) or vehicle (saline, 0.2 ml per animal) was administered intratracheally (i.t.) and the animals allowed to recover.

Lung parenchymal strip: general procedure Rats sensitised to OA, as described above, were challenged either with vehicle (saline) or OA ($0.3 \text{ mg}/\text{kg}^{-1}$) 3 h prior to death by exposure to carbon dioxide. The lungs were perfused in situ via a cannula inserted into the pulmonary artery with 60 ml of 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. The lungs were removed and four slices (10–12 mm long, 3 mm thick) were cut from the left lobe. Tissues 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 1 g. After a stabilisation period of 1 h, during which time the tissues were repeatedly washed, a supramaximal concentration of bethanechol (0.1 mM) was added.

Effects of agonists and antagonists After repeated washing for 1–1.5 h, a single concentration of 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 (1 μM –0.1 mM). Because of the possibility of tachyphylaxis, only one concentration of adenosine receptor ligand was tested per tissue. Antagonists or their vehicles were included in the bath fluid 30 min before the addition of the agonist and again 30 min before the start of the concentration–response curves to 5-HT and bethanechol. Tension changes were expressed relative to the response to bethanechol (0.1 mM).

In vitro challenge with OA The procedure has recently been described in detail (Wolber et al. 2004). In brief, strips were prepared from sensitised, non-challenged animals as described above. After a stabilisation 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 OA ($10 \mu\text{g}/\text{ml}^{-1}$) was added to the bath

followed 30 min later by addition of adenosine (1 mM). After washing the tissue repeatedly during 1 h, a concentration–response curve to 5-HT (100 nM–0.1 mM) was established.

Treatment with pertussis toxin Pertussis toxin (15 µg/kg⁻¹) or saline (vehicle) was administered through a tail vein 48 h prior to removal of the lungs for preparation of the parenchymal strip for in vitro analysis (Carruthers and Fozard 1993).

Materials Aluminium hydroxide was from Merck, Darmstadt, Germany. Forene (isofluran, 100%) was obtained from Abbott, Baar, Switzerland. Ovalbumin was obtained from Fluka, Basel, Switzerland. Bethanechol (carbamyl-β-methyl-choline chloride), disodium cromoglycate, 5-hydroxytryptamine creatinine sulphate, inosine, methacholine chloride, pertussis toxin (from Bordetella pertussis), MRS 1523 (3-propyl-6-ethyl-5-[(ethyl-thio)carbonyl]-2-phenyl-4-propyl-3-pyridine carboxylate), MRS 1191 (3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate) and adenosine hemisulphate were obtained from Sigma, Basel, 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-[-4-[[[(2-aminoethyl)-amino]carbonyl]ethyl]oxy]phenyl]-1,3-dipropylxanthine (XAC), 5'-*N*-ethylcarboxamidoadenosine (NECA), were obtained from Research Biochemicals International, Natick, MA, USA. *N*-(4-cyano-phenyl)-2-[4-(2,6-dioxo-1,3-dipropyl-2,3,4,5,6,7-hexahydro-1*H*-purin-8-yl)-phenoxy]-acetamide (MRS 1754) was synthesised in the Novartis Respiratory Disease Research Centre, Horsham, UK, 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, London, UK. Theophylline was used in the form of aminophylline and was synthesised at Novartis Pharma, Basel, Switzerland. For the in vitro studies adenosine and bethanechol were made up in water. All other drugs were dissolved in DMSO. The final bath concentration of DMSO was 0.2% which did not affect the sensitivity of the tissues.

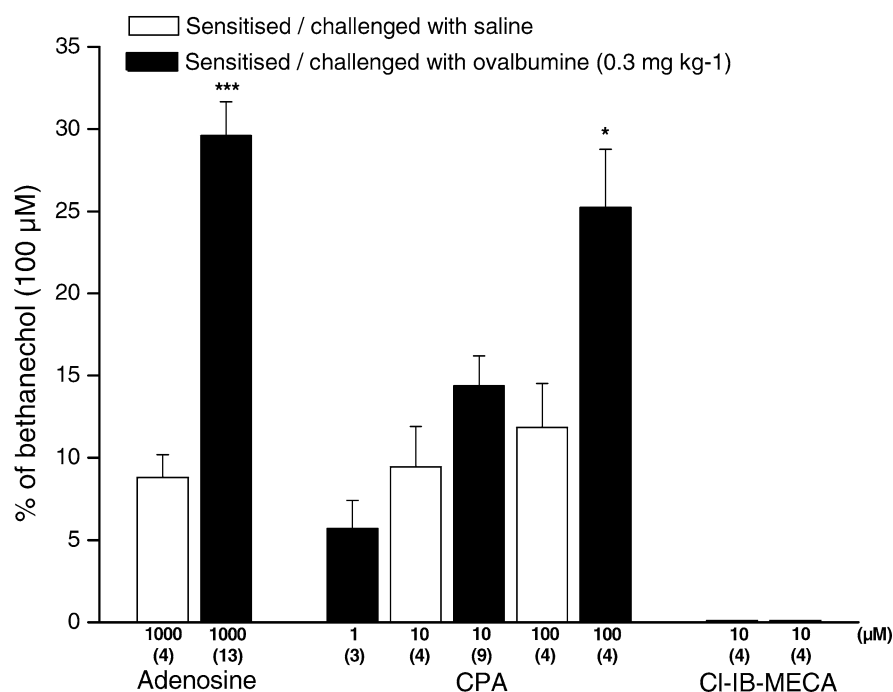
Data processing All data are presented as mean ± SEM. Comparisons were made with control data generated on tissues from the same animals run concurrently and Student's *t* test for two samples assuming unequal variances was used to compare control and test groups. Where more than one test group was compared with the same control, a post-hoc Dunnett's test for multiple comparisons was carried out. The cumulative control data were pooled for ease of presentation. *P* values <0.05 were considered to be significant.

Results

Effects of high concentrations of CPA and CI-IB-MECA on parenchymal strips from sensitised animals challenged with saline or ovalbumin: comparison with adenosine

In confirmation of previous studies (Hannon et al. 2002), contractile responses to adenosine (1 mM) were enhanced on strips prepared from lungs removed from sensitised animals challenged 3 h previously with OA compared to those seen on strips from saline-challenged (control) animals (Fig. 1). CPA (10 and 100 µM) induced small contractions in control strips, which were enhanced on strips removed from allergen-challenged animals (Fig. 1). CI-IB-

Fig. 1 Effect of adenosine, CPA and CI-IB-MECA at the concentrations indicated on lung parenchymal strips prepared from actively sensitised Brown Norway rats 3 h post intratracheal instillation of ovalbumin (0.3 mg/kg⁻¹; filled columns) or saline (open columns). Responses are expressed relative to the response to bethanechol (100 µM), which was taken as 100%. Mean values (± SEM) from the number of individual experiments shown in parentheses are presented. **P*<0.05, ****P*<0.001 vs. the equivalent values in the saline-challenged group.



MECA (10 nM–10 μ M; concentration limited by solubility) did not induce contraction of either control or challenged strips (illustrated for the 10 μ M concentration in Fig. 1). The response to administration of CPA (10 μ M) concomitantly with CI-IB-MECA (10 μ M) on the strips from challenged animals was $11 \pm 4\%$ ($n=3$) of the response to 0.1 mM bethanechol and not significantly different from the response to CPA (10 μ M) alone ($14 \pm 4\%$ [$n=3$] of the response to 0.1 mM bethanechol).

Effects of methysergide, CI-IB-MECA and DPCPX on responses to adenosine and CPA on parenchymal strips from sensitised animals challenged with ovalbumin

Methysergide is a selective 5-HT receptor antagonist which blocks the bronchoconstrictor effect of 5-HT released from the airway mast cells by adenosine (Hannon et al. 2001, 2002). At a concentration of 30 nM, methysergide inhibited the response to adenosine (1 mM) by 74% (Fig. 2). The degree of inhibition was similar to that seen in our earlier studies following 10 nM of methysergide (Hannon et al. 2001) indicating the presence of a residual, small, methysergide-insensitive component in the response to adenosine. Responses to CPA (10 and 100 μ M) were also inhibited by methysergide (30 μ M). However, as with adenosine, there remained a methysergide-insensitive component in the response to CPA which was of similar magnitude at the 10 and 100 μ M concentration levels ($11 \pm 2\%$

[$n=5$] and $14 \pm 5\%$ [$n=4$] of the response to bethanechol [0.1 mM], respectively; Fig. 3).

The potent and selective A_1 receptor antagonist DPCPX was used to define the role of this site in the response to adenosine and CPA. DPCPX induced inhibition of the response to adenosine but only at relatively high concentrations (1, 3 and 10 μ M). The degree of inhibition was small (1 μ M blocked the response to adenosine by 13%) and did not differ significantly for the three concentrations of DPCPX (see Fig. 8). A combination of DPCPX (1 μ M) with methysergide (30 nM) abolished the response to adenosine (Fig. 2). DPCPX (1 μ M) inhibited the response to CPA (100 μ M) by 20%. A combination of methysergide (30 nM) with DPCPX (10 μ M) abolished the response to CPA (Fig. 3).

CI-IB-MECA was tested for antagonist activity against adenosine (1 mM). At a concentration of 10 nM, CI-IB-MECA had no significant effect on the response to adenosine. However, following 100 nM and 1 μ M of CI-IB-MECA, the response to adenosine was reduced significantly and to a broadly similar extent (ca. 60%) at each concentration level (Fig. 4). Blockade was selective in that responses to 5-HT were unchanged (data not illustrated). A combination of methysergide (30 nM) with CI-IB-MECA (1 μ M) did not result in further reduction of the effects of adenosine relative to the effects seen with either drug alone (Fig. 2). However, incubation with DPCPX (1 μ M) in combination with CI-IB-MECA (1 μ M) abolished the response to adenosine (Fig. 2).

Fig. 2 Effect of DPCPX, CI-IB-MECA and methysergide alone or in combination at the concentrations indicated on the response to adenosine (1 mM) on lung parenchymal strips prepared from actively sensitised Brown Norway rats challenged 3 h previously with ovalbumin (0.3 mg/kg^{-1} intratracheally). Responses are expressed relative to the response to bethanechol (100 μ M), which was taken as 100%. Mean values ($\pm$ SEM) from the number of individual experiments shown in parentheses are presented. * $P < 0.05$, *** $P < 0.001$ vs. the equivalent value in the vehicle group.

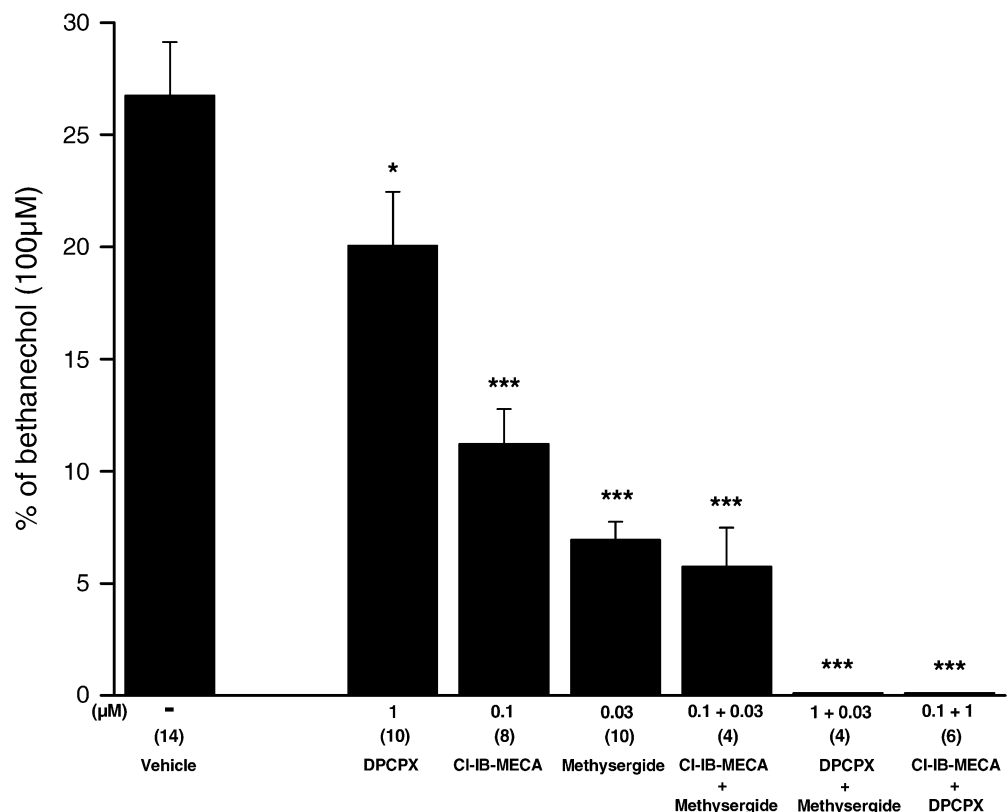
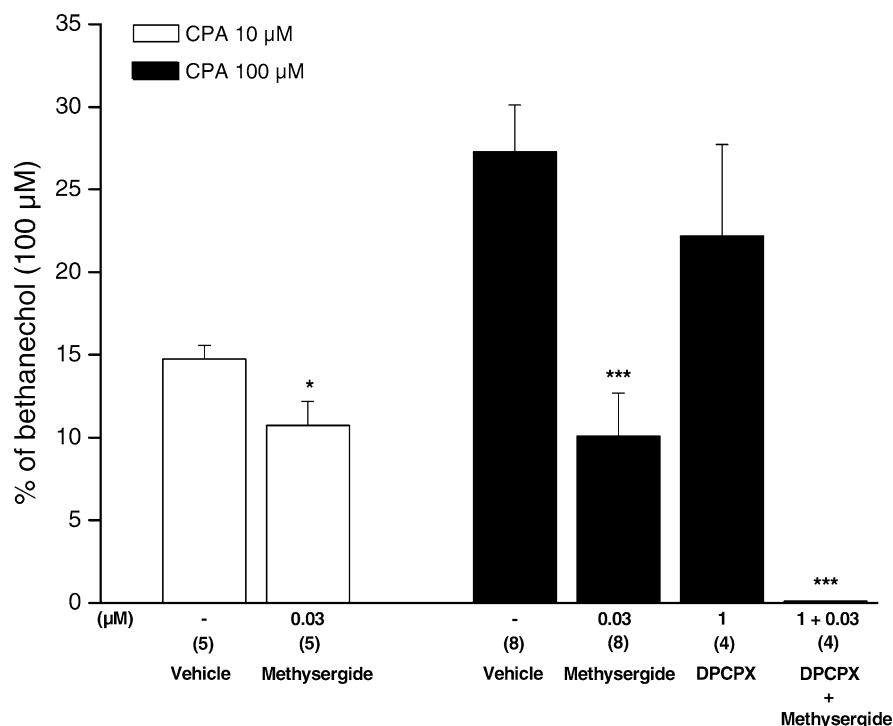


Fig. 3 Effect of methysergide and DPCPX at the concentrations indicated on the response to CPA (10 or 100 μM) on lung parenchymal strips prepared from actively sensitised Brown Norway rats challenged 3 h previously with ovalbumin (0.3 mg/kg^{-1} intratracheally). Responses are expressed relative to the response to bethanechol (100 μM), which was taken as 100%. Mean values ($\pm$ SEM) from the number of individual experiments shown in parentheses are presented. * $P < 0.05$, *** $P < 0.01$ vs. the equivalent values in the relevant vehicle group.



Effects of inosine on parenchymal strips from sensitised animals challenged with saline or ovalbumin

Inosine (0.3–1 mM) had no contractile effects on strips removed from saline-challenged animals. When the strips were prepared from lungs removed from the allergen-challenged animals, inosine induced concentration-related contractile responses (Fig. 5). The response of the challenged strips to 1 mM of inosine was markedly and significantly reduced in the presence of methysergide (30 nM), Cl-IB-MECA (1 μM) or disodium cromoglycate 0.1 mM. Incubation with DPCPX (1 μM) in combination with methysergide (30 nM) abolished the response to inosine (Fig. 5).

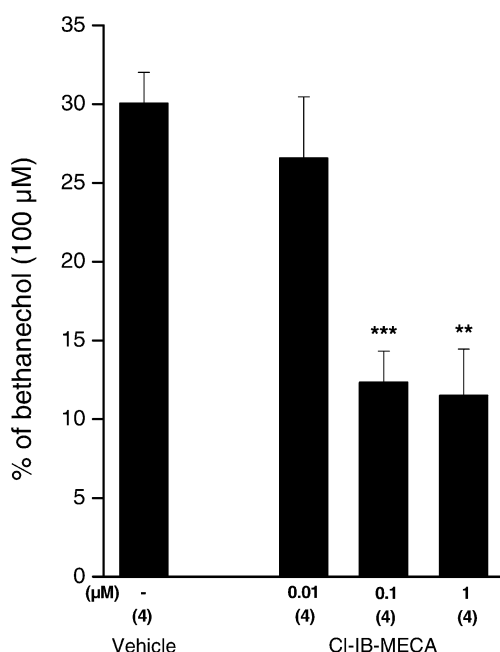


Fig. 4 Effect of Cl-IB-MECA at the concentrations indicated on the response to adenosine (1 mM) on lung parenchymal strips prepared from actively sensitised Brown Norway rats challenged 3 h previously with ovalbumin (0.3 mg/kg^{-1} intratracheally). Responses are expressed relative to the response to bethanechol (100 μM), which was taken as 100%. Mean values ($\pm$ SEM) from the number of individual experiments shown in parentheses are presented. ** $P < 0.01$, *** $P < 0.001$ vs. the equivalent value in the vehicle group.

Effects of pertussis toxin on the contractile responses to adenosine on the parenchymal strip in vitro

Two experiments were carried out. In the first, actively sensitised animals were pretreated with 15 $\mu\text{g}/\text{kg}^{-1}$ of pertussis toxin or vehicle and 45 h later challenged with OA. After a further 3 h, the animals were killed and the parenchymal strip preparations set up. Responses to adenosine (0.1 and 1 mM) were abolished in preparations taken from the pertussis toxin-treated animals. Responses to 5-HT were slightly, but significantly reduced (Fig. 6). In the second experiment, tissues were prepared from lungs removed from sensitised animals treated 48 h prior to death with pertussis toxin. Tissues were challenged in vitro with OA, adenosine and 5-HT as described above. Strips prepared from the pertussis toxin-treated animals re-

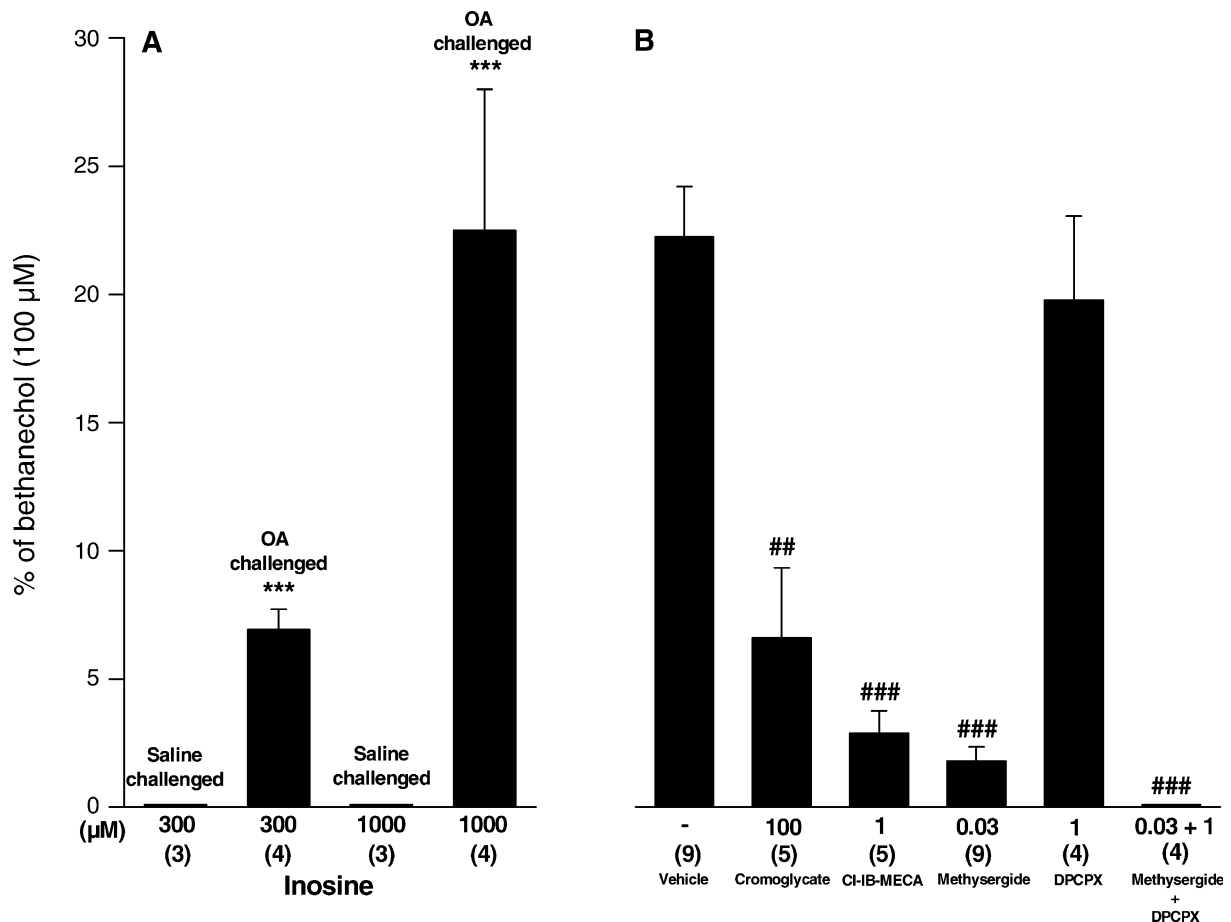


Fig. 5 **a** Effect of inosine on lung parenchymal strips prepared from actively sensitised Brown Norway rats 3 h post intratracheal instillation of ovalbumin (OA, 0.3 mg/kg⁻¹; filled columns) or saline. **b** Effect of methysergide, disodium cromoglycate (*cromoglycate*), CI-IB-MECA, DPCPX or a combination of methysergide and DPCPX at the concentrations indicated on the response to inosine (1 mM) on lung parenchymal strips prepared from actively sensitised

Brown Norway rats challenged 3 h previously with ovalbumin (0.3 mg/kg⁻¹ intratracheally). Responses are expressed relative to the response to bethanechol (100 μM), which was taken as 100%. Mean values (± SEM) from the number of individual experiments shown in parentheses are presented. ****P* < 0.001 vs. the equivalent values in the saline-challenged groups. ##*P* < 0.01, ###*P* < 0.001 vs. the equivalent values in the vehicle-treated group.

sponded normally to OA and 5-HT but the response to adenosine (1 mM) was abolished (Fig. 7).

Effects of adenosine receptor antagonists on the contractile responses of the lung parenchymal strip to adenosine

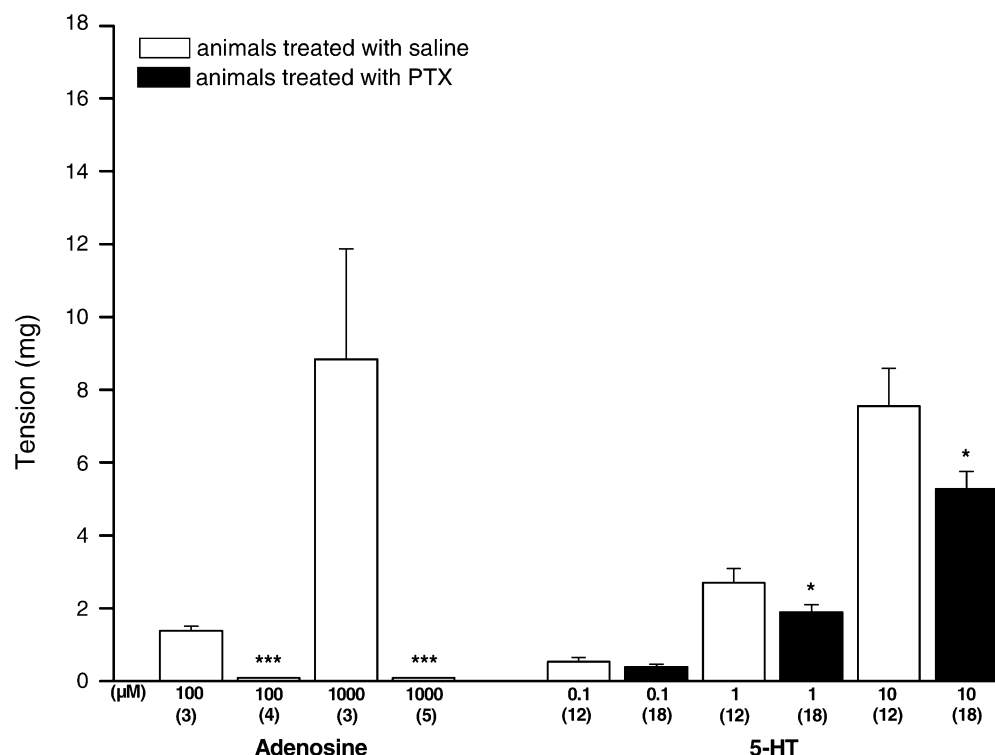
A selection of adenosine receptor antagonists with different selectivities for the individual adenosine receptor subtypes was tested for their effects on the response to adenosine (1 mM) on parenchymal strips prepared from lungs removed from sensitised animals challenged 3 h previously with allergen. When applied to the tissue 30 min prior to adenosine, 8-SPT, CGS 15943, XAC, MRS 1754, DPCPX and theophylline, which inhibit the rat A₁ A_{2A} and/or A_{2B} receptors but, at the concentrations used have negligible affinity for the rat A₃ receptor, induced blockade of the contractile response to adenosine. However, blockade was evident only at relatively high concentrations and limited in scope with the possible exception

of theophylline at 100 μM, the degree of antagonism estimated from the concentration–response curve to adenosine did not exceed threefold with any of these compounds (Fig. 8). Although a trend to inhibition was seen with MRS 1754, this did not achieve statistical significance. MRS 1523 and MRS 1191 showed no antagonist effects towards adenosine at concentrations up to 10 μM. Moreover, MRS 1523 (3 μM) failed to block the response to inosine 1 mM (data not illustrated). There was no change in the sensitivity of the preparations to 5-HT with any compound at any concentration with the exception of theophylline where at a concentration of 100 μM the response to 5-HT was reduced by 27% (data not illustrated).

Discussion

In our earlier studies we showed a marked and selective enhancement of the bronchoconstrictor response to adenosine in actively sensitised BN rats 3 h following challenge with OA, 0.3 mg/kg⁻¹ (Fozard and Hannon 2000; Hannon

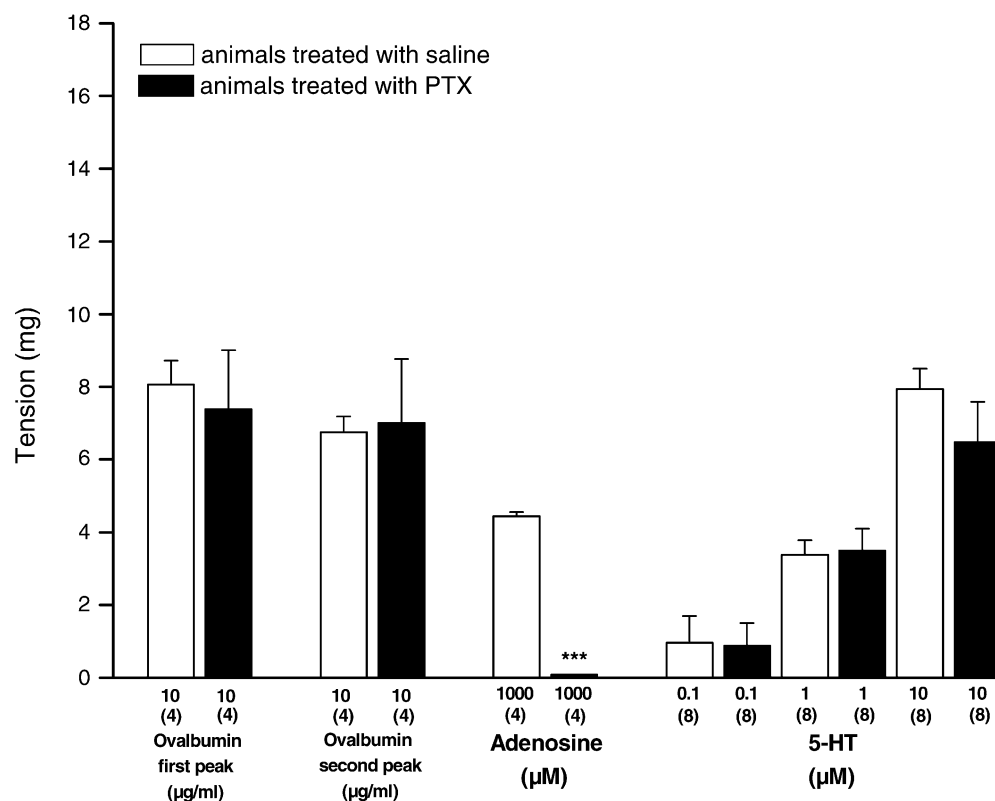
Fig. 6 Effect of pre-treatment with pertussis toxin (PTX) on responses of lung parenchymal strips to adenosine and 5-HT. Actively sensitised rats were given $15 \mu\text{g}/\text{kg}^{-1}$ PTX intravenously (i.v.; or saline) and 45 h later challenged with ovalbumin ($0.3 \text{ mg}/\text{kg}^{-1}$ intratracheally [i.t.]). A further 3 h later the animals were killed and the lung parenchymal strip preparations set up. Responses to adenosine (0.1 and 1 mM) were established on separate strips followed in each case by a concentration–response curve to 5-HT. Mean values ($\pm$ SEM) are presented for the number (n) of individual experiments shown. * $P < 0.05$, *** $P < 0.001$ vs. the equivalent values in the saline-treated group.



et al. 2001). Similar augmented responses to adenosine are seen ex vivo on the lung parenchymal strip removed from challenged animals. Evidence from bronchoalveolar fluid and histological analyses indicate that at this dose of allergen and at this time there are minimal signs of inflam-

mation in the lung (Ellis et al. 2004). Both the bronchoconstrictor response in vivo and the contractile response of the parenchymal strip are mast cell mediated and involve a receptor(s) which in a preliminary pharmacological analysis could not be categorised as one of the four recognised

Fig. 7 Effect of pertussis toxin (PTX) on responses of lung parenchymal strips to ovalbumin, adenosine and 5-HT. Actively sensitised rats were given $15 \mu\text{g}/\text{kg}^{-1}$ PTX i.v. (or saline) and 48 h later the lung parenchymal strip preparations were set up. Responses to ovalbumin ($10 \mu\text{g}/\text{kg}^{-1}$), which were biphasic (see Wolber et al. 2004), and adenosine (1 mM) were established on separate strips followed in each case by a concentration–response curve to 5-HT. Mean values ($\pm$ SEM) are presented for the number (n) of individual experiments shown. *** $P < 0.001$ vs. the equivalent value in the saline-treated group.



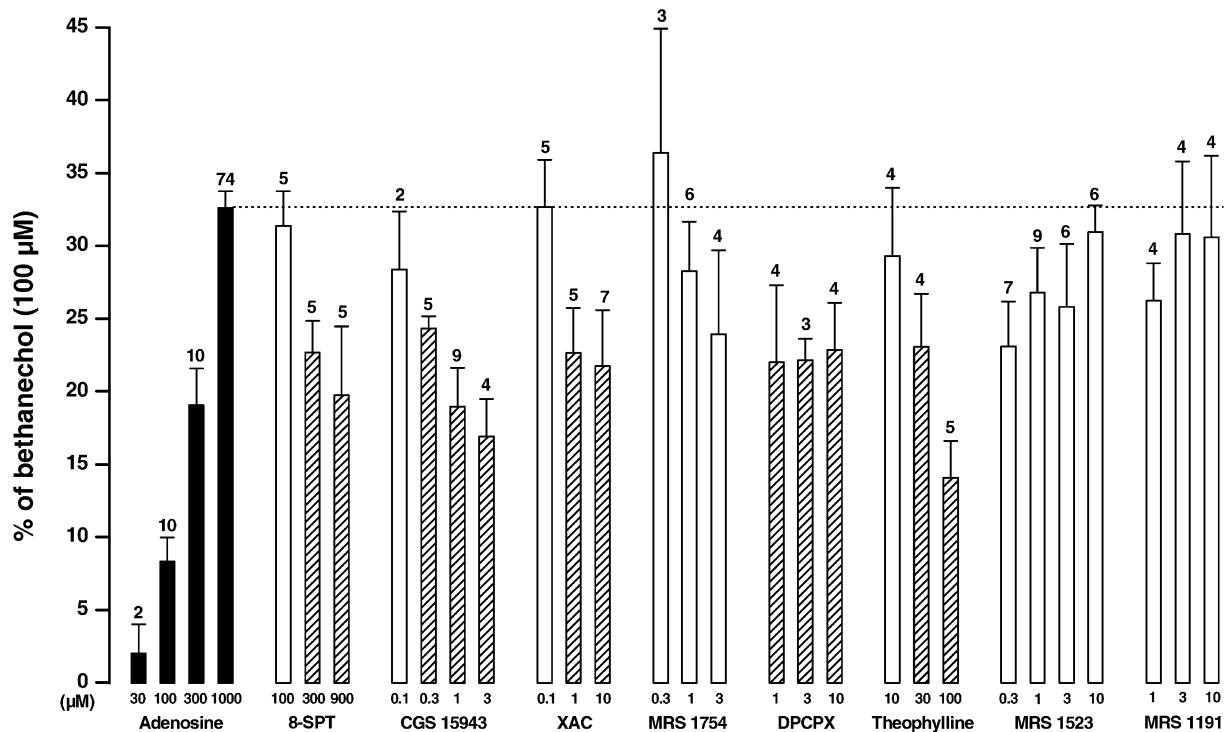


Fig. 8 The effect of the adenosine receptor antagonists, 8-SPT, CGS 15943, XAC, MRS 1754, DPCPX, theophylline, MRS 1523 or MRS 1191 on responses to adenosine (1 mM) on parenchymal strips prepared from lungs removed from Brown Norway rats actively sensitised to OA and challenged 3 h previously with OA (0.3 mg/kg^{-1} i.t.). Responses are expressed relative to the maximal response to bethanechol ($100 \mu\text{M}$). Only one concentration of antagonist was tested on each tissue. The concentration–response relationship for adenosine appears on the left. The results represent the means ($\pm$ SEM) of the number of individual experiments appearing above the columns. Comparisons made were with control

data obtained on tissues from the same animals run concomitantly and Student's *t* test was used to compare control and test groups. The control data for adenosine 1 mM were pooled for ease of presentation. *Hatched columns* indicate a significant difference ($P < 0.05$) from the response to adenosine (1 mM) in the absence of antagonist measured in the same experiment. The data with 8-SPT, CGS 15943 (0.1, 0.3 and 1 mg/kg^{-1}) and MRS 1754 expressed in terms of the response to 5-HT ($100 \mu\text{M}$) were published earlier (Fozard et al. 2003b). They are included here, expressed in terms of the maximal response to bethanechol ($100 \mu\text{M}$), for comparison.

adenosine receptor subtypes (Hannon et al. 2001, 2002; Fozard et al. 2003b). The data presented in this report indicate that the atypical aspect of the receptor mechanism mediating the contractile response to adenosine on the parenchymal strip can be explained by a combined effect of adenosine on the A_1 receptor and a site with similar pharmacology to, but not identical with, the A_3 receptor.

As part of our earlier analysis, we showed that the broad spectrum agonists, adenosine and NECA, induced bronchoconstriction whereas CPA, CGS 21680 and Cl-IB-MECA, agonists with selectivity for the A_1 , A_{2A} and A_3 receptors, respectively, were inactive either alone or in combination despite the use of concentrations 100-fold greater than the K_i values at their receptor subtypes (Hannon et al. 2002; unpublished observations). It was noteworthy, however, that the concentrations of adenosine and NECA required to contract the strip were high (see Introduction for details). On this basis, we decided to re-evaluate the effects of CPA and Cl-IB-MECA at higher concentrations than those used previously.

At concentrations of 10 and $100 \mu\text{M}$, CPA induced small contractile responses on tissues from saline-challenged animals which were increased if the strips were taken from OA-challenged animals. This effect is qual-

itatively similar to that seen with adenosine or NECA (Hannon et al. 2002). The augmented response to adenosine following antigen challenge is mediated in large part by 5-HT released from the airway mast cells (Hannon et al. 2001; Wolber et al. 2004). Blockade by the 5-HT receptor antagonist, methysergide, can be used to define this mechanism (Hannon et al. 2001). Methysergide reduced the augmented response to CPA (by 65%) to that observed on the strips taken from saline-challenged rats. Thus, as is the case for adenosine, the response to CPA enhanced following allergen challenge appears to be in part mast cell mediated. The selective A_1 receptor antagonist, DPCPX, partly blocked the response to CPA and, in combination with methysergide abolished the response. Thus, the response to CPA augmented following allergen challenge, is mediated by an A_1 receptor mechanism and a non- A_1 receptor mechanism that is sensitive to methysergide.

Analysis of the response to adenosine gave qualitatively similar results to those seen with CPA. Thus, methysergide blocked the response to adenosine by around 80%, DPCPX blocked the response by approximately 20% and a combination of methysergide and DPCPX abolished the response. The difference between CPA and adenosine is

therefore in the relative contribution of the two mechanisms to the contractile responses.

The question of the receptor mechanism mediating the methysergide-sensitive, mast cell-dependent component in the response to adenosine and CPA was next addressed. From the literature, the A_3 receptor mediates activation of rat mast cells (Fozard and Hannon 2000). It was therefore surprising that concentrations of the selective A_3 receptor agonist, CI-IB-MECA, as high as 10 μ M did not contract the parenchymal strip. We considered the possibility that CI-IB-MECA might be a low efficacy agonist at the site mediating the response to adenosine and tested the compound for antagonist activity against adenosine. CI-IB-MECA proved a potent antagonist of the contractile response to adenosine although blockade was incomplete. Since combining CI-IB-MECA with methysergide induced no further blockade than was seen with either substance given alone whereas combining CI-IB-MECA with DPCPX abolished the response to adenosine, it can be concluded that CI-IB-MECA and methysergide act on the same component in the response to adenosine. Selective blockade by CI-IB-MECA would be consistent with the A_3 receptor being involved in the response. Our finding that CI-IB-MECA manifests antagonist rather than agonist activity at the A_3 receptor is surprising since from the literature CI-IB-MECA behaves consistently as an agonist; indeed, the intrinsic efficacy of CI-IB-MECA at the human A_3 receptor has been reported to be 99% (Gao et al. 2004).

Supportive evidence for a role for the A_3 receptor was obtained using inosine, a weak but selective A_3 receptor agonist (Jin et al. 1997; Fredholm et al. 2001b; Gao et al. 2001). Inosine was without effect on the tissues taken from saline-challenged animals but induced concentration-dependent contraction of those removed from antigen-challenged animals. The contractile response to inosine was significantly inhibited by disodium cromoglycate and markedly and similarly reduced in the presence of methysergide or CI-IB-MECA consistent with mediation in large part by 5-HT released from mast cells as a result of activation of A_3 receptors. The residual response in the presence of methysergide could be abolished by DPCPX indicating that inosine, like adenosine, has a small A_1 receptor component.

A further piece of evidence consistent with a role for A_1/A_3 receptors in the augmented response to adenosine comes from the use of pertussis toxin. By ADP-ribosylating the α subunit of GTP binding proteins, pertussis toxin interferes with Gi/Go coupled receptor transduction systems (Nuernberg 2000). When parenchymal strips were prepared from lungs removed from pertussis toxin-treated animals which had been challenged with antigen, responses to adenosine and NECA were abolished whereas responses to 5-HT, were reduced, by only ca. 30%. Activation of mast cells through the high affinity IgE receptor, FC ϵ RI, is claimed not to be affected by pertussis toxin (Saito et al. 1987; Bronner et al. 1990; Apgar 1994) and thus the inhibitory effect on adenosine and NECA is unlikely to reflect blockade of the sensitisation step involving allergen challenge 3 h prior to removal of the tissues from the animals. To verify this we carried out experiments

where lungs were removed from sensitised animals treated with pertussis toxin and antigen challenge was carried out entirely *in vitro* (Wolber et al. 2004). Under these conditions, the biphasic response to ovalbumin (which reflects mast cell 5-HT and leukotriene release—Wolber et al. 2004) was unaffected and the response to adenosine, augmented after allergen challenge, was abolished. The data indicate that the contractile response of the parenchymal strip *in vitro* is transduced by a receptor (or receptors) coupled to a pertussis toxin-sensitive G protein. Pertussis toxin-sensitive G proteins include the Gi and Go families and are involved in the signalling pathways linked to A_1 and A_3 receptors (Fredholm et al. 2001a). Indeed, degranulation of pulmonary mast cells from the mouse induced by adenosine A_3 receptor activation is abolished by incubation with pertussis toxin (Zhong et al. 2003). The data with pertussis toxin thus provide further support for a role for the adenosine A_1 and A_3 receptors in the response of the parenchymal strip to adenosine.

We have characterised previously (Fozard et al. 2003b) and in the present work (Fig. 8) a selection of antagonists with different selectivities for the established adenosine receptor sites to further probe the receptor mechanism(s) mediating the contractile response to adenosine on the parenchymal strip. These were the broad spectrum adenosine A_1 , A_{2A} , A_{2B} receptor antagonists, 8-SPT (Jacobson et al. 1999) theophylline (Fredholm et al. 2001a) and CGS 15943 (Kim et al. 1998), the A_1 receptor selective antagonists, XAC (Jacobson et al. 1986) and DPCPX (Bruns et al. 1986; Jacobson et al. 1999), the A_1/A_{2B} receptor selective antagonist, MRS 1754 (Kim et al. 2000) and the selective A_3 receptor antagonists, MRS 1523 and MRS 1191 (Mueller 2003). The fact that high concentrations of adenosine need to be used to induce contraction of the parenchymal strip and that tachyphylaxis occurs rapidly, hampers a classical approach, based on shifts in concentration-response curves, to defining the antagonist potency. Nevertheless, activity can be measured by using different concentrations of antagonists against a single submaximal concentration of adenosine.

In order to facilitate discussion of the significance of the antagonist data, we have assumed based on the above analysis that the contractile response to adenosine involves both A_1 and A_3 receptors with the respective contribution to a response to 1 mM adenosine being approximately 20:80%. In Table 1 we have compared the concentration range of the antagonists within which significant blockade of adenosine on the parenchymal strip first occurs with the affinities of the antagonists at the four recognised rat receptors taken from the literature. The first point to be made is that, with the exception of MRS 1523 and MRS 1191, none of the antagonists has activity at the rat A_3 receptor at the concentrations used and therefore some 80% of the response to adenosine would be expected to be resistant to blockade by these agents. Within the limits of experimental variation, this was in fact the case. (The relatively high potency of theophylline and the concomitant blockade of the response to 5-HT could reflect the relaxant effect on the tissue due to non-selective phos-

Table 1 Affinities of antagonists for rat adenosine receptor subtypes and their potencies as antagonists of the response to adenosine on the lung parenchymal strip. *n.d.* no data

	A ₁ K _i (nM)	A _{2A} K _i (nM)	A _{2B} K _b (nM) ^c	A ₃ K _i (nM)	Lung strip (nM) ^d
8-SPT	4,200 ^b	n.d.	1,680	>100,000	100,000–300,000
Theophylline	11,000	32,000	7,874	>100,000	10,000–30,000
CGS 15943	16	2.5	70	>100,000	100–300
XAC	3	37	74	>100,000	100–1,000
DPCPX	0.3	340	247	>100,000	<1,000
MRS 1754	17	610	19	n.d.	>1,000
MRS 1523 ^a	15,600	2,050	n.d.	316	>10,000
MRS 1191 ^a	40,100	>100,000	n.d.	1420	>10,000

K_i values from binding assays taken from Fredholm et al. (2001b) except ^aMueller (2003) and ^bJacobson et al. (1999)

^cValues from rat colon longitudinal strip functional assay (Fozard et al. 2003a)

^dConcentration range within which significant blockade of the response to adenosine on the parenchymal strip first occurs

phodiesterase inhibition at the concentrations used.) On the other hand, if blockade is seen then this should occur at concentrations adequate to block the A₁ receptors. From Table 1, all the compounds would be expected to block the A₁ receptors at the concentrations which inhibit the response to adenosine. The reason for why high concentrations of the antagonists are needed for blockade could reflect the fact that a concentration of 1 mM adenosine is supramaximal for inducing A₁ receptor activation.

We used the selective, but relatively weak, rat A₃ receptor antagonists, MRS 1523 and MRS 1191, in an attempt to provide further evidence for the involvement of this site in the contractile response to adenosine. The result was surprising in that neither compound was effective against adenosine at 10 µM which exceeds the K_i for the A₃ receptor by 30-fold and 7-fold, respectively. Due to solubility constraints, neither compound could be used at concentrations higher than 10 µM. The lack of effect cannot be attributed to the fact that the response to adenosine was supramaximal since from the concentration–response relationship for adenosine (Fig. 8), a 3-fold blockade would have been easily detected. Moreover, from the literature, similar or lower concentrations of MRS 1523 and MRS 1191 have been shown to be effective in blocking a variety of responses mediated through the rat A₃ receptor (Schneyvays et al. 2000; Jacobson 2001; Hentschel et al. 2003). The data from the antagonist analysis are in agreement with the A₁ receptor contributing to the contractile response. However, they do not support the involvement of the A₃ receptor implied from the agonist analysis.

The parenchymal strip contains several potentially contractile components including small airways, small vessels and alveolar walls (Goldie et al. 1982). The relative contribution of these elements to the contractile response to adenosine in the present experiments is not known. However, 5-HT is a potent contractor of the terminal bronchioles of the rat lung whether released from mast cells by allergen or added directly to the tissue (Wohlsen et al. 2001). It is, therefore likely that the small airways in the parenchymal

strip make a significant contribution to the 5-HT receptor-mediated component of the response to adenosine. The cellular location of the A₁ receptor implicated in the contractile response to adenosine must remain speculative although our data rule out its being the mast cell. A₁ receptors are located on pulmonary C fibres of the rat lung and neuronal activation may underlie the contractile response (see Meade et al. 2001).

In conclusion, the present results support and extend our earlier conclusion that an atypical receptor mechanism mediates adenosine-induced contraction of the parenchymal strip prepared from the lungs of actively sensitised BN rats challenged with allergen. The response arises from a combined effect of adenosine on the A₁ receptor and a receptor with similarities to the A₃ receptor subtype but where Cl-IB-MECA behaves as an antagonist and MRS 1523 and MRS 1191 are inactive at concentrations which substantially exceed their affinities for the rat A₃ receptor.

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