British Journal of Pharmacology (2002) 136, 111–119

www.nature.com/bjp

Airway hyperresponsiveness to adenosine induced by lipopolysaccharide in Brown Norway rats

[1]B. Tigani, [1]J.P. Hannon, [1]C. Rondeau, [1]L. Mazzoni & *,[1]J.R. Fozard

[1]Research Department, Novartis Pharma AG, CH-4002 Basel, Switzerland

1 We have explored the effects of bacterial endotoxin (lipopolysaccharide; LPS) on the response of the airways of Brown Norway (BN) rats to adenosine. Comparisons have been drawn with the effects on responses to methacholine and 5-hydroxytryptamine.

2 In vehicle-challenged animals, adenosine, given i.v. was only a weak bronchoconstrictor. In contrast, 1 h following intratracheal administration of LPS, 0.3 mg kg^{-1}, bronchoconstrictor responses to adenosine were markedly and selectively enhanced. At this time point, there were no significant changes in leukocyte numbers, eosinophil peroxidase and myeloperoxidase activities or protein concentrations in bronchoalveolar lavage (BAL) fluid. Twenty-four hours after challenge, the sensitivity of the airways to both adenosine and methacholine was reduced relative to the earlier time point and there were substantial increases in each marker of inflammation in BAL fluid.

3 The bronchoconstrictor response to adenosine was blocked selectively by methysergide, disodium cromoglycate and the broad-spectrum adenosine receptor antagonist, 8-SPT, but not by DPCPX or ZM 243185, selective antagonists for the A_1 and A_{2A} receptors, respectively.

4 Thus, the response to adenosine augmented following LPS is mast cell mediated and involves a receptor which can be blocked by 8-SPT but not by selective A_1 or A_{2A} receptor antagonists. It thus bears similarity to the augmented response to adenosine induced by allergen challenge in actively sensitized BN rats. Exposure to LPS could be a factor along with allergen in determining the increased sensitivity of the airways of asthmatics to adenosine.

British Journal of Pharmacology (2002) **136,** 111–119

Keywords: Adenosine; airway hyperresponsiveness; LPS; neutrophils; methacholine; disodium cromoglycate; methysergide; TNFα

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)adenosine-5′-N-methyl-carboxamide; CPA, N^6-cyclopentyladenosine; DMSO, dimethylsulphoxide; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; HR, heart rate; 5-HT, 5-hydroxytryptamine; i.t., intratracheal; L-NAME, N^G-nitro-L-arginine methyl ester; LPS, lipopolysaccharide; MABP, mean arterial blood pressure; NECA, 5′-N-ethylcarboxamido adenosine; NO, nitric oxide; OA, ovalbumin; R_L, airway resistance; 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

The markedly enhanced sensitivity of the airways of asthmatic patients to adenosine is one of the most consistent aspects of the condition (Phillips & Holgate, 1995; Meade *et al*., 2001). The mechanism of adenosine-induced bronchoconstriction in asthmatic patients has been well explored and an intermediary role for the mast cell is established (Phillips & Holgate, 1995; Marquardt, 1997; Fozard & Hannon, 2000; Meade *et al*., 2001). The reason for the striking up-regulation of the response in the asthmatic airways remains, however, unknown.

We have previously shown a marked and selective augmentation of the bronchoconstrictor response to adenosine following allergen challenge in actively sensitized, Brown Norway rats (Fozard & Hannon, 2000; Hannon *et al*., 2001). The response occurs against a background of mild pulmonary inflammation and is mast cell mediated. It can be blocked by 8-SPT, a broad spectrum adenosine receptor antagonist, but appears not to be mediated by any of the four recognized cloned adenosine receptor subtypes (Hannon *et al*., 2001; 2002).

In order to define the role and specificity of the inflammatory stimulus which results in hyperreactivity of the airways to adenosine, we have investigated the consequence of inducing non-allergic inflammation with lipopolysaccharide (LPS) on the bronchoconstrictor response to adenosine in non-sensitized BN rats. Our data show a marked and selective augmentation of the bronchoconstrictor response to adenosine following LPS challenge. The augmented response shows similarities to the response to adenosine up-regulated following allergen challenge in actively sensitized animals in being mast cell mediated and showing similar antagonist pharmacology.

Preliminary findings from these studies have been presented to the British Pharmacological Society (Fozard *et al*., 2000).

*Author for correspondence;
E-mail: john_r.fozard@pharma.novartis.com

Methods

Animals

Male Brown Norway (BN) rats (Iffa-Credo, L'Arbresle, France) weighing 250–300 g were used throughout. Groups of up to five animals were housed in sawdust lined drawer cages and kept at an ambient temperature of $22\pm2^{\circ}C$ under 12 h normal phase light–dark cycles. All experiment were carried out according to Swiss federal regulations for animal protection.

LPS exposure

Rats were anaesthetized (4% isofluran) in an anaesthetic chamber, until surgical anaesthesia was achieved. LPS or vehicle (saline, 0.2 ml per animal) was administered intratracheally (i.t.) and the animals allowed to recover. Animals were taken for measurement of lung function or bronchoalveolar lavage (BAL) fluid analysis at various time intervals after challenge.

Bronchoalveolar lavage (BAL) fluid collection and analysis

Animals were challenged with LPS or vehicle (saline 0.2 ml, i.t.) and killed 1 or 24 h later with pentobarbitone (250 mg kg^{-1} i.p.). The lungs were lavaged using three 4 ml aliquots of solution A (Hanks' balanced salt solution $\times 10$, 100 ml; ethylenedaminetetraacetic acid 100 mM, 100 ml; 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid 1 M, 10 ml; double-distilled H_2O 790 ml). The recovered solution was pooled (representative mean recovery 11.3 ± 0.1 ml, $n=55$) and the total volume of recovered fluid adjusted to 12 ml by addition of solution A.

The methods for the determination of total leukocyte numbers and differential cell counts, eosinophil peroxidase activity and protein concentration in the bronchoalveolar lavage fluid have recently been described in detail (Beckmann *et al*., 2001). In brief, leukocyte numbers and differential cell counts were obtained using an automatic cell analysing system (Cobas Helios 5Diff, Hoffmann-La Roche, Axon Lab, Switzerland). Myeloperoxidase activity was measured in a photometric assay based on the oxidation of O-dianiside dihydrochloride by myeloperoxidase in the presence of hydrogen peroxide. Eosinophil peroxidase activity was measured in a photometric assay based on the oxidation of O-phenylenediamine by eosinophil peroxidase in the presence of hydrogen peroxide. Protein concentrations were measured in a photometric assay based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent.

TNFα was measured as follows: 50 μl of the samples and standards, in duplicate, were added to TNFα antibody coated wells; 50 μl of biotinylated anti-TNFα (biotin conjugate) solution was pipetted into each well except the chromogen blanks. The plates were incubated for 90 min at room temperature and then washed four times. We then added 100 μl of streptavidin–HPR to each well, except the chromogen blanks. After incubation for 45 min at room temperature the wells were washed four times and 100 μl of stabilized chromogen were added to each well. The plates were incubated for a further 30 min in the dark at room temperature. The absorbance of each well was read at 450 nm.

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 mean arterial blood pressure (MABP) and into the left jugular vein for drug administration. To suppress spontaneous respiration animals were given an intramuscular injection of vecuronium bromide (Norcuron, 12 mg kg^{-1}). No experiment lasted longer than 90 min, during which time surgical anaesthesia was maintained throughout the experiment 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, v v^{-1}). 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.) as described by Amdur & Mead (1958). From measurements of airflow and transpulmonary pressure, airway resistance (R_L, cm H_2O l^{-1} s) was calculated after each respiratory cycle by use of a digital electronic pulmonary monitoring system (PMS, Mumed, London, U.K.). Mean arterial blood pressure and heart rate (HR) by derivation was recorded from the carotid artery by means of a pressure transducer (P23Dd, Gould, U.S.A.).

Experimental protocols

Effect of methysergide Bronchoconstrictor responses to adenosine (0.3, 1 and 3 mg kg^{-1} i.v.), methacholine (3, 10 and 30 μg kg^{-1} i.v.) and 5-HT (3, 10 and 30 μg kg^{-1} i.v.) were established in groups of animals challenged i.t. 1 h previously with vehicle (saline 0.2 ml) or LPS (0.3 mg kg^{-1}). The interval between adenosine doses was 15 min. Fifteen min after the last adenosine dose, methacholine was administered with a 2 min interval between doses. Finally, after a further restabilization period of 15 min, 5-HT was given with a 2 min interval between doses. Methysergide, at a dose just supramaximal for blockade of 5-HT_2 receptors *in vivo* (30 μg kg^{-1}; Fozard, 1982), or vehicle (saline) was given i.v. 5 min prior to the start of the agonist sequence.

Effect of disodium cromoglycate Bronchoconstrictor responses were established to 5-HT (30 μg kg^{-1} i.v.) and, 5 min later, adenosine (1 mg kg^{-1} i.v.) in groups of animals challenged i.t. 1 h previously with LPS (0.3 mg kg^{-1}). Disodium cromoglycate, at a dose previously shown to block mast cell degranulation in rats *in vivo* (20 mg kg^{-1}, Hannon *et al*., 1995; 2001), or vehicle (saline) was given i.v. 5 min prior to the start of the agonist sequence.

Effects of adenosine receptor antagonists One hour following LPS, (0.3 mg kg^{-1}), animals were given a bolus i.v. injection of either the broad spectrum adenosine A_1, A_{2A}, A_{2B}

receptor antagonist, 8-SPT (40 mg kg^{-}), the adenosine A_1 receptor selective antagonist, DPCPX (100 μg kg^{-1}), the adenosine A_{2A} receptor selective antagonist, ZM 241385 (30 μg kg^{-1}), or their respective vehicles. The animals were given adenosine 5 min later (3 mg kg^{-1} i.v.) and a dose–response curve to 5-HT (3–30 μg kg^{-1} i.v.) was performed 15 min later, the interval between doses being 2 min. After a further 5 min, a dose–response curve to an adenosine receptor agonist corresponding to the selectivity of the antagonist used was established. To this end, NECA was used in conjunction with 8-SPT, CPA with DPCPX and CGS 21680 with ZM 241385.

Materials

Hanks' balanced salt solution (10×), and 4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid were from Gibco BRL, U.K. Ethylenedaminetetraacetic acid (pH 7.4) from Merck, Germany; Pentothal (thiopentalum natricum) and Forene (Isofluran 100%) were obtained from Abbott, Switzerland; Norcuron (vecuronium bromide) was from Organon Teknika, Holland; Methacholine chloride, disodium cromoglycate, 5-hydroxytryptamine creatinine sulphate, atropine sulphate, adenosine hemisulphate, lipopolysaccharide (from Salmonella typhosa) and Bovine Serum albumin (fraction V) were obtained from Sigma, Switzerland. 1,3-dipropyl-8-cyclopentyl xanthine (DPCPX); 8-p-(sulphophenyl) theophylline (8-SPT) 5′-N-ethyl carboxamido adenosine (NECA), 2-[p-(2-carboxyethyl)phenylamino]-5′-N-ethyl carboxamidoadenosine (CGS 21680) and N^6-cyclopentyladenosine (CPA) and 2-chloro-N^6-(3-iodobenzyl)adenosine-5′-N-methyl-carboxamide (2-CI-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-yl amino)ethyl)phenol (ZM 241385) was a gift from AstraZeneca Pharmaceuticals, U.K.). Methysergide was synthesized at Novartis Pharma AG, Basel, Switzerland. The adenosine agonists and antagonists (with the exception of 8-SPT) were dissolved in 50% DMSO in distilled water and diluted immediately before use in 0.9 % w v^{-1} NaCl. All other compounds were made up in 0.9% w v^{-1} NaCl.

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 unpaired 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

Bronchial hyperresponsiveness to adenosine induced by LPS in BN rats: Dose–response relationship, time course and selectivity with respect to methacholine

A series of bronchoconstrictor responses to adenosine (0.3 and 1 mg kg^{-1} i.v.) and methacholine (3 and 10 μg kg^{-1} i.v.) were established in groups of animals challenged intratracheally with vehicle or LPS. The interval between the two adenosine doses was 15 min, and 15 min after the second dose, methacholine was administered with a 2 min interval between doses.

In a first series of experiments, LPS was given i.t. at doses of 0.1, 0.3 or 1 mg kg^{-1} and the sequence of bronchoconstrictor agents was started 1 h later. Vehicle-challenged animals received saline, 0.2 ml i.t., 1 h before the start of the bronchoconstrictor agonist sequence. There were no significant changes in basal airway resistance following any of the doses of LPS (data not illustrated). The bronchoconstrictor effects of adenosine were weak in BN rats challenged with saline. In contrast, responses to adenosine were markedly increased following all doses of LPS. Responses to methacholine were not significantly altered (Figure 1).

In a second series of experiments, animals were challenged with LPS (0.3 mg kg^{-1}) or vehicle (saline, 0.2 ml) and the sequence of bronchoconstrictor agents started 1, 3 or 24 h after LPS (1 h after saline) – the results are shown in Figure 2. At the 1 h time point, responses to adenosine were significantly enhanced by LPS, whereas those to methacholine were unaffected. At the 3 h time point, responses to adenosine had returned to those of control animals. Responses to methacholine were again unaffected by the LPS challenge. At the 24 h time point, significant bronchial hyporesponsiveness to both spasmogens was evident (Figure 2). From these studies, 0.3 mg kg^{-1} of LPS administered i.t. 1 h prior to testing was considered a suitable paradigm to explore the mechanism of the augmented response to adenosine induced by LPS challenge in BN rats.

The effect of 2-Cl-IB-MECA, a selective agonist at adenosine A_3 receptors (Fredholm *et al*., 2001), was investigated in animals treated 3 h previously with LPS, 0.3 mg kg^{-1}. In three animals, 2-Cl-IB-MECA given i.v. at a dose of 0.2 mg kg^{-1} was devoid of bronchoconstrictor activity. The compound did, however, lower blood pressure (by 58±8%) confirming that activation of A_3 receptors occurs at the dose used (Van Schaick *et al*., 1996; Hannon *et al*., 2002).

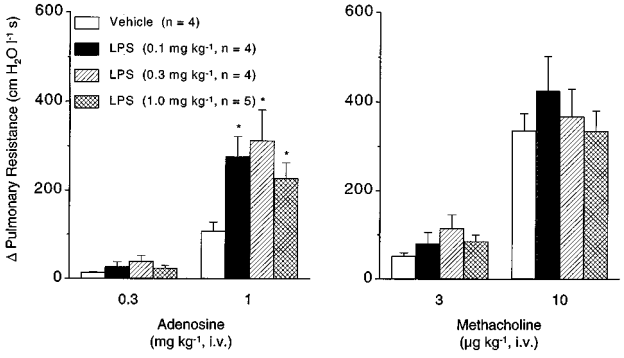

Figure 1 Increases in pulmonary resistance induced by intravenous administration of adenosine and methacholine starting 1 h post intratracheal instillation of lipopolysaccharide (LPS; 0.1, 0.3 or 1 mg kg^{-1}), or vehicle (saline, 0.2 ml), in Brown Norway rats. Results are expressed as means±s.e.means of the number (n) of animals shown in parentheses. *$P<0.05$ indicates a significant difference between the LPS-treated and equivalent vehicle-treated group.

Bronchial hyperresponsiveness to adenosine induced by LPS in BN rats: selectivity with respect to the cardiovascular effects

Bronchoconstrictor and cardiovascular responses to i.v. injections of adenosine (0.3, 1 and 3 mg kg^{-1}; 20 min interval between doses) were established in groups of animals challenged intratracheally (i.t.) 1 h previously with vehicle (saline, 0.2 ml) or LPS (0.3 mg kg^{-1}). These experiments were the control experiments for the study with methysergide shown in Figure 5. In confirmation of the data presented in Figure 1, bronchoconstrictor responses to adenosine were significantly enhanced in animals treated with LPS (Figure 3). Enhancement was selective for the airways since neither the fall in blood pressure (with the exception of the lowest dose) nor the bradycardia induced by adenosine was increased in the LPS-treated animals (Figure 3). There were no significant changes in baseline airway resistance, blood pressure or heart rate following LPS (data not illustrated).

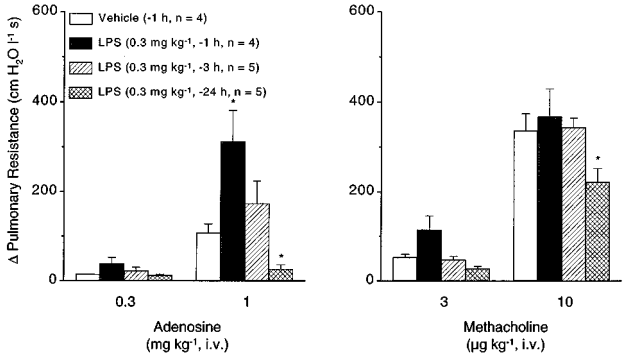

Figure 2 Increases in pulmonary resistance induced by intravenous administration of adenosine and methacholine starting 1, 3 or 24 h post intratracheal instillation of lipopolysaccharide (LPS; 0.3 mg kg^{-1}), or vehicle (saline, 0.2 ml), in Brown Norway rats. Results are expressed as means$\pm$s.e.means of the number (n) of animals shown in parentheses. $^{*}P<0.05$ indicates a significant difference between the equivalent LPS-and vehicle-treated groups.

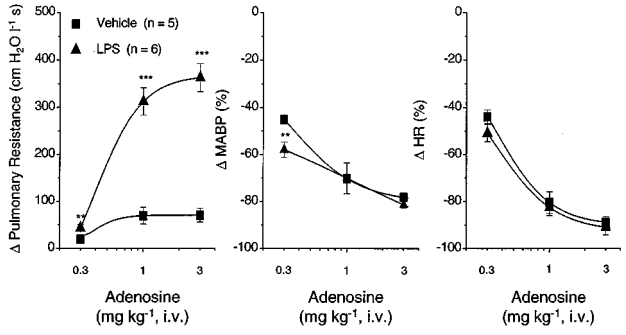

Figure 3 Comparison of the bronchoconstrictor and cardiovascular (mean arterial blood pressure, MABP; heart rate, HR) effects of adenosine in Brown Norway rats challenged intratracheally 1 h previously with vehicle (saline, 0.2 ml) or LPS (0.3 mg kg^{-1}). Results are expressed as means$\pm$s.e.means of the number (n) of animals shown in parentheses. $^{**}P<0.01$, $^{***}P<0.001$ that the value is significantly different from the equivalent value in vehicle-challenged animals.

Effects of treatment with LPS on markers of inflammation in the BAL fluid

BN rats were challenged with LPS (0.3 mg kg^{-1} i.t.) and killed 1 h or 24 h later. Vehicle-challenged animals (saline, 0.2 ml i.t.) were killed 1 h later. The lungs were lavaged and the BAL fluid differential cell counts, eosinophil peroxidase and myeloperoxidase activities and the protein and TNFα concentrations determined, the results are presented in Figure 4. One hour following LPS there were no signs of pulmonary inflammation as monitored by leukocyte numbers, eosinophil peroxidase and myeloperoxidase activities or protein concentrations in the BAL fluid. TNFα concentrations were, however, markedly increased. At 24 h, significant increases in the numbers of eosinophils (2.4 fold), macrophages (2.1 fold) and neutrophils (56 fold), the activities of eosinophil peroxidase and myeloperoxidase (5 and 7 fold, respectively) and the concentrations of protein (1.5 fold) and TNFα (2.6 fold) were evident (Figure 4).

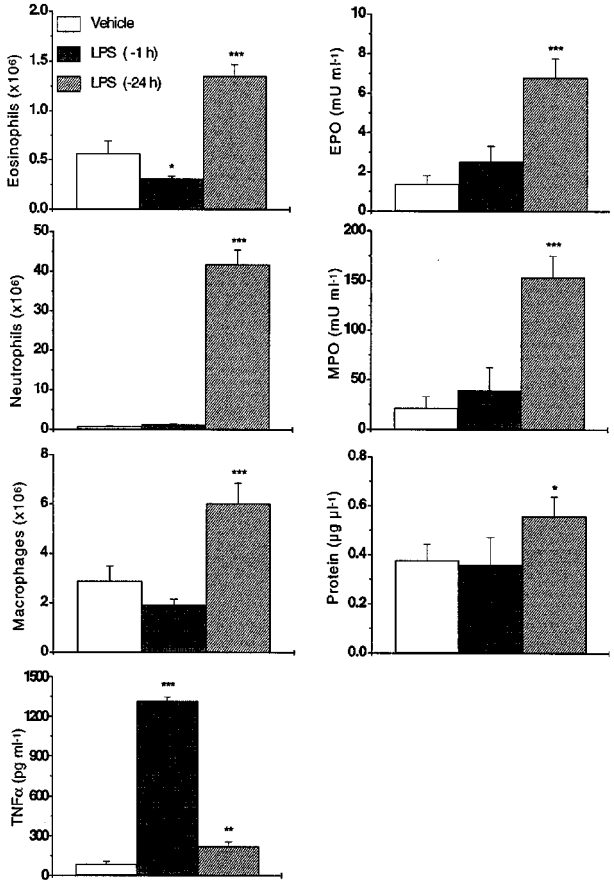

Figure 4 Effect of intratracheal instillation of lipopolysaccharide (LPS, 0.3 mg kg^{-1}) or vehicle (saline, 0.2 ml) on the number of inflammatory cells, the eosinophil peroxidase (EPO) and myeloperoxidase (MPO) activities and the protein and TNFα concentrations in BAL fluid of Brown Norway rats. Animals were killed either 1 or 24 h following LPS administration; vehicle-treated animals were given saline and killed 1 h after treatment. Histograms represent the mean values ($\pm$s.e.means) from 10 animals per group. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ indicates a significant difference from the equivalent value in the vehicle-treated group.

Effects of methysergide and disodium cromoglycate on the augmented bronchoconstrictor response to adenosine induced by LPS

A role for mast cells in the bronchoconstrictor response to adenosine augmented following LPS challenge was explored by evaluating the effects of methysergide, a 5-HT_2 receptor antagonist, and disodium cromoglycate, a mast cell stabilizing agent, on the response.

The design of the experiment with methysergide included 5-HT as a control bronchospasmogen. Sensitivity to 5-HT was unaffected by challenge with LPS, 0.3 mg kg^{-1}, 1 h previously. Pretreatment with methysergide (30 μg kg^{-1} i.v.) induced similar, marked blockade of the bronchoconstrictor responses to both adenosine and 5-HT. In contrast, responses to methacholine were slightly, but significantly, enhanced (Figure 5). Disodium cromoglycate (20 mg kg^{-1} i.v.) had no effect on the bronchoconstrictor response to 5-HT, but induced a marked and significant inhibition of the response to adenosine (Figure 6).

Effects of adenosine receptor antagonists on the augmented response to adenosine induced by LPS challenge in BN rats

The broad-spectrum adenosine receptor antagonist 8-SPT (40 mg kg^{-1} i.v.) induced significant inhibition of the augmented bronchoconstrictor response to adenosine in LPS-challenged animals. In contrast, bronchoconstrictor responses to 5-HT were similar in both 8-SPT and vehicle-treated animals. Testifying to the non-selectivity of 8-SPT, both the bradycardia and hypotension induced by adenosine were blocked by 8-SPT as were the cardiovascular effects of NECA (Figure 7).

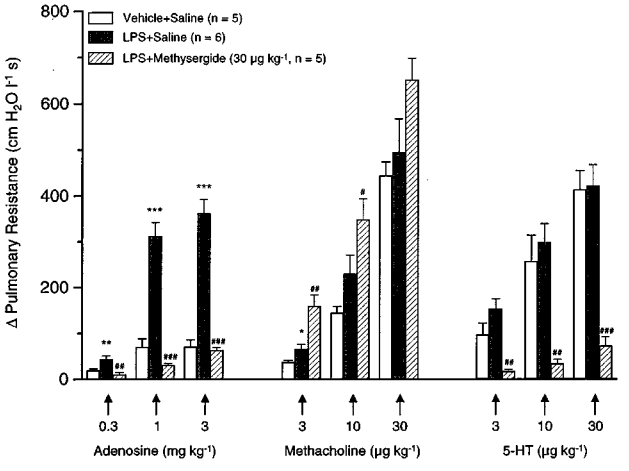

Figure 5 Effect of methysergide (30 μg kg^{-1} given i.v. 5 min prior to the start of the injection sequence) on bronchoconstrictor responses to adenosine, methacholine and 5-HT in Brown Norway rats treated intratracheally 1 h previously with lipopolysaccharide (LPS, 0.3 mg kg^{-1}). Results are expressed as means ± s.e.means of the number (n) of animals shown in parentheses. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ indicates significant difference between animals given LPS plus the vehicle for methysergide (saline) and animals given the vehicle for LPS plus the vehicle for methysergide. $\#P<0.05$, $\#\#P<0.01$, $\#\#\#P<0.001$ indicates significant difference between LPS-challenged animals given methysergide and the corresponding saline-treated control value.

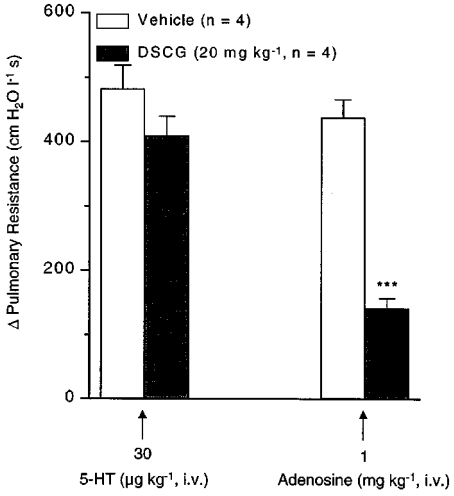

Figure 6 Effect of disodium cromoglycate (DSCG, 20 mg kg^{-1} given i.v. 5 min prior to the start of the injection sequence) on bronchoconstrictor responses to 5-HT and adenosine in Brown Norway rats treated with lipopolysaccharide (LPS, 0.3 mg kg^{-1}). Results are expressed as means ± s.e.means of the number (n) of animals shown in parentheses. $^{***}P<0.001$ indicates significant difference between vehicle- and corresponding DSCG-treated animals.

The selective adenosine 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 8). 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 adenosine A_1 receptor agonist, CPA, was markedly and significantly suppressed in these animals.

The selective adenosine A_{2A} receptor antagonist, ZM 241385 (30 μg kg^{-} i.v.), had no effect on the bronchoconstrictor response to adenosine augmented following treatment with LPS. Unexpectedly, bronchoconstrictor responses to both the low and high doses of 5-HT were significantly inhibited by ZM 241385. 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 adenosine A_{2A} receptor agonist, CGS 21680 (Figure 9).

Discussion

The major finding of the present study is that administration of LPS directly to the lungs of BN rats results in a marked increase in the sensitivity of the airways to the bronchoconstrictor effects of adenosine. The qualitative and quantitative aspects of the change were dependent on the time after challenge that the adenosine was administered. Thus, responses to adenosine were increased markedly and selectively 1 h after LPS administration irrespective of the dose of LPS administered. Selectivity was evident on two grounds: First, with respect to the effects on the airways, since the effects of adenosine on blood pressure and heart rate were minimally affected; second, with respect to the

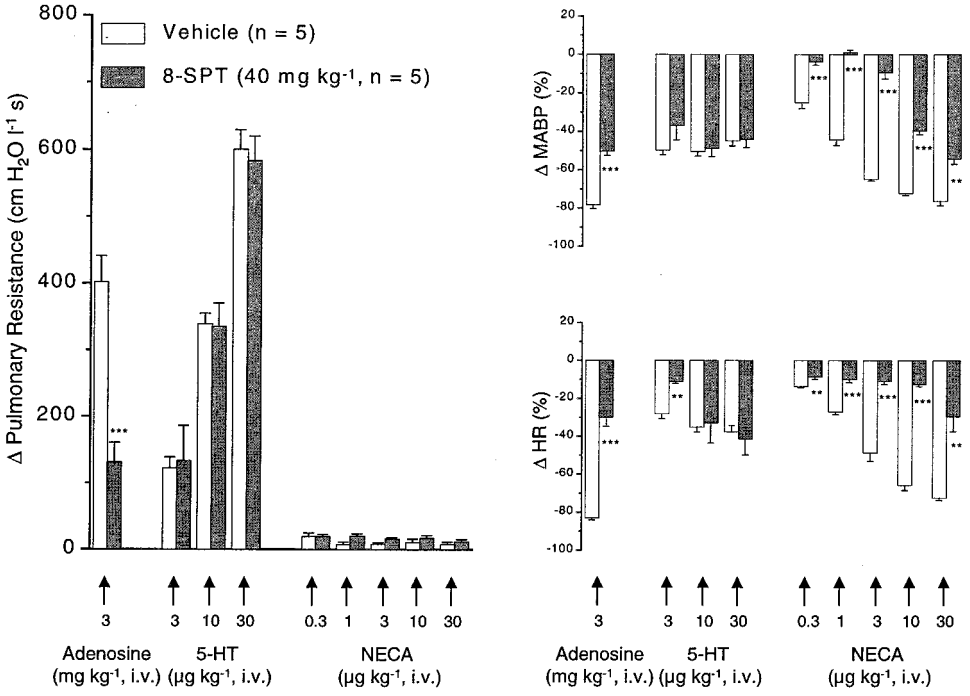

Figure 7 Effect of 8-SPT (40 mg kg^{-1} given i.v. 5 min prior to adenosine) on responses to adenosine, 5-HT and NECA with respect to pulmonary resistance, mean arterial blood pressure (MABP) and heart rate (HR) in Brown Norway rats 1 h post intratracheal instillation of lipopolysaccharide (LPS, 0.3 mg kg^{-1}). Results are expressed as means ± s.e.means of the number (*n*) of animals shown in parentheses. $^{**}P<0.01$, $^{***}P<0.001$ indicates significant difference between vehicle- and corresponding 8-SPT-treated animals.

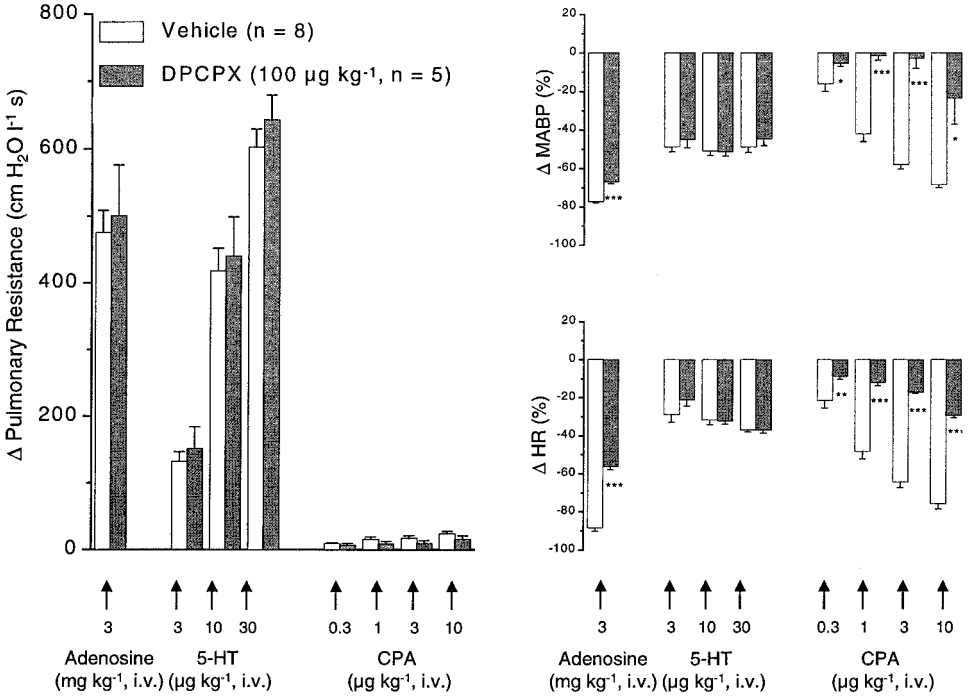

Figure 8 Effect of DPCPX (100 μg kg^{-1} given i.v. 5 min prior to adenosine) on responses to adenosine, 5-HT and CPA with respect to pulmonary resistance, mean arterial blood pressure (MABP) and heart rate (HR) in Brown Norway rats 1 h post intratracheal instillation of lipopolysaccharide (LPS, 0.3 mg kg^{-1}). Results are expressed as means ± s.e.means of the number (*n*) of animals shown in parentheses. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ indicates significant difference between vehicle- and corresponding DPCPX-treated animals.

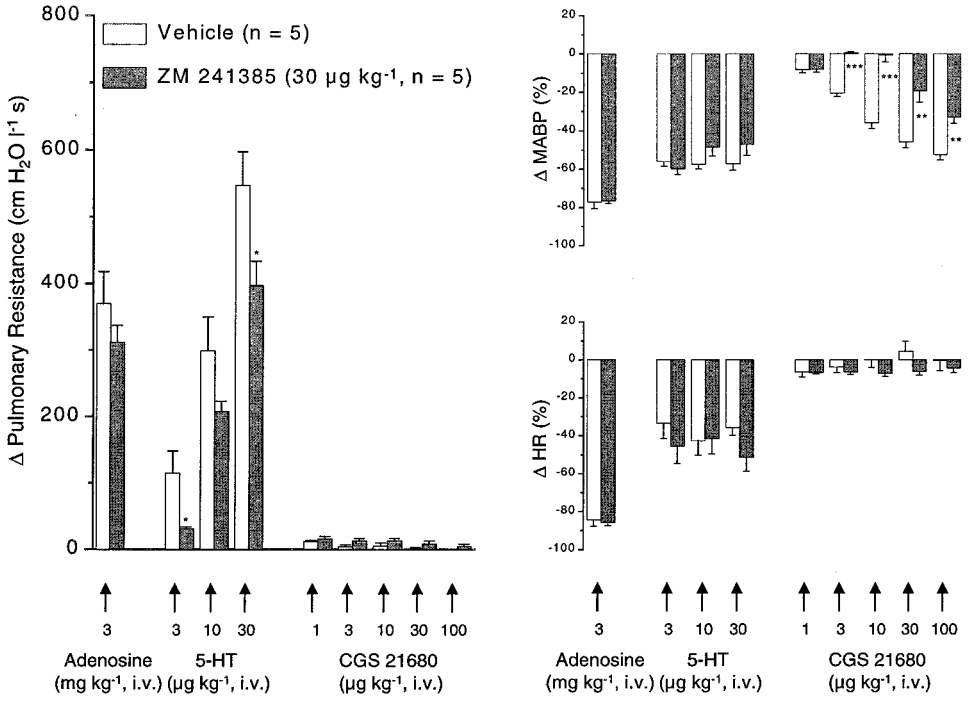

Figure 9 Effect of ZM 241385 (30 μg kg^{-1} given i.v. 5 min prior to adenosine) on responses to adenosine, 5-HT and CGS 21680 with respect to pulmonary resistance, mean arterial blood pressure (MABP) and heart rate (HR) in Brown Norway rats 1 h post intratracheal instillation of lipopolysaccharide (LPS, 0.3 mg kg^{-1}). Results are expressed as means ± s.e.means of the number (n) of animals shown in parentheses. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ indicates significant difference between vehicle- and corresponding ZM 24138-treated animals.

effects on methacholine and 5-HT, which act directly on bronchial smooth muscle (Hannon *et al*., 2001), and which were unaffected by LPS. Such selectivity rules out a pharmacokinetic interaction and/or a non-specific increase in bronchial hyperresponsiveness as explanations for the enhancement of the response to adenosine seen following LPS. The augmentation was short-lived, being greatest 1 h after challenge and absent at 3 h. LPS, 0.3 mg kg^{-1} given 1 h prior to adenosine, was chosen for the analysis of the mechanisms contributing to the augmented response to adenosine.

Airway inflammation is generally considered to be a contributory factor to bronchial hyperresponsiveness (Cockcroft, 1997; O'Connor *et al*., 1999). We therefore investigated the markers of inflammation in the BAL fluid 1 h following treatment with LPS, when the sensitivity to adenosine was markedly increased. In fact, there were no signs of inflammation at this time point as indicated by the leukocyte cell numbers, eosinophil peroxidase and myeloperoxidase activities or protein concentrations in the BAL fluid although, as expected, the TNFα concentrations were elevated. Thus, the present data suggest that LPS-induced inflammation is not the cause of the selective increase in sensitivity to adenosine, but, as noted below, may well contribute to the non-selective decrease in sensitivity observed at the 24 h time point.

It is difficult to make a case for TNFα being the cause of the increase in responsiveness to adenosine. Although TNFα has been implicated in airways hyperresponsiveness to 5-HT in guinea-pigs (Uno *et al*., 1996) and acetylcholine in rats (Kips *et al*., 1992), it appears to play no role in the airways hyperreactivity to methacholine induced by LPS in mice (Lefort *et al*., 1998). Importantly, bronchoconstrictor responses to methacholine and 5-HT in the present studies were unaffected at the time when BAL fluid TNFα concentrations were elevated. Moreover, unlike for LPS (see below) there are no reports of an effect of TNFα on mast cell reactivity, which might result in augmentation of the response to adenosine.

Since challenge with LPS induced minimal short-term effects on the direct acting bronchoconstrictor agents, methacholine and 5-HT, an up-regulation of an indirect mechanism of action comes into focus as the mechanistic basis of the augmented bronchoconstrictor responses to adenosine. Our previous studies established a role for mast cells in the response to adenosine augmented by allergen challenge in actively sensitized BN rats (Hannon *et al*., 2001) by demonstrating blockade with low doses of methysergide, a selective 5-HT$_2$ receptor antagonist (Cohen *et al*., 1985), and with disodium cromoglycate, an inhibitor of mast cell degranulation (Bernstein & Bernstein, 1997). In the present studies, methysergide at a low dose, selective for the 5-HT$_2$ receptors which mediate the response to 5-HT on the airways (Hannon *et al*., 2001), induced marked, quantitatively similar and selective (*vis-à-vis* methacholine) blockade of adenosine and 5-HT. Cromoglycate, used at a dose which in analogous experiments into the mechanism of adenosine induced potentiation following allergen, blocked both the bronchoconstrictor response and the associated mediator release induced by adenosine (Hannon *et al*., 2001), blocked selectively (*vis-à-vis* 5-HT) the bronchoconstrictor response to adenosine. Together, these observations provide compel-

ling evidence that the response to adenosine augmented following LPS is mast-cell mediated.

An intriguing finding was the increased responsiveness to methacholine in the presence of methysergide. A similar trend was seen with a lower dose of methysergide, and also with ketanserin in our earlier experiments to analyse the response to adenosine augmented after allergen challenge (Hannon *et al*., 2001). We have no explanation for the observation.

Again seeking to compare the response to adenosine in animals challenged with LPS with that in sensitized animals challenged with allergen, experiments were carried out to define the receptor subtype(s) implicated in the response. The broad-spectrum adenosine receptor antagonist, 8-SPT, blocked the bronchoconstrictor response to adenosine without affecting those to 5-HT. This observation is 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. However, neither DPCPX nor ZM 241385 was able to block the response to adenosine despite clear evidence from suppression of the cardiovascular effects of subtype-selective agonists that the A_1 and A_{2A} receptors were effectively antagonized in these animals. 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). Our earlier studies established that the dose of 8-SPT used in the present study (40 mg kg^{-1}) does not block A_3 receptor-mediated hypotension in the rat (Fozard & Carruthers, 1993). Moreover, 2-Cl-IB-MECA, a selective A_3 receptor agonist, induced no bronchoconstriction in animals challenged with LPS despite clear evidence from the fall in blood pressure that the A_3 receptors were being activated. Taken together, the findings essentially rule out involvement of the A_3 receptor. Blockade by 8-SPT despite a lack of involvement of A_1, A_{2A} or A_3 receptors resembles the pharmacological profile of the augmented response to adenosine seen following ovalbumin challenge in sensitized BN rats. The receptor site mediating the latter response is atypical in not having the characteristics of any of the four recognized adenosine receptor subtypes (Hannon *et al*., 2002). Our data suggest that a similar site mediates the augmented response to adenosine following LPS challenge.

It should be noted that responses to 5-HT were reduced after ZM 243185, which confirms a similar finding in our earlier study to analyse the response to adenosine augmented following allergen challenge (Hannon *et al*., 2002). We have no explanation for the observation. Importantly, however, the finding does not compromise the conclusion that A_{2A} receptors are not involved in the bronchoconstrictor response to adenosine.

Our analysis of the mechanism by which adenosine is up-regulated following allergen challenge in sensitized animals suggested that 'priming' of a population of mast cells in the lung by the allergen was the key event (Hannon *et al*., 2001). In this context, it is of interest that a direct interaction between LPS and mast cells has recently been described. Thus, intradermal administration of LPS in rats was shown to cause an inflammatory reaction characterized by increased plasma exudation (Iuvone *et al*., 1998). Histological and pharmacological examinations established that the effect was mediated by mast-cell degranulation (Iuvone *et al*., 1999). A direct effect of LPS on mast cells is supported by a further study investigating the mechanisms involved in LPS-induced injury to the neonatal rat colon. Doxantrazole, a non-selective mast-cell stabilizer, dose-dependently reduced the LPS-induced increase in the plasma concentration of mast cell protease-II, a marker of mast cell degranulation (Brown *et al*., 1998). Finally, recent work on the signalling pathway involved in LPS-induced cytokine expression in murine, bone marrow-derived mast cells has revealed the presence of Toll-Like Receptor-4 (TLR-4) expression on mast cells and showed that the existence of functional TLR-4 was essential for LPS-induced mast-cell degranulation (Supajatura *et al*., 2001). Thus, LPS may induce an increase in sensitivity of a discrete lung-based population of mast cells to adenosine in an analogous way to allergen in actively sensitized animals (Hannon *et al*., 2001). However, precise definition of the mechanism(s) involved must await further experimentation.

Responses to adenosine began to decline 3 h after LPS administration and had returned to control values or slightly below at the 24 h time point. Reduced responsiveness at 24 h was not specific to adenosine since responses to the direct acting bronchospasmogen, methacholine, were reduced to a similar extent. BAL fluid analysis at this time point indicated a pronounced neutrophilic pulmonary inflammation. As noted above, airway inflammation is generally considered a contributory factor to bronchial hyperresponsiveness; our findings would, therefore, appear to be anomalous. However, similar hyporesponsiveness has been observed in guinea-pigs (Folkerts *et al*., 1988) and rats (Pauwels *et al*., 1990; Kips *et al*., 1995) $\geqslant$ 12 h following LPS inhalation. Moreover, Kips *et al*. (1995) provided a possible explanation for the phenomenon. They showed that treatment of F344 rats with LPS induced a significant hyporesponsiveness between 9 and 12 h after exposure, which was reversed by L-NAME, an inhibitor of NO synthase. Induction of NO synthase and the consequent over production of NO, a powerful bronchodilator (Nijkamp & Folkerts, 1995), would provide a plausible explanation for the non-selective decline in airway responsiveness induced by LPS in our study.

A marked increase in sensitivity to adenosine of the airways of asthmatic patients is one of the most consistent aspects of the condition (Phillips & Holgate, 1995; Meade *et al*., 2001). The response provides a sensitive marker of disease activity with a close relationship to the inflammatory process (Phillips & Holgate, 1995; Van den Berge *et al*., 2001a; 2001b). We have previously pointed out the striking similarities between the bronchoconstrictor response to adenosine seen following allergen challenge in the actively sensitized BN rat and that on the airways of asthmatics (Fozard & Hannon, 2000; Hannon *et al*., 2001). The present data suggest exposure to LPS may produce the same effect. LPS is ubiquitous in the environment (Kline *et al*., 1999) and in particular is present in high concentrations in organic dusts (Rylander *et al*., 1985) and in house dust (Siraganian *et al*., 1979). It is also generated in the course of airway infections. Exposure to LPS could be a factor which, along with allergen, determines the increased sensitivity of the airways of asthmatics to adenosine.

It is a pleasure to thank E. Schaeublin and F. Baur for their excellent technical support.

References

AMDUR, M.O. & MEAD, J. (1958). Mechanics of respiration in unanesthetized guinea-pigs. *Am. J. Physiol.,* **192,** 364 – 368.

BECKMANN, N., TIGANI, B., EKATODRAMIS, D., BORER, R., MAZZONI, L. & FOZARD, J.R. (2001). Pulmonary edema induced by allergen challenge in the rat: Non-invasive assessment by magnetic resonance imaging. *Magn. Reson. Med.,* **45,** 88 – 95.

BERNSTEIN, J.A. & BERNSTEIN I. L. (1997). Cromones. In: *Asthma*. eds Barnes, P.J., Grunstein, M.M., Leff, A.R. & Woolcock, A.J., pp. 1647 – 1665. Lippincott – Raven: New York.

BROWN, J.F., CHAFEE, K.A. & TEPPERMAN, B.L. (1998). Role of mast cells, neutrophils and nitric oxide in endotoxin-induced damage to the neonatal rat colon. *Br. J. Pharmacol.,* **123,** 31 – 38.

COCKCROFT, D.W. (1997). Airway responsiveness. In: *Asthma*. eds Barnes, P.J., Grunstein, M.M., Leff, A.R. & Woolcock, A.J., pp. 1253 – 1266. Lippincott – Raven: New York.

COHEN, M.L., SCHENCK, K.W., COLBERT, W. & WITTENAUER, L. (1985). Role of 5-HT2 receptors in serotonin-induced contractions of non-vascular smooth muscle. *J. Pharmacol. Exp. Ther.,* **232,** 770 – 774.

FOLKERTS, G., HENRICKS, P.A J., SLOOTWEG, P.J. & NIJKAMP, F.P. (1988). Endotoxin-induced inflammation and injury of the guinea pig respiratory airways cause bronchial hyporeactivity. *Am. Rev. Respir. Dis.,* **137,** 1441 – 1448.

FOZARD, J.R. (1982). Mechanism of the hypotensive effect of ketanserin. *J. Cardiovasc. Pharmacol.,* **4,** 829 – 838.

FOZARD, J.R. & CARRUTHERS, A.M. (1993). The cardiovascular effects of selective adenosine A_1 and A_2 receptor agonists in the pithed rat: no role for glibenclamide-sensitive potassium channels. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **347,** 192 – 196.

FOZARD, J.R. & HANNON, J.P. (2000). Species differences in adenosine induced bronchoconstrictor responses. *Clin. Exp. Allergy,* **30,** 1213 – 1220.

FOZARD, J.R., RONDEAU, C.M., HANNON, J.P., TIGANI, B. & MAZZONI, L. (2000). Mechanism of airway hyperreactivity to adenosine induced by LPS challenge Brown Norway rats. *Br. J. Pharmacol.,* **129,** 94P.

FREDHOLM, B.B., IJZERMAN, A.P., JACOBSON, K.A., KLOTZ, K.N. & LINDEN, J. (2001). International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol. Rev.,* **53,** 527 – 552.

HANNON, J.P., PFANNKUCHE, H.-J. & FOZARD, J.R. (1995). A role for mast cells in adenosine A_3 receptor-mediated hypotension in the anaesthetised rat. *Br. J. Pharmacol.,* **115,** 945 – 952.

HANNON, J.P., TIGANI, B., WOLBER, C., WILLIAMS, I., MAZZONI, L., HOWES, C. & FOZARD, J.R. (2002). Evidence for an atypical receptor mediating the augmented bronchoconstrictor response to adenosine induced by allergen challenge in actively sensitised Brown Norway rats. *Br. J. Pharmacol.,* **135,** 685 – 696.

HANNON, J.P., TIGANI, B., WILLIAMS, I., MAZZONI, L. & FOZARD, J.R. (2001). Mechanism of airway hyperresponsiveness to adenosine induced by allergen challenge in actively sensitized Brown Norway rats. *Br. J. Pharmacol.,* **132,** 1509 – 1523.

IUVONE, T., D'ACQUISTO, F., VAN DEN OSSELAER, N., DI ROSA, M., CARNUCCIO, R. & HERMAN, A.G. (1998). Evidence that inducible nitric oxide synthase is involved in LPS-induced plasma leakage in rat skin through the activation of nuclear factor-kappaB. *Br. J. Pharmacol.,* **123,** 1325 – 1330.

IUVONE, T., VAN DEN BOSSCHE, R., D ACQUISTO, F., CARNUCCIO, R. & HERMAN, A.G. (1999). Evidence that mast cell degranulation, histamine and tumour necrosis factor α release occur in LPS-induced plasma leakage in rat skin. *Br. J. Pharmacol.,* **128,** 700 – 704.

JI, X.-D., VON LUBITZ, D., OLAH, M.E., STILES, G.L. & JACOBSON, K.A. (1994). Species differences in ligand affinity at central A_3 adenosine receptors. *Drug Dev. Res.,* **33,** 51 – 59.

KIPS, J.C., CUVELIER, C.A. & PAUWELS, R.A. (1992). Effect of acute and chronic antigen inhalation on airway morphology and responsiveness in actively sensitized rats. *Am. Rev. Respir. Dis.,* **145,** 1306 – 1310.

KIPS, J.C., LEFEBVRE, R.A., PELEMAN, R.A., JOOS, G.F. & PAUWELS, R.A. (1995). The effect of a nitric oxide synthase inhibitor on the modulation of airway responsiveness in rats. *Am. J. Respir. Crit. Care Med.,* **151,** 1165 – 1169.

KLINE, J.N., COWDEN, J.D., HUNNINGHAKE, G.W., SCHUTTE, B.C., WATT, J.L., WOHLFORD-LENANE, C.L., POWERS, L.S., JONES M.P. & SCHWARTZ, D.A. (1999). Variable airway responsiveness to inhaled lipopolysaccharide. *Am. J. Respir. Crit. Care Med.,* **160,** 297 – 303.

LEFORT, J., SINGER, M., LEDUC, D., RENESTO, P., NAHORI, M.A., HUERRE, M., CRÉMINON, C., CHIGNARD, M. & VARGAFTIG, B.B. (1998). Systemic administration of rndotoxin induces bronchopulmonary hyperreactivity dissociated from TNF-α formation and neutrophil sequestration into the murine lungs. *J. Immunol.,* **161,** 474 – 480.

MARQUARDT, D.L. (1997). Adenosine. In: *Asthma*. eds Barnes, P.J., Grunstein, M.M., Leff, A.R. & Woolcock, A.J., pp. 585-59. Lippincott – Raven: New York.

MEADE, C.J., DUMANT, I. & WORRALL, L. (2001). Why do asthamatic subjects respond so strongly to inhaled adenosine? *Life Sci.,* **69,** 1225 – 1240.

NIJKAMP, F.P. & FOLKERTS, G. (1995). Nitric oxide and bronchial hyperresponsiveness. *Arch. Int. Pharmacodyn.,* **329,** 81 – 96.

O'CONNOR, B.J., CROWTHER, S.D., COSTELLO, J.F. & MORLEY, J. (1999). Selective airway responsiveness in asthma. *Trends Pharmacol. Sci.,* **20,** 9 – 11.

PAUWELS, R.A., KIPS, J.C., PELEMAN, R.A. & VAN DER STRAETEN, M.E. (1990). The effect of endotoxin inhalation on airway responsiveness and cellular influx in rats. *Am. Rev. Resp. Dis.,* **141,** 540 – 545.

PHILLIPS, G.D. & HOLGATE, S.T. (1995). Adenosine and the airways in asthma. In: *Asthma and Rhinitis*. eds Busse, W.W. & Holgate, S.T., pp. 851 – 857. Blackwell Scientific: Oxford.

RYLANDER, R., HAGLIND, P. & LUNDHOLM, M. (1985). Endotoxin in cotton dust and respiratory function decrement among cotton workers in an experimental cardroom. *Am. Rev. Respir. Dis.,* **131,** 209 – 213.

SUPAJATURA, V., USHIO, H., NAKAO, A, OKUMURA, K, RA, C. & OGAWA, H. (2001). Protective roles of mast cells against enterobacterial infection are mediated by Toll-Like Receptor 4. *J. Immunol.,* **167,** 2250 – 2256.

SIRAGANIAN, R.P., BAER, H., HOCHSTEIN, H.D. & MAY, J.C. (1979). Allergen and biologic activity of commercial preparation of house dust extract. *J. Allergy Clin. Immunol.,* **64,** 526.

UNO, T., TANAKA, H. & NAGAI, H. (1996). Cysteinyl leukotrienes do not mediate lipopolysaccharide-induced airway hyperresponsiveness in guinea pigs. *Prostaglandins,* **52,** 447 – 461.

VAN DEN BERGE, M., KERSTJENS, H.A.M., MEIJER, R.J., DE REUS, D.M., KOETER, G.H., KAUFFMAN, H.F. & POSTMA, D.S. (2001a). Corticosteroid-induced improvement in the PC_{20} of adenosine monophosphate is more closely associated with reduction in airway inflammation than improvement in the PC_{20} of methacholine. *Am. J. Respir. Crit. Care Med.,* **164,** 1127 – 1132.

VAN DEN BERGE, M., MEIJER, R.J., KERSTJENS, H.A.M., DE REUS, D.M., KOETER, G.H., KAUFFMAN, H.F. & POSTMA, D.S. (2001b). PC_{20} AMP is more closely associated with airway inflammation in asthma than PC_{20} methacholine. *Am. J. Respir. Crit. Care Med.,* **163,** 1546 – 1550.

VAN GALEN, P.J.M., VAN BERGEN, A.H., GALLO-RODRIGUEZ, C., MELMAN, N., OLAH, M.E., IJZERMAN, A.P., STILES, G.L. & JACOBSON, K.A. (1994). A binding site model and structure-activity relationships for the rat A_3 adenosine receptor. *Mol. Pharmacol.,* **45,** 1101 – 1111.

VAN SCHAICK, E.A., JACOBSON, K.A., KIM, H.O., IJZERMAN, A.P. & DANHOF, M. (1996). Haemodynamic effects and histamine release elicited by the selective adenosine A_3 receptor agonist 2-Cl-IB-MECA in conscious rats. *Eur. J. Pharmacol.,* **308,** 311 – 314.

(Received December 14, 2001
Revised February 4, 2002
Accepted February 20, 2002)