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EVIDENCE FOR STEREOSPECIFICITY OF THE P_1-PURINOCEPTOR

C. BROWN[1], G. BURNSTOCK, N.J. CUSACK[2], P. MEGHJI & C.J. MOODY

Department of Anatomy & Embryology and Centre for Neuroscience, University College London, Gower Street, London WC1E 6BT

1 The effects of adenosine, 5'-N-ethylcarboxamidoadenosine (NECA), 2-chloroadenosine, 2-azidoadenosine, and their L-enantiomers were examined on driven left atria, trachea and transmurally stimulated ileum of the guinea-pig.

2 In each tissue the order of potency of the D-enantiomers for producing inhibitory effects was NECA > 2-chloroadenosine ≥ 2-azidoadenosine > adenosine.

3 The log concentration-response curve of each agonist was shifted to the right in the presence of the P_1-purinoceptor antagonist, theophylline.

4 Dipyridamole, which blocks adenosine uptake, potentiated the effects of adenosine but not those of the D-enantiomers of adenosine analogues.

5 The greater potency of the adenosine analogues therefore, is at least partly due to their resistance to tissue uptake and subsequent enzymatic destruction.

6 The L-enantiomers of adenosine and its analogues did not produce inhibitory responses in the driven left atria or transmurally stimulated ileum. At high concentrations relaxations of the tracheal muscle were obtained, with the potency series L-NECA > 2-chloro-L-adenosine > 2-azido-L-adenosine > L-adenosine.

7 It is concluded that the postsynaptic P_1-purinoceptors in the guinea-pig atria and trachea and the presynaptic P_1-purinoceptors on cholinergic nerve terminals in guinea-pig ileum are stereospecific for the D-enantiomers of adenosine and its analogues.

Introduction

During the last decade there has been considerable interest in the nature of purine receptors, and recently accumulated evidence has suggested that there is a basis for a subdivision in their classification (Spedding & Weetman, 1976; Burnstock, 1978; Bartlett, Stewart & Nakatsu, 1979). Burnstock (1978) has proposed that the receptors can be subdivided into P_1- and P_2-purinoceptors, according to their different agonist potency series and the selectivity of antagonists. The P_1-purinoceptor is characterized by the agonist potency sequence of adenosine ≥ adenosine 5'-monophosphate (AMP) > adenosine 5'-diphosphate (ADP) ≥ adenosine 5'-triphosphate (ATP), and is selectively antagonized by the methylxanthines. In contrast, the P_2-purinoceptor has an agonist potency sequence of ATP ≥ ADP > AMP ≥ adenosine and is blocked, though not selectively, in certain tissues by high concentrations of quinidine and the 2-substituted imidazolines, 2'2-pyridylisatogen and apamin.

The relationship between agonists and their receptors is generally highly specific. In the adrenergic and cholinergic systems this is demonstrated by the differences in activity found between optical isomers of noradrenaline and acetyl-β-methylcholine. The naturally occurring laevo-isomers of noradrenaline and adrenaline are 50–500 times more potent than the dextro isomers, the potency ratios depending on the preparations on which they are tested (Bowman & Rand, 1980), while in the isolated rat intestine the D-isomer of acetyl-β-methylcholine is less than one hundredth as active as the L-isomer (Ellenbroek & Van Rossum, 1960).

In the present study we have investigated the stereospecificity of the P_1-purinoceptor in the guinea-pig atria and trachea which possess postsynaptic purine receptors, and in the guinea-pig ileum which has presynaptic purine receptors present on the cholinergic nerve terminals, by comparing the actions of the D- and L-enantiomers of adenosine and some analogues. The D- and L-enantiomers of the same compounds have recently been tested on the

[1] Present address: Bioscience II Department, ICI, Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire.

[2] Present address: Department of Pharmacology, King's College London, University of London, Strand, London WC2R 2LS

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purine receptors in the guinea-pig taenia coli (Cusack & Planker, 1979) and on the adenosine receptor present in human platelets (Cusack, Hickman & Born, 1979; Cusack & Hourani, 1981).

Methods

Guinea-pigs (300–600 g) of either sex were killed by a blow to the head.

Atria

The hearts were excised and the left atria were dissected free in cold bicarbonate-buffered physiological salt solution (Blinks, 1966) which was gassed continuously with a mixture of 95% O_2 and 5% CO_2. The left atrium was mounted on a punctate electrode (Blinks, 1965) and then was transferred to a 10 ml bath maintained at 32.5°C. An initial load of 0.5 g was applied to the preparation. The punctate electrode was used as a cathode (with a distant anode) to deliver electrical stimuli to the muscle (2.5 Hz, 5 ms duration) at twice threshold voltage. The mechanical activity was recorded isometrically by means of a Grass FT 03C force transducer and a Grass model 79D polygraph. Cumulative concentration-effect curves were obtained for the various agonists. Theophylline and dipyridamole were added to the organ baths and the tissues were allowed to equilibrate for 30 min before the effects of these drugs were investigated.

Trachea

The trachea was excised and a strip was prepared by first cutting through the ventral cartilage and then by making suitable transverse cuts as in the method of Emmerson & Mackay (1979). The resulting zig-zag preparation could be cut to provide two preparations from each trachea. Threads were tied to the ends of each zig-zag strip and these were suspended in a 30 ml overflow organ bath containing modified Krebs solution at 37°C of the following composition (mM): NaCl 133, KCl 4.7, NaH_2PO_4 1.4, $NaHCO_3$ 16.3, $MgSO_4$ 2.5 and glucose 7.8; when this solution had been saturated with a gas mixture of 95% O_2 and 5% CO_2, 2.5 mM $CaCl_2$ was added. The tracheal strip was initially placed under 1 g resting tension. Relaxations were recorded by an isotonic lever (ADG Instruments) and displayed on a Grass polygraph. The preparation was allowed to equilibrate for at least 15 min; the basal tone was then increased by the application of a sub-maximal dose of carbachol (0.5 μM); and the experiments were started after a 45 min interval. In the experiments where dipyridamole or theophylline were used the drugs were added to the bathing fluid 30 min before the start of the experiment.

The tracheal strip recovered very slowly from application of inhibitory agonists; therefore cumulative concentration-response curves were obtained for the various agonists and responses were measured in millimetre relaxations.

Ileum

The abdominal cavity was opened and the ileum was freed of mesentery. Sections about 3 cm in length were taken from the region midway between the stomach and ileo-caecal junction. Tissues were placed in a 25 ml organ bath containing modified Krebs solution (Bülbring, 1953) bubbled with 5% CO_2 in O_2 at 37°C (Paton, 1955). Responses to transmural stimulation were recorded via a Grass FT03 isometric transducer connected to a Grass polygraph. Stimulation was achieved using a Grass SD9 stimulator which delivered pulses of 0.5 ms duration at 0.2 Hz and 25–30 V.

Tissues were left to equilibrate for an hour, by which time twitch responses to transmural stimulation had settled to a steady control height. Drugs were added to the bath by automatic pipette in volumes less than 0.1 ml and washed out by overflow when the maximum effect had occurred. Theophylline and dipyridamole were made up in bathing Krebs solution and allowed to equilibrate for 30 min before their effects were investigated.

Statistical methods

The mean and standard error of the mean were calculated for each group. The means were compared using unpaired *t* tests. *P* values of 0.05 or less were considered to be significant.

Drugs

Adenosine, 2-chloroadenosine, theophylline and carbamylcholine chloride (carbachol) were obtained from Sigma, London. Dipyridamole was obtained from Boehringer Ingelheim. 9-(-L-Ribofuranosyl)adenine (L-adenosine) was synthesized by the method of Acton, Ryan & Goodman (1964). 5′-N-ethylcarboxamidoadenosine (NECA) was prepared from adenosine as described by Prasad & Tietje (1978), and 5′-N-ethylcarboxamido-L-adenosine (L-NECA) was similarly synthesized from L-adenosine (Cusack & Hourani, 1981). 2-Azidoadenosine was synthesized from 2-chloroadenosine (Schaeffer & Thomas, 1958). 2-Chloro-9-(-L-ribofuranosyl)adenine (2-chloro-L-adenosine) and 2-azido-9-(-L-ribofuranosyl)adenine (2-azido-L-adenosine) were synthesized as previously described (Cusack *et al.*, 1979).

Results

Guinea-pig atria

Relative potency of adenosine and its analogues Adenosine (4–100 μM), 2-chloroadenosine (0.1–1 μM), 2-azidoadenosine (0.3–1.5 μM) and NECA (0.02–0.3 μM) caused a concentration-dependent reduction in the force of contraction of the guinea-pig atria. The potency series for the negative inotropic effect was NECA > 2-chloroadenosine > 2-azidoadenosine > adenosine.

Substitution of the adenosine moiety can result in analogues that are resistant to tissue uptake and/or enzymatic degradation, therefore the log concentration-response curves to adenosine and its analogues were repeated in the presence of dipyridamole (0.5 μM) which blocks adenosine uptake. The concentration-response curves which were obtained under these conditions are illustrated in Figure 1. Incubation with dipyridamole (0.5 μM) for 30 min significantly potentiated ($P < 0.001$) the negative inotropic response to adenosine but not to the adenosine analogues. Maximum response to adenosine was reached by 22 ± 2.8 s; the analogues took significantly longer, 2-chloroadenosine taking 35 ± 2.2 s, 2-azidoadenosine 33.5 ± 1.7 s and NECA 36 ± 1.4 s ($P < 0.01$). The potency series for the negative inotropic effect in the presence of dipyridamole was NECA > 2-chloroadenosine > adenosine = 2-azidoadenosine. EC_{50} values are given in Table 1.

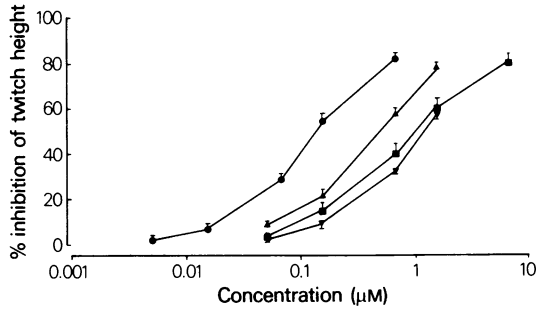

Figure 1 Guinea-pig driven left atria: log concentration-response curves to adenosine and its analogues in the presence of dipyridamole 0.5 μM. (■) Adenosine; (▼) 2-azidoadenosine; (▲) 2-chloroadenosine; (●) 5′-N-ethylcarboxamidoadenosine. Each point is the mean of 6 or more observations from at least 6 different animals. Vertical bars show s.e.mean. Log concentration-response curves were constructed from the mean ± s.e.mean of the responses for a given drug concentration.

Sensitivity of responses to theophylline The adenosine antagonist theophylline was used in order to check that the responses of the guinea-pig atria to adenosine and its analogues were mediated via P_1-purinoceptors. In the presence of theophylline (100 μM) log concentration-response curves to adenosine and its analogues were shifted 10–16 fold to the right (see Tables 1 and 2 for EC_{50} values and concentration-ratios).

Table 1 EC_{50} values for adenosine and various adenosine analogues with 95% confidence intervals

Driven left atria	*Adenosine* (μM)	*2-Azidoadenosine* (μM)	*2-Chloroadenosine* (μM)	*NECA* (μM)
Control	18.9(27.9,12.8)	1.0(1.2,0.9)	0.4(0.5,0.3)	0.1 (0.11,0.08)
Dipyridamole	1.0(0.7,0.6)**	1.1(1.3,1.03)	0.5(0.6,0.3)	0.12(0.15,0.10)
Theophylline	202.4(450.0,90.9)**	10.3(17.5,6.0)**	6.8(13.3,3.5)**	1.0 (2.1,0.5)**
Trachea	(μM)	(μM)	(μM)	(μM)
Control	12.6(21.7,7.3)	2.3(8.4,0.6)	2.2(6.3,0.8)	0.6(2.0,0.2)
Dipyridamole	3.9(6.2,2.5)*	1.3(6.4,0.3)	5.5(9.0,3.4)	0.4(2.1,0.1)
Dipyridamole + theophylline	51.9(95.6,28.2)**	34.8(69.4,15.5)**	30.3(57.4,16.0)**	9.3(15.2,5.7)**
Transmurally stimulated ileum	(nM)	(nM)	(nM)	(nM)
Control	64.1(97.5,42.1)	4.3(7.0,2.7)	4.3(10.3,0.6)	0.5(0.9,0.3)
Dipyridamole	4.5(7.6,2.7)**	3.7(5.4,2.5)	1.6(2.7,0.9)*	0.3(0.5,0.2)
Theophylline	544.6(906.8,327.0)**	54.1(154.5,18.9)**	42.1(106.7,16.6)**	4.2(7.3,2.4)**

* $P < 0.05$; ** $P < 0.001$.

In order to test for significant differences between the groups the data were converted to their $\log_e$ form. A normal distribution was thus obtained. Arithmetic values of mean EC_{50}s and 95% confidence limits (in parentheses) are given in the table.

Stereospecificity of the P_1-purinoceptors The L-enantiomers of adenosine and its analogues were tested on the guinea-pig atria and the resulting responses compared with the results for the corresponding D-isomers. The L-enantiomers did not produce a negative inotropic response at concentrations up to 50 μM. However, at higher concentrations (65–150 μM) a small increase in the force of contraction was observed. The results obtained with two test concentrations of the L-enantiomers of adenosine and analogues are given in Table 3. The potency series for the increase in the force of contraction of the guinea-pig atria was 2-azido-L-adenosine > L-NECA = 2-chloro-L-adenosine > L-adenosine which was different from the series obtained with the D-enantiomers for the negative inotropic response.

Guinea-pig trachea

Relative potency of adenosine and analogues Adenosine (1–300 μM), 2-chloroadenosine (0.5–100 μM), 2-azidoadenosine (0.3–300 μM) and NECA (0.1–10 μM) produced slowly developing relaxations of the guinea-pig trachea which took approximately 5 min to reach maximum relaxation. Log concentration-response curves to the inhibitory agonists were constructed. The potency series for relaxing the tracheal musculature was NECA > 2-chloroadenosine > 2-azidoadenosine > adenosine. EC_{50} values are given in Table 1.

As cumulative log concentration-response curves were constructed for the inhibitory agonists, the possibility that appreciable tissue uptake of adenosine could alter the potency series was considered. Thus the log concentration-response curves were reconstructed in the presence of dipyridamole (0.5 μM). The log concentration-response curves which were obtained under these new conditions are illustrated in Figure 2. Incubation with dipyridamole significantly ($P<0.05$) potentiated the responses to adenosine, but not to adenosine analogues. Thus, the potency series for relaxing the tracheal muscle became NECA > 2-chloroadenosine > adenosine > 2-azidoadenosine (see Table 1). The following experiments were undertaken in the presence of dipyridamole because there appeared to be appreciable uptake of adenosine.

Sensitivity of responses to theophylline The response of the trachea to adenosine and its analogues was tested for theophylline sensitivity in order to ascertain whether all the compounds were acting through a P_1-purinoceptor. The responses to all four compounds were antagonized by theophylline (100 μM). EC_{50} values and potency-ratios are given in Tables 1 and 2.

Stereospecificity of the P_1-purinoceptor The L-enantiomers of adenosine and its analogues were tested on the guinea-pig tracheal strip and the responses that were obtained were compared to those of the corresponding D-enantiomers. The L-enantiomer of adenosine was almost inactive at high concentrations, while the L-enantiomers of the adenosine analogues were considerably less potent

Table 2 Potency-ratios at EC_{50} responses with 95% confidence intervals*

Driven left atria	*Adenosine*	*2-Azidoadenosine*	*2-Chloroadenosine*	*NECA*
Control	1.00	0.05(0.08,0.04)	0.02(0.03,0.01)	0.005(0.008,0.003)
Dipyridamole	1.00	1.12(1.82,0.68)	0.45(0.76,0.27)	0.120(0.200,0.072)
Theophylline	1.00	0.05(0.12,0.02)	0.03(0.08,0.01)	0.005(0.012,0.002)
Trachea				
Control	1.00	0.15(0.55,0.04)	0.15(0.45,0.05)	0.043(0.130,0.014)
Dipyridamole	1.00	0.29(1.06,0.08)	1.35(2.62,0.70)	0.087(0.302,0.025)
Dipyridamole + Theophylline	1.00	0.62(1.45,0.27)	0.54(1.24,0.24)	0.168(0.355,0.080)
Transmurally stimulated ileum				
Control	1.00	0.06(0.12,0.04)	0.06(0.14,0.03)	0.008(0.014,0.004)
Dipyridamole	1.00	0.77(1.59,0.37)	0.33(0.72,0.15)	0.067(0.140,0.032)
Theophylline	1.00	0.09(0.26,0.03)	0.07(0.18,0.03)	0.007(0.015,0.004)

*Confidence intervals for the potency ratios were calculated from $\log_{10}$ transformations of EC_{50} values followed by a bias correction for converting the data back to its original form.

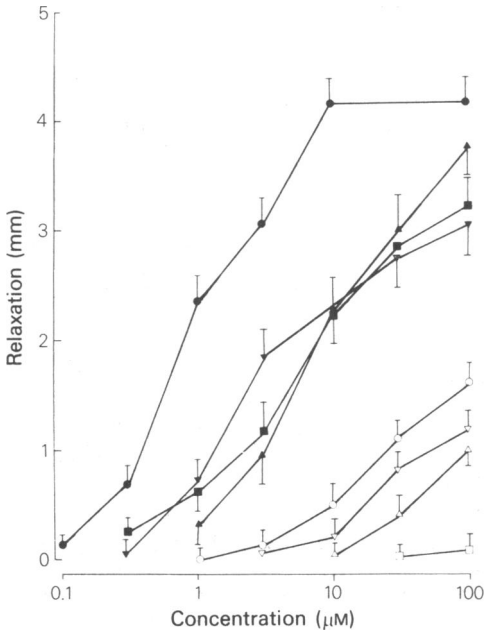

Figure 2 Guinea-pig isolated tracheal chain: log concentration-response curves to adenosine and its analogues in the presence of dipyridamole (0.5 μM). (■) Adenosine; (▼) 2-azidoadenosine; (▲) 2-chloroadenosine; (●) 5'-N-ethylcarboxamidoadenosine (NECA); (□) L-adenosine; (▽) 2-azido-L-adenosine; (△) 2-chloro-L-adenosine; (○) L-NECA. Each point is the mean of 5 or more observations from at least 5 different animals. Vertical bars show s.e.mean. Log concentration-response curves were constructed from the mean ± s.e.mean of the responses for a given drug concentration.

than their corresponding D-enantiomers (Figure 2). The potency series of the L-enantiomers for relaxing the tracheal muscle was L-NECA > 2-chloro-L-adenosine > 2-azido-L-adenosine > L-adenosine.

Guinea-pig ileum

Relative potency Adenosine (0.3–1.0 μM), 2-chloroadenosine (0.02–0.07 μM), 2-azidoadenosine (0.02–0.1 μM) and NECA (0.002–0.009 μM) all caused a concentration-dependent inhibition of twitch height in the transmurally stimulated guinea-pig ileum. The potency series for inhibition of twitch height was NECA > 2-chloroadenosine = 2-azidoadenosine > adenosine. EC_{50} values are given in Table 1.

As in the atria and trachea, the log concentration-response curves for the inhibitory agonists were reconstructed in the presence of dipyridamole (0.05–0.5 μM). The log concentration-response curve to adenosine was then significantly potentiated ten fold ($P < 0.001$), while the curves to the analogues were not significantly altered (Figure 3). The potency series for inhibition of the guinea-pig ileum twitch height in the presence of dipyridamole was NECA > 2-chloroadenosine = 2-azidoadenosine = adenosine (see Table 1). The time taken to reach a maximum response was similar for adenosine (31 ± 2.4 s), 2-chloroadenosine (36 ± 4.5 s) and NECA (39 ± 4.5 s), whereas 2-azidoadenosine (45 ± 4.5 s) took significantly ($P < 0.05$) longer in comparison with adenosine.

Sensitivity of responses to theophylline The inhibitory responses of the guinea-pig ileum to adenosine and its analogues were all reduced after incubation with theophylline (50 μM) for 30 min. Log concentration-response curves were shifted 9–13 fold to the right.

Stereospecificity of the P_1-purinoceptor The L-enantiomers did not cause any inhibition of twitch height of the transmurally stimulated ileum. However, at concentrations of 3 μM and over, the L-enantiomers of 2-chloroadenosine, 2-azidoadenosine and NECA increased the size of the twitch response (see Table 3).

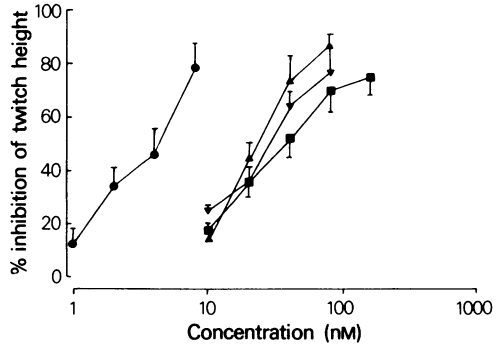

Figure 3 Transmurally stimulated guinea-pig ileum: log concentration-response curves to adenosine and its analogues in the presence of dipyridamole (0.05 μM). (■) Adenosine; (▼) 2-azidoadenosine; (▲) 2-chloroadenosine; (●) 5'-N-ethylcarboxamidoadenosine. Each point is the mean of 6 or more observations from at least 6 different animals. In preparations from 2 guinea-pigs a higher concentration (0.5 μM) of dipyridamole was used. Vertical bars show s.e.mean. Log concentration-response curves were constructed from the mean ± s.e.mean of the responses for a given drug concentration.

Table 3 Excitatory effects of the L-enantiomers of adenosine and its analogues

Compound	*Driven left atria* No. of tissues (No. of animals)	% increase in force of contraction (mean ± s.e.mean) 65 μM	150 μM	*Transmurally stimulated ileum* No. of tissues (No. of animals)	% increase in twitch height (mean ± s.e.mean) 3 μM	10 μM
L-Adenosine	4(4)	0	2±1.2	3(2)	0	0
2-Azido-L-adenosine	4(4)	5±0.6	14±2.2	4(3)	16±6.6	27±12.8
2-Chloro-L-adenosine	4(4)	3±2.0	8±1.6	6(6)	11±1.6	23± 6.1
L-NECA	4(4)	4±1.6	8±1.7	3(3)	35±7.5	48± 6.4

Discussion

The results of the present investigation have demonstrated the potent inhibitory effects of adenosine and its C^2 (2-chloroadenosine, 2-azidoadenosine) and 5′(5′-N-ethylcarboxamide adenosine) substituted analogues on three isolated preparations from the guinea-pig. Previous investigations have shown that at postsynaptic sites in the guinea-pig taenia coli (Satchell & Maguire, 1975) and presynaptic sites in the rat vas deferens (Muller & Paton, 1979) the C^2-substituted analogues of adenosine are more potent than the parent compound. The results of our study agree with these reports which show that 2-chloroadenosine is the more potent of the two analogues substituted in this position. It is interesting to observe that the relative potency of the C^2-substituted analogues when compared to adenosine, showed considerable variation within the three preparations studied. 2-Chloroadenosine was approximately 50 times more potent than adenosine in the guinea-pig atria, yet only 4 times more active in the tracheal preparation. The tissue variation was considerably attenuated after dipyridamole treatment and therefore may represent differing rates of adenosine uptake and deamination between tissues.

Many analogues of adenosine, modified in the 5′-position, were found to be inactive on the adenosine receptor which is present in platelets (Haslam & Cusack, 1981). However, NECA has been shown to be 5–10 times more potent than is adenosine as an inhibitor of human platelet aggregation (Cusack & Hourani, 1981). This 5′-substituted analogue has also been found to be a highly potent coronary vasodilator in the dog, and has been found to be 22,000 times more potent than adenosine (Raberger, Schütz & Kraupp, 1977). Our results show that NECA is 20–200 times more potent than adenosine in the three preparations from the guinea-pig. Potency ratios for adenosine, NECA and the C^2-substituted analogues varied considerably from one preparation to another. The ratios were greatest in the guinea-pig atria and least in the trachea.

In the presence of dipyridamole, which blocks adenosine uptake, the responses to adenosine in all three preparations were selectively potentiated, while no significant alteration in the responses to the analogues was noted, with the exception of responses to 2-chloroadenosine in the ileum. Thus, the greater potency of the C^2- and 5′-substituted analogues appeared to be due, at least partly, to their resistance to tissue uptake and subsequent enzymatic destruction. In all three preparations the potency sequence for adenosine and its analogues in the presence of dipyridamole was NECA > 2-chloroadenosine ≥ 2-azidoadenosine = adenosine.

Since the adenosine antagonist, theophylline, reduced the inhibitory effects of adenosine and its analogues, all four agonists appeared to be acting through the same adenosine receptor.

The present study provides evidence for the stereospecificity of both the postsynaptic P_1-purinoceptors in the guinea-pig atria and trachea, and the presynaptic P_1-purinoceptor on cholinergic nerve terminals in the guinea-pig ileum. Marked differences in potency between the L- and D-enantiomers of adenosine and its analogues have been established, and are comparable to those established for receptors mediating responses to acetylcholine and to noradrenaline (Ellenbroek & Van Rossum, 1960; Bowman & Rand, 1980). The evidence that the adenosine receptor shows stereospecificity is supported by similar findings for the external adenosine receptor linked to the human platelet adenylate cyclase system (Cusack *et al.*, 1979; Cusack & Hourani, 1981) and the adenosine receptor in the guinea-pig taenia coli (Cusack & Planker, 1979).

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