Evidence for the involvement of cyclic GMP in adenosine-induced, age-dependent vasodilatation

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1 Adenosine-induced dilatation of rat aorta was present in aorta taken from 4 week-old rats, attenuated with increase in age of rats to 8 weeks, and was virtually absent in the aorta from 12 week-old rats.

2 Removal of the endothelium by mechanical rubbing attenuated adenosine-induced dilatation.

3 Haemoglobin and methylene blue partly reversed the adenosine-induced endothelium-dependent dilatation.

4 The order of potency of adenosine derivatives was 5'-(N-ethylcarboxamido)adenosine (NECA) > 2-phenylaminoadenosine (CV-1808) > 2-chloroadenosine > N^6-([R]-[−]-phenylisopropyl)adenosine (R-PIA) > adenosine > N^6-cyclohexyladenosine (CHA) > N^6-([S]-[+]-phenylisopropyl)adenosine (S-PIA), indicating that adenosine receptors mediating the dilatation are of the A_2 subtype.

5 [^{3}H]-NECA bound to preparations of membranes from rats of 4 weeks old; it was displaced more effectively by NECA and the A_2 ligand CV-1808 than by the A_1 ligands CHA and S-PIA.
ligands CHA and S-PIA.

6 The number but not affinity of specific binding sites for NECA decreased considerably with increase in age of rats to 8 weeks, and binding sites for [^{3}H]-NECA were hardly detected in membrane preparations from rats of 20 weeks old.

7 Adenosine caused a marked increase in cyclic GMP production, but did not induce an increase in the cyclic AMP level.

8 This increase in cyclic GMP production induced by adenosine was abolished by methylene blue or 8-phenyltheophylline, or by removal of the endothelium.

9 The age-associated decrease in adenosine-induced dilatation was found to be associated with a reduction in the formation of cyclic GMP, but not of cyclic AMP.

10 These results suggest that adenosine causes dilatation via A_2 receptors by inducing production of an endothelium-derived relaxing factor (EDRF), which in turn stimulates soluble guanylate cyclase, and so increases production of cyclic GMP. It is also suggested that the main reason for the age-associated decrease in adenosine-induced dilatation is a decrease in the number of A_2-receptors or the ability of the endothelium to produce EDRF, leading to decreased production of cyclic GMP.

Introduction

There are many studies showing that adenosine plays an important role in the regulation of cardiovascular functions (Kukovetz et al., 1978; Berne, 1980; Silver et al., 1984; White & Angus, 1987). Adenosine has been proposed to promote relaxation of vessels by increasing adenosine 3':5'-cyclic monophosphate (cyclic AMP) formation (Kukovetz et al., 1978) and adenosine receptors in several systems have been classified into A_1/R_i or A_2/R_a on the basis of whether adenosine stimulates or inhibits adenylate cyclase (van Calker et al., 1979; Londos et al., 1980). On the other hand, a dissociation between adenosine-induced dilatation and an increase in the cyclic AMP level (Herlihy et al., 1976; Verhaeghe, 1977) has also been demonstrated.

Adenosine has been shown to cause either endothelium-dependent (Gordon & Martin 1983; Kennedy & Burnstock, 1985; Rubanyi & Vanhoutte, 1985) or independent (Collis & Brown, 1983; Kennedy et al., 1985; Mathieson & Burnstock, 1985; White & Angus, 1987) dilatation of various isolated vessels. There are, however, data showing that adenosine caused scarcely any dilatation of rat thoracic aorta (White et al., 1985), or was not as effective as adenosine 5'-triphosphate (ATP) in dilating the coronary artery (Gordon & Martin, 1983; White & Angus, 1987). We have observed that dilatation of rat aorta in response to histamine decreased with an increase in age (Moritoki et al., 1986; 1988), and that the ability of the endothelium to produce endothelium-derived relaxing factor (EDRF) in response to histamine, and to form cyclic GMP decreased with age (Moritoki et al., 1988). These

findings suggested that the responsiveness of rat aorta to adenosine might depend on the age of the animals.

In the present study, we examined the mechanism of the dilator effect of adenosine on rat thoracic aorta. We also investigated the effect of the age of rats on adenosine-induced dilatation in relation to guanosine 3':5'-cyclic monophosphate (cyclic GMP) production, and the affinity and number of adenosine receptors.

A preliminary account of some of this work was presented at the International Symposium of Neurotransmitter Receptors, in Hiroshima, in 1987 (Moritoki & Tanioka, 1987), and the International Symposium on Biosignalling in Cardiac and Vascular Systems, in Kyoto, in 1988 (Moritoki et al., 1989).

Methods

Organ bath experiments

Male Wistar rats aged 4 weeks (25–31 days, 127 ± 10 g), 8 weeks (8–10 weeks, 282 ± 6 g), 12 weeks (12–15 weeks, 371 ± 6 g) and 20 weeks (20–22 weeks, 454 ± 8 g) were used. The rats were killed by a blow on the head and cervical dislocation, and bled. Segments of their thoracic aorta, 3 mm in length, were cut with parallel razors and freed from adjacent tissues under a dissecting microscope. These ring segments were set up in a 10 ml organ bath containing Krebs solution of the following composition (mM): NaCl 115.3, KCl 4.9, $CaCl_2$ 1.4, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 11.1. This

solution was maintained at 34°C and bubbled with 95% O_2 and 5% CO_2.

The preparations were maintained at a resting tension of 1.0 g in the bath for 120 min before experiments. Responses were recorded isometrically with a force displacement transducer (Nihon Kohoden SB 1TH). For measurement of dilatation, the arteries were first contracted with noradrenaline at a concentration corresponding to the EC_{80}. Concentration-response curves were constructed by adding adenosine cumulatively to the 10 ml bath in a volume of approximately 20 μl, and dilatations were plotted as a % of the contraction induced by noradrenaline.

The endothelium was removed by rubbing the lumen of the artery with cotton thread, and its removal was confirmed by checking that the dilator response to acetylcholine (ACh) was lost.

Pure haemoglobin was prepared as described by Martin *et al.* (1985). Commercial haemoglobin at a concentration of 1 mM was reacted with 10 mM sodium hydrosulphite and then the reducing agent was removed by dialysis at 4°C.

Adenosine receptor binding assay

Segments of rat thoracic aorta were minced with a pair of scissors in 10 to 20 volumes of 50 mM Tris-Cl buffer (pH 7.4). The mixture was then homogenized 3 times for 30 s periods with 30 s intervals in a Polytron homogenizer at setting 6 in an ice-water bath, and then rehomogenized 5 times with a Potter glass-glass homogenizer. The homogenate was centrifuged at 105,000 g for 60 min, and the pellet was resuspended in 50 mM Tris-Cl buffer by 5 strokes of a teflon glass homogenizer for binding assay. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Aliquots (400 μl) of membrane suspension were incubated in triplicate with various concentrations of [^{3}H]-5'-(N-ethylcarboxamido)adenosine ([^{3}H]-NECA), with or without 50 μM NECA, in a total volume of 500 μl at 25°C to determine specific and non-specific binding. After incubation for 60 min, the membrane bound ligand was separated from unbound ligand by vacuum-filtration through a glass fibre filter (GF/C, Whatman). The filter was then washed repeatedly with 10 ml of ice-cold buffer and dried in a vial at room temperature, and the radioactivity trapped on the filter was counted in a liquid scintillation spectrophotometer (Aloka LSC-700). Specific binding was calculated as the difference between total and non-specific bindings.

Competition assays were performed as described above except that a final concentration of 50 nM [^{3}H]-NECA was added to mixture containing membrane and various concentrations of NECA (0.1 nM–1 μM) or N^6-cyclohexyladenosine (CHA) (10 nM–1 μM), and the mixture was incubated for a further 40 min. Data were analysed by the method of nonlinear least-squares by use of a computer programme (Taira & Terada, 1985).

Assay of cyclic nucleotides

Segments of aorta were equilibrated in Krebs solution at 34°C bubbled with 95% O_2 and 5% CO_2 for 2 h before addition of 0.3 μM noradrenaline. Then the preparations were incubated with 10 μM adenosine. After various times of incubation with adenosine, the preparations were quickly frozen in liquid nitrogen and homogenized in ice-cold 6% trichloroacetic acid in a Potter-glass homogenizer in ice. The homogenates were centrifuged at 1700 g for 15 min at 4°C, and the supernatants were extracted with 3 volumes of water-saturated ether. Cyclic nucleotides (cyclic AMP and cyclic GMP) were measured by radioimmunoassay (Honma *et al.*, 1977) with commercially available kits. Briefly, cyclic nucleotides in the supernatant were succinylated and incubated with [^{125}I]-succinyl cyclic GMP tyrosine methyl ester or [^{125}I]-succinyl cyclic AMP tyrosine methyl ester and antisera for 18 h at 4°C. Then dextran-coated charcoal was added to the reaction medium to

terminate the reaction. The radioactivity in the supernatant was counted in a gamma spectrometer (Aloka ARC-301). Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Each experimental group consisted of 4 to 6 preparations from different rats.

Statistical analysis

Values are expressed as means ± s.e.mean. The statistical significance of differences was analysed by Student's unpaired *t* test, and *P* values of less than 0.05 were considered to be significant.

Materials

The drugs used were adenosine, 5'-(N-ethylcarboxamido)adenosine (NECA), N^6-([R]-[−]-phenylisopropyl) adenosine (R-PIA), N^6-([S]-[+]-phenylisopropyl) adenosine (S-PIA), 2-chloroadenosine, N^6-cyclohexyl-adenosine, 8-phenyl-theophylline, acetylcholine chloride, noradrenaline hydrochloride, methylene blue, indomethacin, haemoglobin (all from Sigma Chemical Co., St Louis, MO, U.S.A.), 2-phenylaminoadenosine (CV-1808, Funakoshi Chemical Co., Tokyo, Japan), dipyridamole (Boehringer Ingelheim, FRG), dilazep (Kowa Co., Tokyo, Japan), cilostazole (Otsuka Pharmaceutical Co., Tokushima, Japan) and 5'-N-ethyl-carboxamido [8-^{3}H]-adenosine (specific activity, 0.74–1.5 TBeq mmol^{-1}, Amersham Japan). Kits for radioimmunoassay of cyclic GMP and cyclic AMP were from Yamasa Shoyu Co. Ltd. (Choshi, Japan).

Results

Dilatation and aging

Adenosine at concentrations up to 100 μM did not cause dilatation of the thoracic aorta from rats of 12 weeks old (Figure 1). However, in the arteries from rats of 4 weeks old it caused dose-dependent dilatation at concentrations of above 0.3 μM. The maximal dilatation of 79.4 ± 5.5% (*n* = 9) was observed at 300 μM adenosine and the apparent log EC_{50} value was 5.63 ± 0.08.

With an increase in the age of the rats to 8 weeks, about a 30 times higher concentration of adenosine was necessary to produce comparable dilatations to those observed with the arteries from 4 week-old rats, while with aortae from 12 week-old rats, the threshold concentration of adenosine for dila-
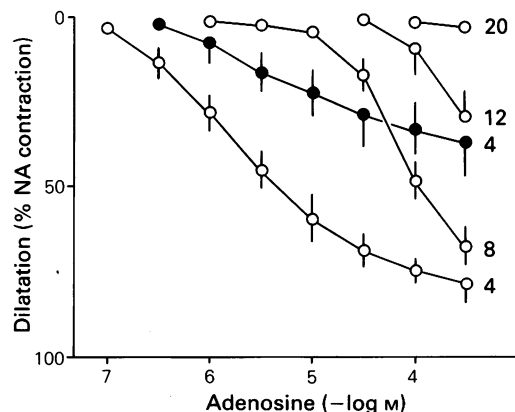

Figure 1 Age-associated change in the dilator response of the rat thoracic aorta to adenosine. The arteries were contracted with noradrenaline at concentrations corresponding to the EC_{80} values. Adenosine was added cumulatively. Dilator responses are expressed as percentages of the noradrenaline-induced contraction. Ages of rats are shown in weeks. (●) Responses of preparations without endothelium from rats of 4 weeks old. Values are means and vertical lines s.e.mean of preparations from 5 to 12 rats.

tation was as high as $100\,\mu M$. With a further increase in age to 20 weeks, the aorta no longer responded to adenosine at concentrations of up to $300\,\mu M$ or even to a single dose of a high concentration of adenosine $(300\,\mu M)$. In addition, the stable adenosine derivatives NECA and 2-phenylaminoadenosine (CV-1808) at concentrations of up to $30\,\mu M$ caused scarcely any dilatation. Inhibitors of adenosine uptake and adenosine deaminase, such as dipyridamole (Afonso & O'Brien, 1967) and dilazep (Fujita et al., 1980), at concentrations of up to $0.3\,\mu M$ did not restore the responsiveness to adenosine. Under these conditions, however, arteries dilated in response to histamine and ACh $(100\,\mu M,$ each), although to a somewhat lesser extent than arteries from rats of 8 weeks old; $100\,\mu M$ histamine and ACh induced dilatations of $63.9 \pm 4.5\%$ (vs $93.6 \pm 5.4\%$ at 4 weeks old) and $67.6 \pm 7.8\%$ (vs $87.2 \pm 7.2\%$ at 4 weeks old), respectively $(n = 6)$.

Characteristics of adenosine-induced dilatation

For characterization of adenosine-induced dilatation, the arteries from rats of 4 weeks old were used. We examined whether the endothelium of the vessels contributes to the dilator response of the artery to adenosine. For this, after confirming the dilator response to ACh of the aortae of 4 week-old rats, the endothelium was removed by rubbing the lumen of the arteries with cotton thread. Then 60 min later, loss of endothelial function was confirmed by checking loss of ACh-induced dilatation. In these aortae without endothelium, the adenosine-induced dilatation was significantly attenuated but not abolished: the concentration-response curve was shifted about 30 times to the right (Figure 1). Dipyridamole and dilazep, at concentrations of up to $0.3\,\mu M$, did not enhance or inhibit the endothelium-independent dilator response of the artery to adenosine.

Haemoglobin at concentrations above 10 nM reversed adenosine-induced dilatation of the aorta with endothelium (Figure 2). In contrast, in arteries without endothelium, haemoglobin failed to reverse the adenosine-induced dilatation. In addition, dilatation induced by the cyclic AMP phosphodiesterase inhibitor cilostazole $(1\,\mu M)$ used as a reference was hardly affected by haemoglobin at concentrations of up to $100\,\mu M$.

Pretreatment with the guanylate cyclase inhibitor methylene blue (MB, Gruetter et al., 1981) at insufficient concentrations to attenuate cilostazole-induced dilatation, caused
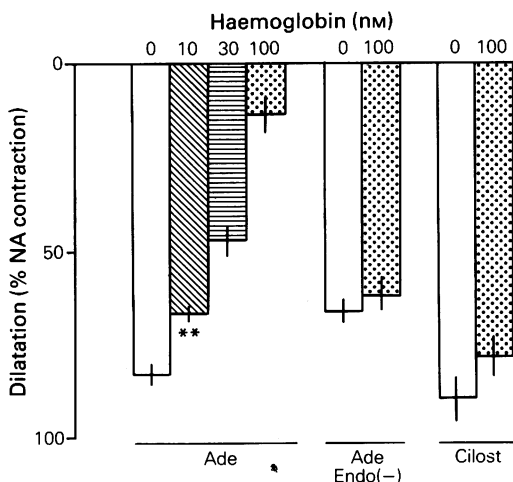
a dose-dependent shift of the concentration-response curve for adenosine to the right (Figure 3); in the presence of 0.1, 0.3 and $1\,\mu M$ MB, the log EC_{50} value of adenosine decreased from the control value of 5.62 ± 0.18 to 5.31 ± 0.25, 4.88 ± 0.19 and 4.21 ± 0.16 $(n = 7)$, respectively. However, $3\,\mu M$ MB did not shift the concentration-response curve further, the log EC_{50} value being 4.17 ± 0.21. In contrast, the adenosine-induced dilatation remaining after removal of the endothelium was not reversed by application of MB at concentrations of up to $3\,\mu M$.

In arteries that had been treated with $100\,\mu M$ indomethacin for 60 min and then washed for 30 min, the dilator effect of adenosine was not affected irrespective of whether the endothelium was intact (apparent log EC_{50} values; 5.50 ± 0.08 vs 5.58 ± 0.19, $n = 4$) or removed $(3.93 \pm 0.14$ vs $3.90 \pm 0.23)$.

Next, the adenosine receptor subtype responsible for the dilator response of the arteries from rats of 4 weeks old was characterized by use of several adenosine derivatives (Figure 4). The rank order of the potencies of adenosine derivatives in inducing dilatation was NECA (apparent log EC_{50} value and maximal response: 7.56 ± 0.18, $\alpha = 1.0$) > CV-1808
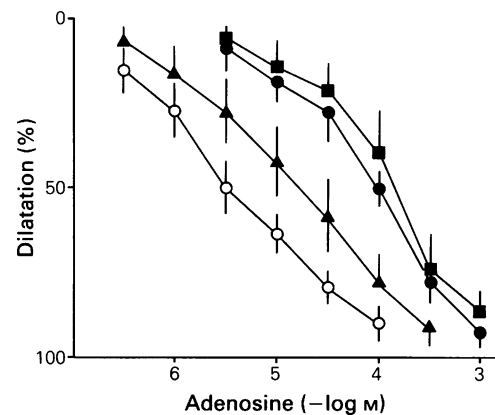
Figure 3 Inhibitory effect of methylene blue on the dilator response of the arteries from rats of 4 weeks old to adenosine. The arteries were treated with the EC_{80} concentration of noradrenaline to produce tone. Methylene blue (MB) was added for 20 min before addition of noradrenaline. Then the dilator response to adenosine was tested. (○), control; (▲), with $0.3\,\mu M$ MB; (●), with $1\,\mu M$ MB; (■), with $3\,\mu M$ MB. Vasodilatations are expressed as percentages of the noradrenaline-induced contractions. Points are means and vertical lines are s.e.mean $(n = 7)$.

Figure 2 Reversal by haemoglobin of adenosine-induced dilatation of the aorta. To study the reversal by haemoglobin, dilatations were induced either by $30\,\mu M$ adenosine or $1\,\mu M$ cilostazole for endothelium intact preparations, and $100\,\mu M$ adenosine for endothelium removed preparations. Dilatations were expressed as percentages of the contractions induced by the EC_{80} concentration $(0.3\,\mu M)$ of noradrenaline. Concentrations of haemoglobin are shown in nM. Ade, adenosine; Endo $(-)$, endothelium removed preparation; Cilost, cilostazole. Columns show mean values of preparations from 6 rats; vertical lines indicate s.e.mean. $**P < 0.01$, compared with control value.
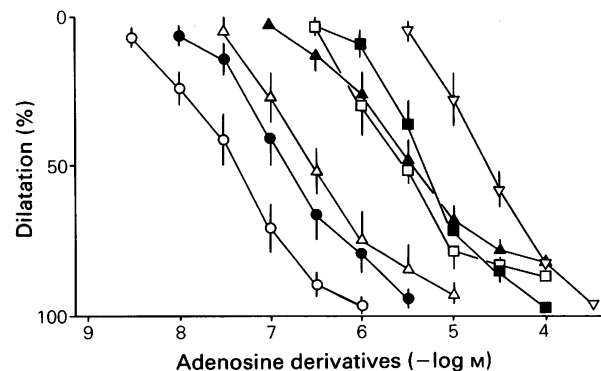
Figure 4 Concentration-response curves for adenosine derivatives showing their order of potency in causing dilatations of thoracic aorta of 4 week-old rats. The ordinate scale shows dilatations of the arteries as percentages of the initial tone induced by the EC_{80} concentration of noradrenaline. (○), $5'$-(N-ethylcarboxamido)adenosine; (△), 2-chloroadenosine; (●), CV-1808; (▲), adenosine; (□),N^6-([R]-[−]-phenylisopropyl)adenosine (R-PIA); (▽), S-PIA, (■), N^6-cyclohexyladenosine. Values are means of preparations from 5 to 9 rats, and vertical lines indicate s.e.mean.
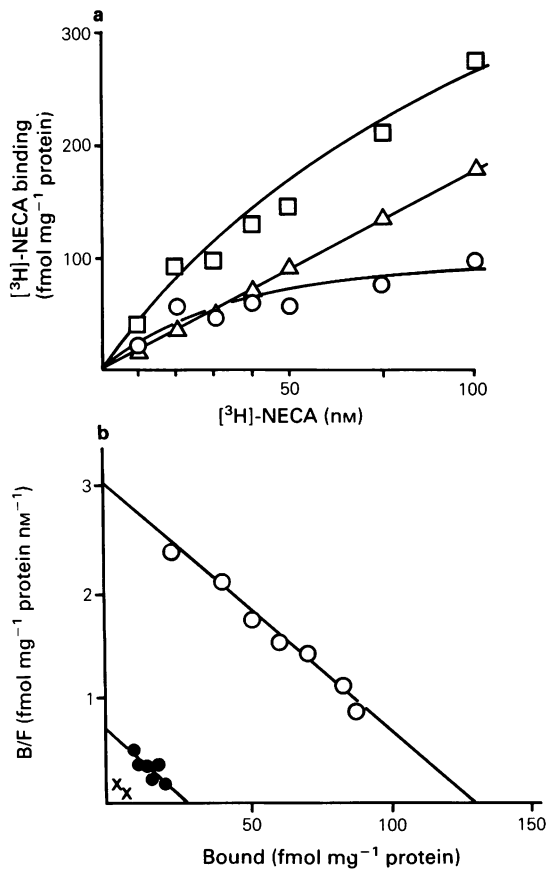
Figure 5 Saturation of [³H]-5'-(N-ethylcarboxamido)adenosine ([³H]-NECA) binding to aortic membranes from rats of 4 weeks old (a). Specific binding (○) was calculated by subtracting non-specific binding (△), determined in the presence of 10 μM NECA, from total binding (□). (b) Scatchard plot of specific binding of [³H]-NECA and effect of aging on the binding. (○), 4 weeks old; (●), 8 weeks old; (×), 20 weeks old. Each point represents the mean of triplicated determination from two experiments.

$(6.73 \pm 0.11, \alpha = 1.0) > 2$-chloroadenosine $(6.48 \pm 0.29, \alpha = 1.0) > $ R-PIA $(5.57 \pm 0.18, \alpha = 0.87) > $ adenosine $(5.55 \pm 0.09, \alpha = 0.84) > $ CHA $(5.36 \pm 0.11, \alpha = 1.0) > $ S-PIA $(4.66 \pm 0.12, \alpha = 1.0)$ $(n = 5-9)$; NECA was about 800 times more potent than S-PIA, and R-PIA was about 8 times more potent than S-PIA.

Binding assay of adenosine receptors

For further characterization of adenosine receptors, a binding assay for adenosine receptors was carried out with [³H]-NECA as a ligand. [³H]-NECA was found to bind to the membrane preparation of the aorta from rats of 4 weeks old, with a dissociation constant (K_d) of 43.1 nM and a receptor number (B_{max}) of 129.2 fmol mg⁻¹ protein (Figure 5).

NECA, CV-1808, R-PIA, S-PIA and CHA inhibited the specific binding of [³H]-NECA (Figure 6), with apparent K_i values of 0.21, 1.51, 758.5, 446.7 and 467.7 μM, respectively $(n = 4)$. The non-selective A_1, A_2 agonist NECA was about 2200 times more effective, and the A_2-selective ligand CV-1808 was about 300 times more effective than the A_1-selective ligand CHA in displacing [³H]-NECA from its binding sites. In addition, the non-selective antagonist 8-phenyltheophylline (8-PT) also inhibited the binding of [³H]-NECA with a K_i value of 2.09 μM.

The specific binding of [³H]-NECA was less in the membrane preparation from 8 week-old rats (Figure 5). Scatchard analysis showed a marked reduction in the number of binding sites for [³H]-NECA to 30.0 fmol mg⁻¹ protein. However, the K_d value of the binding sites was 41.4 nM, which was not sig-
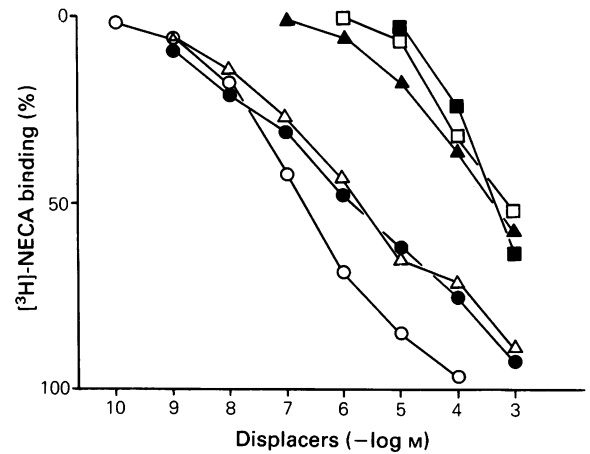
Figure 6 Inhibition by adenosine-related compounds of [³H]-5'-(N-ethylcarboxamide)adenosine ([³H]-NECA) binding to aorta membranes from rats of 4 weeks old. Membrane preparations were incubated with 50 μM [³H]-NECA in the presence of increasing concentrations of unlabelled compounds. (○), NECA; (●), CV-1808; (△), 8-phenyltheophylline; (▲), N⁶-cyclohexyladenosine; (□),N⁶-([R] -[−]-phenylisopropyl)adenosine (R-PIA); (■), S-PIA. Data are representative of triplicate determinations in two experiments.

nificantly different from that in preparations from 4 week-old rats (43.1 nM). With a further increase in age of the rats to 20 weeks, the number of binding sites decreased to an undetectable level.

Effect of adenosine on the production of cyclic nucleotides

We examined whether the dilatation was due to an increase in cyclic nucleotides. As shown in Figure 7, adenosine at a concentration of 10 μM, which caused 60–70% dilatation of the artery, increased the cyclic GMP level in 15 s from the basal level of 1.04 ± 0.33 to 3.73 ± 0.37 pmol mg⁻¹ protein $(n = 9)$.

After removal of the endothelium the basal level of cyclic GMP decreased to 0.16 pmol mg⁻¹ protein, and was not increased by incubation with 10 μM adenosine $(0.09 \pm 0.02$ pmol mg⁻¹ protein in 15 s, $n = 4)$.

In the presence of 0.3 μM MB, which caused slight, but not significant, reduction of basal cyclic GMP production $(1.04 \pm 0.33$ to 0.55 ± 0.20 pmol mg⁻¹ protein in 15 s, $n = 7)$, the adenosine-stimulated cyclic GMP production decreased to nearly the basal level $(0.73 \pm 0.10$ pmol mg⁻¹ protein, $n = 7)$.

8-PT itself did not alter the basal level of cyclic GMP. However, in the presence of 10 μM 8-PT, 10 μM adenosine did not stimulate cyclic GMP production $(0.82 \pm 0.08$ in its presence compared to 0.83 ± 0.19 in its absence, $n = 5)$.

With an increase in the age of the rats to 8 weeks, the basal level of cyclic GMP decreased to 0.73 ± 0.14 pmol mg⁻¹ protein, and the adenosine-stimulated cyclic GMP production decreased to 2.09 ± 0.15 pmol mg⁻¹ protein $(n = 8)$. In arteries from rats of 20 weeks old, adenosine-stimulated production of cyclic GMP decreased to 0.75 ± 0.25 pmol mg⁻¹ protein with a reduction in the basal level $(0.35 \pm 0.04$ pmol mg⁻¹ protein, $n = 6)$.

In contrast to the marked increase in the cyclic GMP level, the cyclic AMP level was not significantly affected by 10 μM adenosine, irrespective of the age of the rats (Figure 7).

Discussion

In the present study, adenosine was found to induce little discernible dilatation of thoracic aorta of rats of above 20 weeks old, as has been previously observed (White et al., 1985). The inability of adenosine to dilate the arteries was not due to its dislocation from its site of action through its uptake mechanism, or to its degradation by adenosine deaminase, because
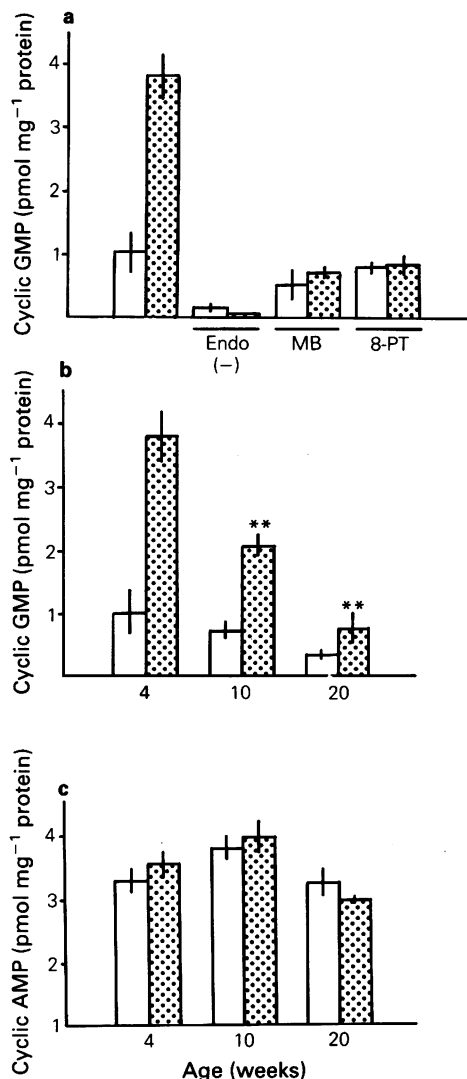
Figure 7 Effects of adenosine on the production of cyclic nucleotides in thoracic aorta from rats of 4 weeks old (a), and effect of aging on the production of cyclic GMP (b) and cyclic AMP (c). Amounts of cyclic nucleotides were measured after incubation with the EC_{80} concentration of noradrenaline $(0.3\,\mu M)$ for 2 min and then with and without $10\,\mu M$ adenosine for 15 s for cyclic GMP and for 2 min for cyclic AMP. Open columns, control; stippled columns, with $10\,\mu M$ adenosine. For study of the effect of methylene blue or 8-PT, these agents were added 20 min before and during incubation with adenosine. MB, with $1\,\mu M$ methylene blue; 8-PT, with $10\,\mu M$ 8-phenyltheophylline; Endo $(-)$, without endothelium. Ages of rats are shown in weeks. Columns represent the mean values $(n = 4-9)$ of preparations from 5 to 7 rats. Vertical lines indicate s.e.mean. $**P < 0.01$, compared with value of the preceding age (unpaired t test).

even in the presence of the inhibitors of adenosine uptake and adenosine deaminase, dipyridamole (Afonso & O'Brien, 1967) and dilazep (Fujita et al., 1980), adenosine had no effect on these preparations, and also the stable adenosine derivatives NECA and CV-1808 caused little dilatation of the arteries. Moreover, the inability of adenosine to cause dilatation of the arteries was not due to rapid development of desensitization due to its cumulative application, because even a single dose of a high concentration of adenosine had no effect on these preparations.

In previous studies, we found that aging decreased the dilatation of rat arteries induced by histamine (Moritoki et al., 1986; 1988). Therefore, in the present study we examined whether the inability of adenosine to dilate the thoracic aorta of rats of 20 weeks old could be due to the age of the rats (Figure 1). We found that adenosine did cause dose-dependent dilatation of the arteries from rats of 4 weeks old, but that with an increase in age of the rats to 8 weeks, the dilator response to adenosine decreased considerably, and that in preparations from rats of over 12 weeks of age a response was no longer detectable. Rats are usually used for experiments on their vasculature at 8 to 15 weeks old. Twelve weeks of age may be borderline for a response of the aorta to adenosine. At this age, the responsiveness to adenosine varied greatly: some preparations did not respond to adenosine, whereas others showed comparable responses to those of the aorta of 4 week-old rats.

The A_2 agonist NECA was more potent than the A_1 agonists R-PIA and CHA in inducing dilatation of rat thoracic aorta, and the order of potency of the adenosine derivatives (NECA > CV-1808 > 2-chloroadenosine > R-PIA > adenosine > CHA > S-PIA) was almost similar to their order of potency in dilating guinea-pig aorta (Collis & Brown, 1983) and in accumulating cyclic AMP in cultured brain cells (van Calker et al., 1979). On the basis of the structure-activity relationship, the adenosine receptors mediating the dilatation of arteries with intact endothelium were judged to be of the A_2 subtype. However, it is unknown whether the maximal dilatations caused by adenosine and R-PIA were less than those induced by NECA and S-PIA.

For further characterization of the adenosine receptor subtype responsible for dilatation, we carried out binding studies of adenosine receptors with $[^3H]$-NECA as a ligand. We demonstrated the presence of specific binding sites for $[^3H]$-NECA in the arteries from rats of 4 weeks old (Figure 5). $[^3H]$-NECA was used as an A_2 ligand, but it has also been shown to bind to A_1 receptors and to lack binding specificity (Schütz et al., 1982; Lohse et al., 1988). However, its binding to rat aortic membranes was displaced more effectively by the so-called A_2 ligand CV-1808 (Daly et al., 1987) and NECA than by the A_1 ligand CHA (Figure 6), suggesting that the binding sites are probably of the A_2 subtype.

With a decrease in the dilator response of the aorta to adenosine with age, the B_{max} decreased with no change in the K_d value (Figure 5). These results indicate that during aging the number of binding sites for $[^3H]$-NECA (A_2 receptors) decreased without a change in their affinity for the agonist. However, it is still not known whether the loss of $[^3H]$-NECA binding sites reflects a reduction in the absolute number (density) of binding sites or is secondary to a reduction of endothelial cells. Furthermore, because the membrane preparations used in the present experiments contained membranes of both the endothelium and smooth muscle, it is unknown whether A_2 receptors are localized in the vascular endothelium or smooth muscle, and which of the receptors (in the endothelium or in smooth muscle) contribute to the adenosine-induced dilator effect which disappears during aging. Nevertheless, our finding that the adenosine-induced dilator response decreased after removal of the endothelium suggests that at least some binding sites are present in the endothelium.

The effects of adenosine have been shown to be caused by mechanisms linked to the adenylate cyclase system (Kukovetz et al., 1978; Rosiers & Nees, 1987), and thus to the 2nd messenger cyclic AMP (Londos et al., 1980) and cyclic AMP-dependent protein kinase (Silver et al., 1984). Subtypes of adenosine receptor in the vasculature have been classified as A_1 and A_2; a classification which was originally proposed on the basis of whether they stimulated or inhibited adenylate cyclase (Van Calker et al., 1979). Functional evidence for the presence of A_2-receptors that stimulate cyclic AMP production in cultured coronary endothelial cells has been presented (Rosiers & Nees, 1987). Therefore, the dilator action of adenosine in the rat thoracic aorta could be mediated through an increase in cyclic AMP accumulation. But this is unlikely, because we found that adenosine did not increase the cyclic AMP level significantly. Furthermore, we found that aging did not alter the cyclic AMP level (Figure 7).

In general, the vasodilator effect of adenosine has been found not to depend on the endothelium (De May & Van-

Kennedy et al., 1985; Mathieson & Burnstock, 1985; White & Angus, 1987), but in some vessels adenosine caused endothelium-dependent dilatation (Frank & Bevan, 1983; Gordon & Martin, 1983; Kennedy & Burnstock, 1985; Rubanyi & Vanhoutte, 1985). We found that in rat aorta, removal of the endothelium considerably attenuated the dilator response to adenosine (Figure 1), as has been shown for the rabbit central ear artery (Kennedy & Burnstock, 1985). In addition, haemoglobin (Martin et al., 1985), which inhibits the effects of endothelium-derived relaxing factor (EDRF), reversed adenosine-induced dilatation without affecting the dilatation caused by the cyclic AMP phosphodiesterase inhibitor cilostazole (Umekawa et al., 1984). From these results and considerations, we suggest that the response of rat thoracic aorta to adenosine is in part mediated by the endothelium and thus EDRF which stimulates soluble guanylate cyclase (Rapoport & Murad, 1983).

In the present study, the endothelium-dependent portion of adenosine-induced dilatation of the aorta from rats of 4 weeks old was found to be abolished or reversed by the soluble guanylate cyclase inhibitor MB at low concentrations that were insufficient to affect cilostazole-induced dilatation, suggesting that soluble guanylate cyclase mediates the adenosine-induced vasodilatation. Furthermore, we found that adenosine increased formation of cyclic GMP, and that removal of the endothelium, besides significantly decreasing the basal level of cyclic GMP, almost completely abolished the adenosine-induced increase in cyclic GMP formation (Figure 7). In addition, the soluble guanylate cyclase inhibitor MB was found to abolish the adenosine-stimulated production of cyclic GMP.

The adenosine antagonist 8-PT (Griffith et al., 1981; Kennedy & Burnstock, 1985) was found to abolish the increase in adenosine-stimulated cyclic GMP production (Figure 7). This observation confirms the idea that cyclic GMP production by adenosine is mediated via adenosine-receptors.

We examined whether the age-associated decrease or loss of dilator response to adenosine was due to a reduction of cyclic GMP formation. In fact, we found that cyclic GMP production decreased with age (Figure 7), and that in the aortae from rats of 20 weeks old, which showed scarcely any dilator response to adenosine, the adenosine-stimulated production of cyclic GMP was less than the resting level in preparations from 4 week-old rats. These results suggest that a reduction in cyclic GMP formation during aging is, in part, responsible for the loss of the dilator response to adenosine.

In addition, as has been shown for vasodilatation induced by nitroprusside (Moritoki et al., 1988), reduction of guanylate cyclase activity itself in the vascular smooth muscle seems to be the cause of the age-associated decrease in adenosine-induced vasodilatation.

In the endothelium-removed preparations, adenosine could activate particulate guanylate cyclase in smooth muscle to produce cyclic GMP, just as has been found for atrial natriuretic peptide (Waldman et al., 1984; Winquist et al., 1984). Recently, adenosine was shown to stimulate particulate guanylate cyclase in cultured vascular smooth muscle cells (Kurtz, 1987). Although our findings in rat aorta differ from those of Kurtz in that cyclic GMP formation was endothelium-dependent and was mediated by soluble guanylate cyclase, his results are consistent with our idea that cyclic GMP, but not cyclic AMP, is responsible for adenosine-induced dilatation. However, this possibility seems unlikely, because adenosine did not elevate the cyclic GMP level in preparations without endothelium.

With respect to an action of adenosine which is independent of cyclic nucleotides, there are data suggesting that adenosine acts at an intracellular site and that its effect appears to be mediated by its metabolite inosine through some metabolic processes (Collis et al., 1986). However, this was not the case in the present study, because dipyridamole (Collis & Brown, 1983) and dilazep (Fujita et al., 1980) which block the access of adenosine to an intracellular site, did not inhibit the dilator effect of adenosine.

Adenosine could dilate the arteries through release of prostanoids, as has been found for some vasodilators (Boeynaems & Galand, 1983; Förstermann et al., 1986; Gryglewski et al., 1986). However, indomethacin did not affect adenosine-induced dilatation, indicating that metabolites of the arachidonic acid cascade such as prostacyclin are unlikely to be candidates for mediating the action of adenosine.

In this work, contrary to the well established concept that effects of adenosine are mediated by cyclic AMP, we found that the vasodilator effect of adenosine is in part mediated by cyclic GMP; adenosine acts on A_2-receptors on the endothelium to produce EDRF, which in turn stimulates soluble guanylate cyclase in vascular smooth muscle, thus producing cyclic GMP and inducing dilatation. In addition, the age-associated loss of dilator response of the rat thoracic aorta to adenosine is suggested to be due to (1) a reduction in the number of A_2-receptors in the endothelium, (2) a decrease in the ability of the endothelium to produce EDRF, and (3) a reduction of guanylate cyclase activity, and thus a decrease in the cyclic GMP level. However, the possibility that a reduction in the number of A_2 receptors in the smooth muscle is likely to contribute partly to the age-associated reduction of the dilator response to adenosine cannot be ruled out. The dilator response to adenosine disappeared earlier in life (during development rather than aging) than the responses to other dilators such as ATP, ACh and histamine.

References

AFONSO, S. & O'BRIEN, G.S. (1976). Enhancement of coronary vasodilator action of adenosine triphosphate by dipyridamole. Circ. Res., 20, 403–408.

BERNE, R.M. (1980). The role of adenosine in the regulation of coronary blood flow. Circ. Res., 47, 807–813.

BOEYNAEMS, J.M. & GALAND, N. (1983). Stimulation of vascular prostacyclin synthesis by extracellular ADP and ATP. Biochem. Biophys. Res. Commun., 112, 290–296.

COLLIS, M.G. & BROWN, C.M. (1983). Adenosine relaxes the aorta by interacting with an A_2 receptor at an intracellular site. Eur. J. Pharmacol., 96, 61–69.

COLLIS, M.G., PALMER, D.B. & BAXTER, G.S. (1986). Evidence that the intracellular effects of adenosine in the guinea-pig aorta are mediated by inosine. Eur. J. Pharmacol., 121, 141–145.

DALY, J.W., UKENA, D. & JACOBSON, K.A. (1987). Analogues of adenosine, theophylline, and caffeine: selective interactions with A1 and A2 adenosine receptors. In Topics and Perspectives in Adenosine Research. ed. Gerlach, E. & Becker, B. pp. 21–36. Berlin: Springer-Verlag.

DE MAY, J.G. & VANHOUTTE, P.M. (1981). Role of the intima in cholinergic and purinergic relaxation of isolated canine femoral arteries. J. Physiol., 316, 347–355.

FÖRSTERMANN, U., HERTTING, G. & NEUFANG, B. (1986). The role of endothelial and non-endothelial prostaglandins in the relaxation of isolated blood vessels of the rabbit induced by acetylcholine and bradykinin. Br. J. Pharmacol., 87, 521–532.

FRANK, G.W. & BEVAN, J.A. (1983). Vasodilatation by adenosine-related nucleotides is reduced after endothelial destruction in basilar, lingual and pulmonary arteries. In Regulatory Function of Adenosine, ed. Berne, R.M., Stone, T.W. & Rubio, R. pp. 511–512. The Hague, Boston, London: Martinus Nijhoff.

FUJITA, S., ISHIDA, Y., IZUMI, K., MORITOKI, H., O'HARA, M. & TAKEI, M. (1980). Potentiation by dilazep of the negative inotropic effect of adenosine on guinea-pig atria. Br. J. Pharmacol., 68, 343–349.

FURCHGOTT, R.F., ZAWADZKI, J.V. & CHERRY, P.D. (1981). Role of endothelium in the vasodilator response to acetylcholine. In Vasodilatation, ed. Vanhoutte, P.M. & Leusen, I. pp. 49–66. New York: Raven Press.

GORDON, J.L. & MARTIN, W. (1983). Endothelium-dependent relax-

ation of the pig aorta: relationship to stimulation of ^{86}Rb efflux from isolated endothelial cells. Br. J. Pharmacol., 79, 531–541.

GRIFFITH, S.G., MEGHJI, P., MOODY, C.J. & BURNSTOCK, G. (1981). 8-Phenyltheophylline: a potent P_1-purinoceptor antagonist. Eur. J. Pharmacol., 75, 61–64.

GRUETTER, C.A., KADOWITZ, P.J. & IGNARRO, L.J. (1981). Methylene blue inhibits coronary arterial relaxation and guanylate cyclase activation by nitroglycerin, sodium nitrite, and amyl nitrite. Can. J. Physiol. Pharmacol., 59, 150–162.

GRYGLEWSKI, R.J., MONCADA, S. & PALMER, R.M.J. (1986). Bioassay of prostacyclin and endothelium-derived relaxing factor (EDRF) from porcine aortic endothelial cells. Br. J. Pharmacol., 87, 685–694.

HARDEBO, J.E., HANKO, J. & OWMAN, C. (1983). Purine P_1 and P_2 receptors in the cerebral circulation. Blood Vessels, 20, 196.

HERLIHY, J.T., BOCKMAN, E.L., BERNE, R.M. & RUBIO, R. (1976). Adenosine relaxation of isolated vascular smooth muscle. Am. J. Physiol., 230, 1239–1243.

HONMA, M., SATOH, T., TAKEZAWA, J. & UI, M. (1977). An ultrasensitive method for the simultaneous determination of cyclic AMP and cyclic GMP in small-volume samples from blood and tissue. Biochem. Med., 18, 257–273.

KENNEDY, C. & BURNSTOCK, G. (1985). ATP produces vasodilatation via P_1 purinoceptors and vasoconstriction via P_2 purinoceptors in the isolated rabbit central ear artery. Blood Vessels, 22, 145–155.

KENNEDY, C., DELBRO, D. & BURNSTOCK, G. (1985). P_2-purinoceptors mediate both vasodilatation (via the endothelium) and vasoconstriction of the isolated rat femoral artery. Eur. J. Pharmacol., 107, 161–168.

KUKOVETZ, W.R., POCH, G., HOLZMANN, S., WURM, A. & RINNER, I. (1978). Role of cyclic nucleotides in adenosine-mediated regulation of coronary flow. Adv. Cyclic Nucleotide Res., 9, 397–409.

KURTZ, A. (1987). Adenosine stimulates guanylate cyclase activity in vascular smooth muscle cells. J. Biol. Chem., 262, 6296–6300.

LOHSE, M.J., ELGER, B., LINDENBORN-FOTINOS, J., KLOTZ, K-N. & SCHWABE, U. (1988). Separation of solubilized A_2 adenosine receptors of human platelets from non-receptor [^{3}H]-NECA binding sites by gel filtration. Naunyn-Schmiedebergs Arch. Pharmacol., 337, 64–68.

LONDOS, C., COOPER, D.M.F. & WOLFF, J. (1980). Subclasses of external adenosine receptors. Proc. Natl. Acad. Sci. U.S.A., 77, 2551–2554.

LOWRY, O.H., ROSEBROUGH, N.J., FALL, A.L. & RANDALL, R.A. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193, 265–275.

MARTIN, W., VILLANI, G.M., JOTHIANANDAN, D. & FURCHGOTT, R.F. (1985). Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by hemoglobin and by methylene blue in the rabbit aorta. J. Pharmacol. Exp. Ther., 232, 708–716.

MATHIESON, J.J.I. & BURNSTOCK, G. (1985). Purine-mediated relaxation and constriction of isolated rabbit mesenteric artery are not endothelium-dependent. Eur. J. Pharmacol., 118, 221–229.

MORITOKI, H., HOSOKI, E. & ISHIDA, Y. (1986). Age-related decrease in endothelium-dependent dilator response to histamine in rat mesenteric artery. Eur. J. Pharmacol., 126, 61–67.

MORITOKI, H. & TANIOKA, A. (1987). Possible involvement of cGMP

in the dilator response of rat thoracic aorta to adenosine and ATP. Abstract of The International Symposium of Neurotransmitter Receptors – Receptor Mechanisms of Neurotransmitters and Neuropeptides. ed. Kitou, S. (Hiroshima University) p. 72. Hiroshima, October 6–9.

MORITOKI, H., TANIOKA, A., MAESHIBA, Y., IWAMOTO, T., ISHIDA, Y. & ARAKI, H. (1988). Age-associated decrease in histamine-induced vasodilatation may be due to reduction of cyclic GMP formation. Br. J. Pharmacol., 95, 1015–1022.

MORITOKI, H., TANIOKA, A. & UEDA, H. (1989). Possible mechanisms of age-associated changes in vascular functions. In Biosignaling in Cardiac and Vascular Systems. ed. Fujiwara, M., Narumiya, S. & Miwa, S. pp. 248–287. Oxford: Pergamon Press.

RAPOPORT, R.M. & MURAD, F. (1983). Agonist-induced endothelium-dependent relaxation in rat thoracic aorta may be mediated through cGMP. Circ. Res., 52, 352–357.

ROSIERS, C.D. & NEES, S. (1987). Functional evidence for the presence of adenosine A_2 receptors in cultured coronary endothelial cells. Naunyn-Schmiedebergs Arch. Pharmacol., 336, 94–98.

RUBANYI, G. & VANHOUTTE, P.M. (1985). Endothelium-removal decreases relaxations of canine coronary arteries caused by β-adrenergic agonists and adenosine. J. Cardiovasc. Pharmacol., 7, 139–144.

SCHUTZ, W., STEURER, G. & TUISL, E. (1982). Functional identification of adenylate cyclase-coupled adenosine receptors in rat brain microvessels. Eur. J. Pharmacol., 85, 177–184.

SILVER, P.L., WALUS, K. & DISALVO, J. (1984). Adenosine-mediated relaxation and activation of cyclic AMP-dependent protein kinase in coronary arterial smooth muscle. J. Pharmacol. Exp. Ther., 228, 342–347.

TAIRA, Z. & TERADA, H. (1985). Specific and non-specific ligand binding to serum albumin. Biochem. Pharmacol., 34, 1999–2005.

UMEKAWA, H., TANAKA, T., KIMURA, Y. & HIDAKA, H. (1984). Purification of cyclic adenosine monophosphate phosphodiesterase from human platelets using new-inhibitor sepharose chromatography. Biochem. Pharmacol., 33, 3339–3344.

VAN CALKER, D., MULLER, M. & HAMPRECHT, B. (1979). Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. J. Neurochem., 33, 999–1005.

VERHAEGHE, R.H. (1977). Action of adenosine and adenine nucleotides on dog's isolated veins. Am. J. Physiol., 233, H114–H121.

WALDMAN, S.A., RAPOPORT, R.M. & MURAD, F. (1984). Atrial natriuretic factor selectively activates particulate guanylate cyclase and elevates cyclic GMP in rat tissues. J. Biol. Chem., 259, 14332–14334.

WHITE, T.D. & ANGUS, J.A. (1987). Relaxant effects of ATP and adenosine on canine large and small coronary arteries in vitro. Eur. J. Pharmacol., 143, 119–126.

WHITE, T.D., CHAUDHRY, A., VOHRA, M.M., WEBB, D. & LESLIE, R.A. (1985). Characteristics of P_2 (nucleotide) receptors mediating contraction and relaxation of rat thoracic aortic strips: possible physiological relevance. Eur. J. Pharmacol., 118, 37–44.

WINQUIST, R.J., FAISON, E.P., WALDMAN, S.A., SCHWARTZ, K., MURAD, F. & RAPOPORT, R.M. (1984). Atrial natriuretic factor elicits an endothelium independent relaxation and activates particulate guanylate cyclase in vascular smooth muscle. Proc. Natl. Acad. Sci. U.S.A., 81, 7661–7664.