Br. J. Pharmac. (1982), **76**, 381–387

EVIDENCE FOR AN A_2/R_a ADENOSINE RECEPTOR IN THE GUINEA-PIG TRACHEA

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1 An attempt was made to determine whether the extracellular adenosine receptor that mediates relaxation in the guinea-pig trachea is of the A_1/R_i or A_2/R_a subtype.

2 Dose-response curves to adenosine and a number of 5′- and N^6-substituted analogues were constructed for the isolated guinea-pig trachea, contracted with carbachol.

3 The 5′-substituted analogues of adenosine were the most potent compounds tested, the order of potency being 5′-N-cyclopropylcarboxamide adenosine (NCPCA) > 5′-N-ethylcarboxamide adenosine (NECA) > 2-chloroadenosine > L-N^6-phenylisopropyladenosine (L-PIA) > adenosine > D-N^6-phenylisopropyladenosine (D-PIA).

4 The difference in potency between the stereoisomers D- and L-PIA on the isolated trachea was at the most five fold.

5 Responses to low doses of adenosine and its analogues were attenuated after treatment with either theophylline or 8-phenyltheophylline. The responses to 2-chloroadenosine were affected to a lesser extent than were those to the other purines.

6 Adenosine transport inhibitors, dipyridamole and dilazep, potentiated responses to adenosine, did not affect those to NCPCA, NECA, L-PIA and D-PIA but significantly reduced the responses to high doses of 2-chloroadenosine.

7 Relaxations evoked by 9-β-D-xylofuranosyladenosine which can activate intracellular but not extracellular adenosine receptors, were attenuated by dipyridamole but unaffected by 8-phenyltheophylline.

8 The results support the existence of an extracellular A_2/R_a subtype of adenosine receptor and an intracellular purine-sensitive site, both of which mediate relaxation.

Introduction

Purine nucleosides are potent relaxants of tracheal musculature (Coleman, 1976; Farmer & Farrar, 1976) and according to the classification of Burnstock (1978) appear to mediate their effects through an extracellular adenosine (P_1) receptor (Farmer & Farrar, 1976; Christie & Satchell, 1980). A subclassification of adenosine receptors has been proposed by two independent groups based on their observations of the adenylate cyclase system. Londos, Cooper, Schlegel & Rodbell (1978) have proposed that R_a-receptors mediate activation and R_i-receptors inhibition of adenylate cyclase. Van Calker, Muller & Hamprecht (1979) designated A_1-receptors as those which inhibit cyclic AMP accumulation and A_2-receptors as those which stimulate its accumulation. Both classifications rely on the relative potency of certain adenosine analogues in particular N^6-substituted analogues such as N^6-phenylisopropyladenosine (PIA) and 5′-substituted analogues such as 5′-N-ethylcarboxamide adenosine (NECA). At the A_2/R_a-receptor, the order of potency is NECA > adenosine > PIA, while the reverse is true at the A_1/R_i-receptor. Furthermore, at A_1-receptors the action of PIA is stereospecific, L-PIA being up to 100 times more potent than D-PIA. At the A_2-receptor the two stereoisomers of PIA are nearly equipotent, with D-PIA being only five times less potent than its L-isomer (Bruns, Daly & Snyder, 1980). Both receptor subtypes are antagonized by the methylxanthines, such as theophylline. In addition to these two subclasses of extracellular adenosine receptor, an intracellular site (P-site) at which adenosine, 2-chloroadenosine and certain ribose modified analogues may interact has also been identified (Londos & Wolff, 1977). This site is methylxanthine-insensitive. The object of the present study was to investigate the adenosine receptor in the guinea-pig trachea and to determine to which sub-class of P_1-receptor it belonged.

0007-1188/82/070381-07 $01.00

Methods

Guinea-pigs (300–500 g) of either sex were killed by cervical dislocation and the trachea removed and placed in physiological salt solution (PSS). Tracheal strips were prepared by the method of Emmerson & Mackay (1979) and placed in 20 ml organ baths containing PSS at 37°C and gassed with a mixture of 95% O_2 and 5% CO_2. The composition of the PSS was as follows (mmol/l): NaCl 118.4, KCl 4.7, KH_2PO_4 1.2, $CaCl_2 2H_2O$ 2.5, $NaHCO_3$ 25.0, glucose 5.5, $MgSO_4$ 1.2 and $CaNa_2$ EDTA 0.03. The tracheal strips were connected to Pioden UFI isometric strain gauge transducers (range ± 56 g) and the resting tension adjusted to 1 g. After an equilibration period of 45–60 min the tracheae were contracted with carbachol (0.5 μM) and left until a steady tone had developed. Cumulative concentration-effect curves to the agonists were then constructed utilizing a 4 min exposure period. Responses to the purines were expressed as a percentage of the maximal relaxation evoked by noradrenaline. In those experiments where the effect of purine transport blockers or receptor antagonists were studied, the drugs were incubated with the tissues for 30 min and the trachea then recontracted to the control level of tone with carbachol.

Drugs used: adenosine (Sigma), (−)-noradrenaline bitartrate ((−)-arterenol bitartrate, Sigma), 2-chloroadenosine (Sigma), dilazep hydrochloride (Asta-Werke), dipyridamole (persantin, Boehringer Ingelheim), isoprenaline sulphate (Sigma), L-N^6-phenylisopropyladenosine (Boehringer Mannheim), 8-phenyltheophylline (Calbiochem), theophylline (Sigma). The following compounds were synthesized by J. Preston & R. Maisey, Pharmaceuticals Division, ICI Alderley Park: 5′-N-cyclopropylcarboxamide adenosine, 5′-N-ethylcarboxamide adenosine, D-N^6-phenylisopropyladenosine and 9-β-D-xylofuranosyladenosine. All compounds were made up in aqueous solution except 8-phenyltheophylline in which case a stock solution of 10 mM was made up in 80% v/v methanol containing 0.2 M NaOH, and aqueous dilutions of this used. In those experiments where 8-phenyltheophylline was used, all control responses were measured in the presence of an equivalent amount of solvent.

Analysis of results and statistics

All results are expressed as the mean ± s.e.mean. Significance of difference in the experimental results was calculated by a paired *t* test and assumed significant at the 5% probability level. The ED_{20} was used for comparison of control and drug-treated data as this was in the mid portion of the dose-response curves.

Results

Effect of NCPCA, NECA, 2-chloroadenosine, adenosine, L- and D-PIA

Potency of agonists All the purine nucleosides relaxed the guinea-pig trachealis muscle in a dose-dependent manner. The 5′-substituted analogues of adenosine, 5′-N-cyclopropylcarboxamide adenosine (NCPCA) and NECA were the most potent of the adenosine analogues tested (Figure 1). The order of agonist potency for relaxing the trachea was NCPCA > NECA > 2-chloroadenosine > L-PIA ≥ adenosine > D-PIA (Table 1). High doses of 2-chloroadenosine (30–200 μM) elicited greater responses than did NECA and NCPCA. However, poor solubility prevented full dose-response curves to D- and L-PIA being constructed. The maximum response obtained to D-PIA was often less than the 20% response level which was normally used for comparison, thus the ED_{15} was employed (Table 1).

Effect of dipyridamole In the presence of dipyridamole (5 μM) which blocks purine transport, sub-maximal responses to adenosine were potentiated (Table 1) but those to the other agonists were not. Under these conditions the order of agonist potency was NCPCA > NECA > 2-chloroadenosine = adenosine > L-PIA > D-PIA (Figure 2). In addition the responses to high doses of 2-chloroadenosine were significantly reduced after dipyridamole treatment, while responses to high doses of adenosine, the other purines and isoprenaline were unaffected (Table 1). The effect of another purine transport blocker, dilazep, was compared with dipyridamole. This drug also potentiated responses to adenosine, and reduced the response to high doses of 2-chloroadenosine (Table 1) without significantly affecting responses to high doses of isoprenaline.

Effect of theophylline The effect of theophylline (100 μM) on the response of the trachea to adenosine and its analogues was tested in the presence of dipyridamole (5 μM). Theophylline antagonized the responses to low doses of adenosine and its analogues (Table 2), with the exception of 2-chloroadenosine which was not significantly affected by the methylxanthine (Figure 3, Table 2). Responses to D-PIA were attenuated by theophylline (D-PIA(100 μM), control: 23.8 ± 3.8%; plus theophylline (100 μM): 10.6 ± 0.9%) but as the response to the highest dose of D-PIA (100 μM) was very small, these values were

not included in Table 2. Responses to high doses of the purines, were often slightly potentiated by theophylline as were responses to isoprenaline (Table 2). 8-Phenyltheophylline (10 μM) attenuated responses to both adenosine and 2-chloroadenosine (Figure 4) without having any significant effect on responses to isoprenaline (Table 2). However, the shift in the 2-chloroadenosine dose-response curve was significantly less than that observed with adenosine.

Effect of the P-site agonist 9-β-D-xylofuranosyladenosine

9-β-D-Xylofuranosyladenosine elicited small relaxations of the guinea-pig trachea when applied in high concentrations. This effect was attenuated in the presence of dipyridamole (5 μM), but was not significantly affected by 8-phenyltheophylline (10 μM) (Figure 5).

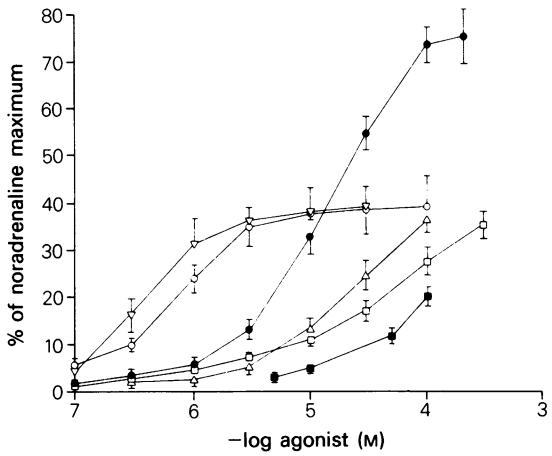

Figure 1 The effect of adenosine and analogues on the guinea-pig isolated trachea: 5′-N-cyclopropylcarboxamide adenosine (▽), 5′-N-ethylcarboxamide adenosine (○), 2-chloroadenosine (●), L-N^6-phenylisopropyladenosine (△), adenosine (□) and D-N^6-phenylisopropyladenosine (■). Each point represents the mean of at least eight experiments, vertical lines are s.e.mean.

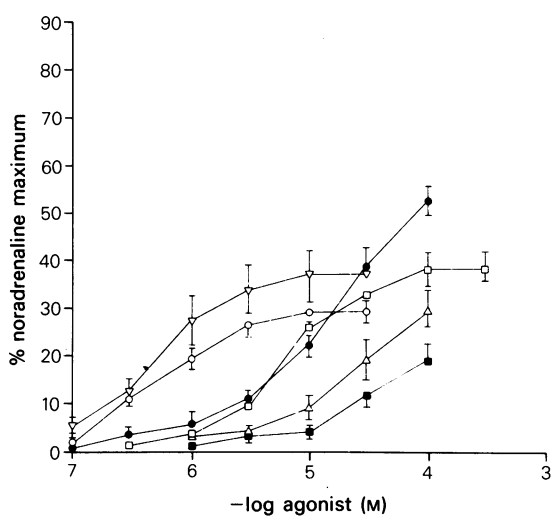

Figure 2 The effect of adenosine and analogues on the guinea-pig isolated trachea in the presence of dipyridamole (5 μM): 5′-N-cyclopropylcarboxamide adenosine (▽), 5′-N-ethylcarboxamide adenosine (○), 2-chloroadenosine (●), adenosine (□), L-N^6-phenylisopropyladenosine (△) and D-N^6-phenylisopropyladenosine (■). Each point represents the mean of at least eight experiments; vertical lines are s.e.mean.

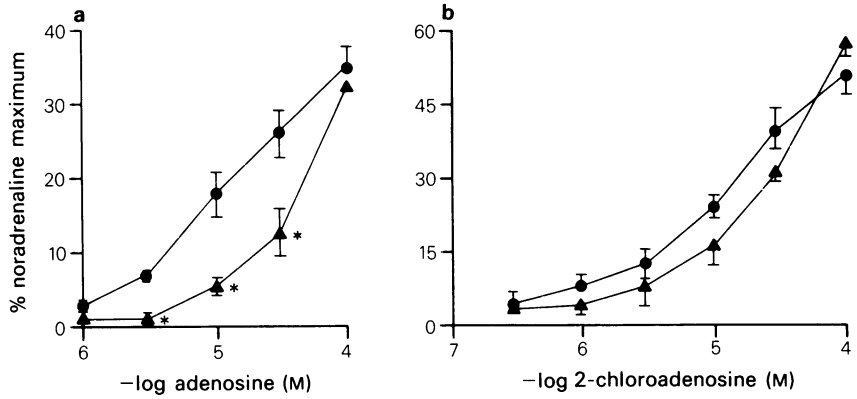

Figure 3 The effect of theophylline (100 μM) on the response of the guinea-pig isolated trachea to (a) adenosine and (b) 2-chloroadenosine. In each graph, control (●), theophylline (▲) and each point represents the mean of at least six experiments; vertical lines are s.e.mean. $*P < 0.05$.

Discussion

Since adenosine receptors have not yet been isolated or chemically characterized, their classification must depend on the application of pharmacological procedures. Thus selected agonists and antagonists have been used to reveal the similarities or differences in these receptors. The 5′ and N^6 substituted analogues of adenosine have been used to classify adenosine receptors into A_1/R_i and A_2/R_a sub-types (Londos *et*

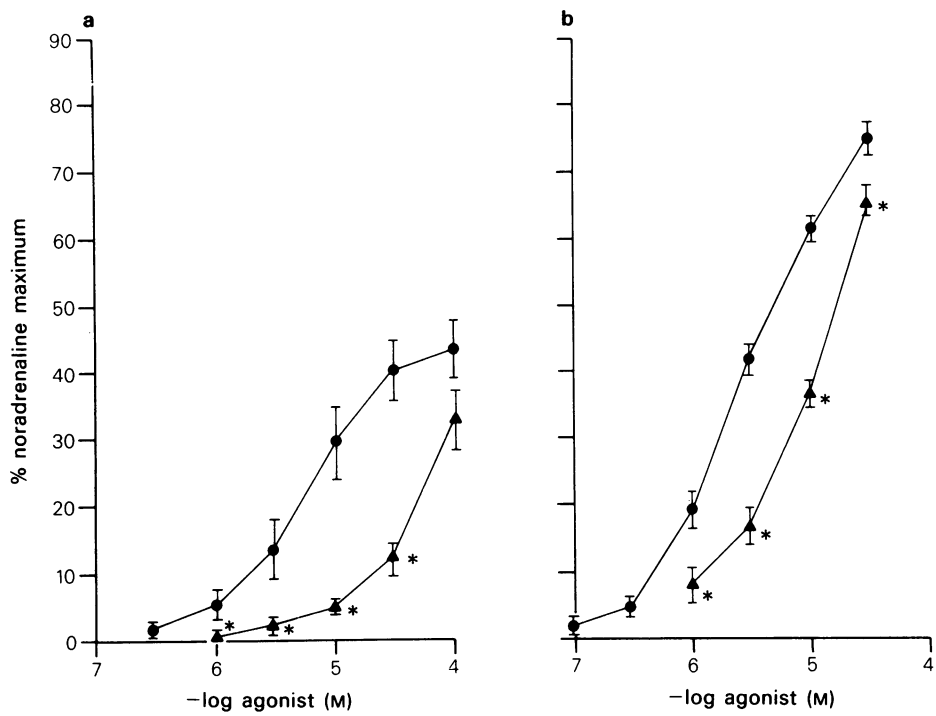

Figure 4 The effect of 8-phenyltheophylline (10 μM) on the response of the guinea-pig isolated trachea to (a) adenosine and (b) 2-chloroadenosine. In each graph, control (●), 8-phenyltheophylline (▲), and each point represents the mean of at least eight experiments; vertical lines are s.e.mean; $*P < 0.05$.

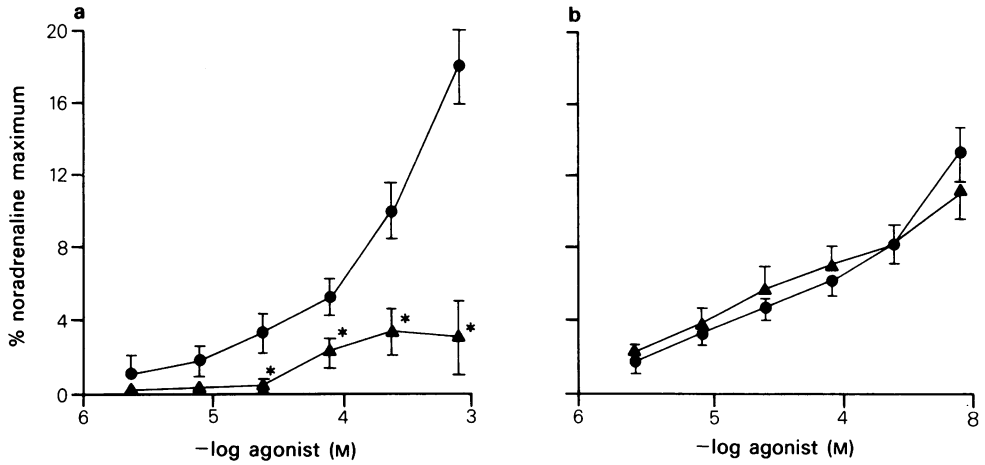

Figure 5 The effect of (a) dipyridamole (5 μM) and (b) 8-phenyltheophylline (10 μM) on the response of the guinea-pig isolated trachea to 9-β-D-xylofuranosyladenosine. In each graph, control (●), drug-treated (▲) and each point represents the mean of at least four experiments; vertical lines are s.e.mean; $*P < 0.05$.

al., 1978; Van Calker *et al.*, 1979).

The extracellular adenosine receptor in the guinea-pig trachea that mediates relaxation appears to be of the A_2/R_a sub-type. This conclusion is prompted by two lines of evidence. Firstly, the 5'-substituted analogues of adenosine (NCPCA, and NECA) were significantly more potent than the N^6-substituted analogues (L- and D-PIA). These analogues exhibit a similar order of potency as stimulants of adenylate cyclase in cell cultures, which is an A_2/R_a mediated effect (Londos *et al.*, 1978; Van Calker *et al.*, 1979). Secondly, the difference in the potency of D- and L-PIA in the trachea was at most five fold. Ligand binding and isolated tissue studies have demonstrated a difference in potency of about one hundred fold between these isomers at A_1-receptors, but only a five fold difference at the A_2-receptor (Bruns *et al.*, 1980; Paton, 1981).

The apparent order of potency of adenosine and its analogues on the trachea could be influenced by tissue uptake and metabolism. Compounds that inhibit purine transport such as dipyridamole (Stafford, 1966; Baer & Vriend, 1981) reduce both transport and metabolism, and are the most effective mechanism of preventing purine disposition. Dipyridamole potentiated responses evoked by adenosine but not those evoked by the 5'- and N^6-substituted analogues. Thus the relative potency of these adenosine analogues in the guinea-pig trachea was not altered.

The order of agonist potency can be used to characterize receptor types if these agents mediate their effects via the same receptor. Both A_1/R_i and A_2/R_a-receptors are competitively antagonized by methylxanthines (Londos *et al.*, 1978; Bruns *et al.*, 1980). In the absence of specific antagonists for these receptor sub-types it cannot be conclusively proved that the agonists used interact solely with A_2/R_a-receptors in the trachea. However, methylxanthines can be used to determine whether these agonists have their effects via adenosine (P_1) receptors or via other purine-sensitive sites (P_2-receptors, intracellular P-site). Responses evoked by low concentrations of NECA, NCPCA, D- and L-PIA and adenosine were inhibited to the same extent by theophylline, implying that their effects were mediated via extracellular adenosine receptors. Responses to high concentrations of these agonists were affected to a lesser extent. This latter observation probably represents inhibition of phosphodiesterase by theophylline (Amer & Kreighbaum, 1975) since responses to isoprenaline were also potentiated. It is known that phosphodiesterase inhibitors devoid of adenosine receptor antagonism potentiate responses to purines in this tissue (Coleman, 1980). 8-Phenyltheophylline is a more potent P_1-antagonist than theophylline but has less effect on phosphodiesterase (Smellie, David, Daly & Wells, 1979). This compound caused a parallel shift to the right of the dose-response curve to adenosine and did not affect responses to isoprenaline.

High doses of 2-chloroadenosine elicited greater responses than did the other agonists. Responses evoked by these high doses of 2-chloroadenosine were depressed by dipyridamole and another purine transport inhibitor, dilazep. This indicates that there may be an intracellular site at which 2-chloroadenosine can act to evoke relaxation of the

trachealis muscle. Responses evoked by 2-chloroadenosine were not significantly attenuated by theophylline (100 μM). 8-Phenyltheophylline did antagonize responses to 2-chloroadenosine but caused a greater blockade of adenosine-induced responses. These results also suggest that the relaxant effects of 2-chloroadenosine are not due solely to interaction with an extracellular adenosine receptor. Londos & Wolff (1977) have proposed the existence of an intracellular purine-sensitive site associated with the adenylate cyclase enzyme. 9-β-D-Xylofuranosyladenosine activates this intracellular site without having any effect on extracellular adenosine receptors. 9-β-D-Xylofuranosyladenosine

Table 1 The effect of dipyridamole (5 μM) and dilazep (10 μM) on the response of the guinea-pig isolated trachea to adenosine and analogues

		Control	*Dipyridamole* (5 μM)		*Control*	*Dilazep* (10 μM)
Adenosine	$-\log ED_{20}$	4.31 ± 0.11	5.06 ± 0.12*	$-\log ED_{20}$	4.28 ± 0.16	5.34 ± 0.8*
	response at 300 μM	35.5 ± 2.9	38.8 ± 3.2	response at 300 μM	37.6 ± 3.4	42.1 ± 4.7
2-chloroadenosine	$-\log ED_{20}$	5.30 ± 0.06	5.14 ± 0.08	$-\log ED_{20}$	5.18 ± 0.29	5.16 ± 0.11
	response at 100 μM	73.9 ± 3.4	53.1 ± 2.9*	response at 300 μM	75.1 ± 10.6	59.6 ± 4.2*
NCPCA	$-\log ED_{20}$	6.37 ± 0.18	6.24 ± 0.22	–	–	–
	response at 30 μM	39.00 ± 6.6	37.0 ± 5.3			
NECA	$-\log ED_{20}$	5.89 ± 0.15	5.61 ± 0.14	–	–	–
	response at 30 μM	38.9 ± 5.8	30.0 ± 2.5			
L-PIA	$-\log ED_{20}$	4.56 ± 0.13	4.46 ± 0.12	–	–	–
	response at 100 μM	36.8 ± 4.3	30.0 ± 3.8			
D-PIA†	$-\log ED_{15}$	4.33 ± 0.08	4.34 ± 0.14	–	–	–
	response at 100 μM	21.2 ± 1.9	22.5 ± 3.6			
Isoprenaline	$-\log ED_{20}$	7.94 ± 0.05	8.0 ± 0.09	$-\log ED_{20}$	8.18 ± 0.08	8.30 ± 0.09
	response at 0.3 μM	87.6 ± 3.9	89.3 ± 2.9	response at 0.3 μM	84.4 ± 3.2	84.4 ± 3.7

†ED_{15} rather than ED_{20} see text. *$P < 0.05$

Data expressed as the mean ± s.e.mean. Response to agonists expressed as a % of the maximum relaxation to noradrenaline. n = at least 8.

NCPCA = 5′-N-cyclopropylcarboxamide adenosine; NECA = 5′-N-ethylcarboxamide adenosine; LPIA L-N^6-phenylisopropyladenosine; DPIA = D-N^6-phenylisopropyladenosine.

Table 2 The effect of theophylline (100 μM) and 8-phenyltheophylline (10 μM) on the $-\log ED_{20}$ for adenosine and analogues on the guinea-pig trachea

	Control	*Theophylline* (100 μM)	*Control*	*8-phenyltheophylline* (10 μM)
Adenosine	4.83 ± 0.14	4.24 ± 0.17*	5.27 ± 0.16	4.30 ± 0.10*
2-chloroadenosine	5.21 ± 0.07	5.03 ± 0.14	5.01 ± 0.07	4.44 ± 0.07*
NCPCA	6.47 ± 0.12	5.86 ± 0.12*	–	–
NECA	5.59 ± 0.17	5.22 ± 0.11*	–	–
L-PIA	4.60 ± 0.19	4.16 ± 0.05*	–	–
Isoprenaline	8.00 ± 0.09	8.15 ± 0.13*	7.97 ± 0.14	7.98 ± 0.17

*$P < 0.05$.

Data expressed as the mean ± s.e.mean. n = at least 6.

NCPCA = 5′-N-cyclopropylcarboxamide adenosine; NECA = 5′-N-ethylcarboxamide adenosine; L-PIA = L-N^6-phenylisopropyladenosine.

in high concentrations evoked relaxations of the guinea-pig trachea, which were attenuated after dipyridamole treatment. 8-Phenyltheophylline did not attenuate responses to 9-β-D-xylofuranosyladenosine; consequently the relaxant effects of this compound appear to be independent of extracellular adenosine receptor activation.

In conclusion, the guinea-pig trachea possesses both extracellular and intracellular purine-sensitive sites that mediate relaxation. The intracellular site exhibits some of the characteristics of the P-site described by Londos & Wolff (1977). The extracellular receptor appears to be of the A_2/R_a sub-type. However, definitive proof of the A_1/R_i, A_2/R_a subclassification of adenosine receptors must await the development of specific antagonists for these receptors.

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(Received February 26, 1982.)