Evidence that A₂ purinoceptors are involved in endothelium-dependent relaxation of the rat thoracic aorta

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- 1 The effect of adenosine and some adenosine analogues on the isolated thoracic aorta from rats was compared with the effect of adenosine 5'-triphosphate (ATP) and adenosine 5'-diphosphate (ADP).
- 2 Both ATP and adenosine analogues caused relaxation of the noradrenaline (30 nM)-contracted thoracic aorta.
- 3 The order of potency for adenosine analogues was 5'-(N-ethyl) carboxamidoadenosine (NECA) > L-N⁶-phenylisopropyladenosine (L-PIA), adenosine 5'-monophosphate (AMP), adenosine indicating the presence of adenosine A₂ receptors.
- 4 Removal of the endothelium or prior treatment with haemoglobin (10 µM) attenuated relaxant responses to both ATP and NECA, attenuation being greater for ATP than NECA.
- 5 8-Phenyltheophylline (10 µM) reduced relaxant responses to NECA but not to ATP in the intact tissue.
- 6 These results provide evidence that there are two components to relaxation of the rat thoracic aorta induced by purinoceptor agonists. The first is an endothelium-dependent mechanism involving release of endothelium-derived relaxant factor (EDRF) and the second is due to a direct effect on smooth muscle.

Introduction

It has been recognized for many years that the purine nucleoside adenosine and nucleotides adenosine 5'-diphosphate (ADP) and adenosine 5'-triphosphate (ATP) are potent vasoactive substances. More recent work has shown that ATP can act as both a vasodilator or vasoconstrictor in isolated vascular preparations (Kennedy & Burnstock, 1985; White *et al.*, 1985; Houston *et al.*, 1987). The vasodilator activity is generally dependent on the presence of an intact endothelium (Furchgott, 1981; De Mey & Vanhoutte, 1982; 1983; Cocks & Angus, 1983; Angus *et al.*, 1983; Martin *et al.*, 1985; White & Angus, 1987), although relaxation to ATP has been demonstrated in the rabbit mesenteric artery (Mathieson & Burnstock, 1985) and portal vein (Kennedy & Burnstock, 1985) due to stimulation of P_{2Y} receptors located on the smooth muscle. Vasoconstrictor activity is principally accepted to be due to direct activation of a receptor present on vascular smooth muscle (Kennedy & Burnstock, 1985; White *et al.*, 1985; Houston *et al.*, 1987).

In a review of the literature Burnstock & Kennedy (1985) suggested that on the basis of rank order of agonist potency, two receptors for purine nucleotides could be identified in blood vessels. The receptors that mediated vasodilatation were present on the endothelial cells and were designated P_{2Y} while receptors mediating vasoconstrictor actions were on the smooth muscle and designated P_{2X}.

The location of the vascular receptors for adenosine is not so well defined. While it is generally agreed that the vasodilator action of adenosine is mediated by the A₂ subclass of the P₁ purinoceptor (Collis & Brown, 1983; Edvinsson & Fredholm, 1983; Kusachi *et al.*, 1983; Ramagopal *et al.*, 1988) some workers suggest that the receptor is located on vascular smooth muscle (Collis & Brown, 1983; White & Angus, 1987), whilst others have shown that removal of the endothelium attenuates vasodilator responses to adenosine (Gordon & Martin, 1983; Rubanyi & Vanhoutte, 1985). The presence of A₂ adenosine receptors on cultures of coronary endothelial cells has also been reported (Des Rosiers & Nees, 1987; Schmidt & Kukovetz, 1986).

The aim of this study was to compare the vasodilator actions of adenosine and ATP in the rat thoracic aorta and using a stable analogue of adenosine, 5'-(N-ethyl)carboxamidoadenosine (NECA), investigate the location of the

vascular A₂ receptor. The mechanism of A₂ receptor-induced vasodilatation was also studied.

Methods

Male Wistar rats weighing 250–350 g were killed by a blow to the head and bled out. The thoracic aorta was excised and rings of 7 mm length were placed in 10–20 ml organ baths filled with Krebs Henseleit solution (composition mM: NaCl 118, KCl 4.7, NaHCO₃ 25, glucose 11, CaCl₂·2H₂O 2.5, MgSO₄·7H₂O 0.45, KH₂PO₄ 1.2, EDTA 0.03, sodium ascorbate 0.09) kept at 37°C and bubbled with 95% O₂: 5% CO₂. Rings of thoracic aorta were mounted horizontally by inserting two wires into the lumen and maintained at 2 g tension. Care was exercised to preserve the integrity of the endothelium on the preparation. After an equilibration period of 45 min a concentration-response curve to noradrenaline (NA) was established. The preparation was then washed out and allowed to recover. The tissue was then contracted with a sub-maximal concentration of NA (30 nM) and allowed to reach maximum tension before cumulative additions of purine analogues. The duration of each concentration-response curve to purine analogues did not exceed 12 min. Results are expressed as a percentage of the maximum contraction before the addition of purines.

The endothelium of some thoracic aortae was removed by gently rubbing the luminal surface with cotton soaked in Krebs Henseleit solution. Absence of relaxation to acetylcholine was used to indicate the complete removal of endothelial cells. In experiments where haemoglobin, 8-phenyltheophylline (8-PT) or indomethacin were used, the tissue was equilibrated for 30 min before recommencement of purine concentration-response curves.

Drugs

Noradrenaline ((-)-arterenol bitartrate), acetylcholine chloride, adenosine 5'-triphosphate (Na salt), 8-phenyltheophylline, adenosine 5' monophosphate (Na salt), 5'-(N-ethyl)carboxamidoadenosine, L-phenylisopropyladenosine, adenosine (Na salt), indomethacin, sodium nitroprusside, haemoglobin and 8-phenyltheophylline were obtained from Sigma.

All compounds were made up in aqueous solution with dilutions made in saline, except 8-phenyltheophylline, indomethacin and haemoglobin. 8-Phenyltheophylline was made up to a concentration of 10 mM in 80% v/v methanol containing 0.2 M NaOH and diluted as required in saline and indomethacin was dissolved in 1% sodium carbonate solution. Haemoglobin was prepared as described by Martin *et al.* (1985).

Statistical analysis

The data are expressed as mean $\pm$ s.e.mean. Concentration-response curves to noradrenaline were studied by least square regression line analysis (LINUS, Apple IIe computer). Significance of differences was calculated by Student's *t* test. *P* values of less than 0.05 were considered to represent significant differences.

Results

Concentration-response curves to noradrenaline

Noradrenaline elicited concentration-dependent contractions in both intact aortic rings and in rings from which the endothelium had been removed. Concentration-response curves in endothelium-denuded segments were not significantly different ($P > 0.05$) from those in control segments with respect to the slope of the curve (58.5 ± 7.0 intact; 44.6 ± 9.2 denuded; LINUS programme) (Figure 1) though the maximum tension developed to NA at a concentration of $10 \mu\text{M}$ was significantly greater ($P < 0.05$) in endothelium-denuded tissues (1.16 ± 0.09 g) compared to endothelium-intact tissues (0.81 ± 0.1 g). The results are summarized in Figure 1 (inset histogram). For experiments where the relaxant effects of the purines were studied the submaximal concentration of 30 nM NA was used to precontract the tissue. The mean contraction produced by 30 nM NA was 0.89 ± 0.09 g in intact aortic rings and 0.98 ± 0.11 g in endothelium-denuded rings. At this concentration of NA there was no significant difference in the tension developed ($P > 0.05$).

Figure 1 Noradrenaline (NA) concentration-response curve on rat thoracic aorta with the endothelium present (Δ , $n = 12$) and absent ($\blacktriangle$, $n = 12$). Data expressed as mean with s.e.mean shown by vertical bars. The inset histogram represents the grams tension exerted after 2 min exposure at each concentration for endothelium intact (open columns) and denuded (hatched columns) aorta. *Indicates a significant difference between groups: $P < 0.05$, Student's *t* test.

Effects of purine receptor agonists on precontracted aortic rings

ATP induced concentration-dependent relaxation of the precontracted rat thoracic aorta (Figure 2, upper panel). Removal of the endothelial cells almost abolished the relaxant effect of ATP. NECA and L-PIA (0.01 – $30 \mu\text{M}$) caused concentration-dependent relaxations of intact vessel segments. Figure 2 shows traces from an experiment which compares the relaxant actions of NECA (lower panel) to those of the P₂ agonist ATP (upper panel). AMP and adenosine (0.1 – $300 \mu\text{M}$) also produced vessel relaxation (Figure 3a). To correlate the relative potencies of the P₁ receptor agonists the degree of relaxation of the NA (30 nM) contracted tissue to purine agonists at concentration $10 \mu\text{M}$ were compared. On the contracted rat thoracic aorta the nonselective P₁ receptor agonist NECA had a greater potency than the A₁ receptor agonist L-PIA. Adenosine and AMP were equipotent in relaxing the tissue. In endothelium-denuded rings the relaxant effects of all four agonists were significantly ($P < 0.05$) less than in intact ring segments (Figure 3b). Removal of the endothelium from these tissues did not alter the relaxant responses to the endothelium-independent vasodilator, sodium nitroprusside (data not shown).

Effect of haemoglobin on the relaxant responses to purines

In the endothelium intact NA (30 nM) contracted rat thoracic aorta, haemoglobin ($10 \mu\text{M}$) induced a further $13.7 \pm 4.7\%$ increase in tension (data not shown). Prior treatment with haemoglobin ($10 \mu\text{M}$) caused a significant ($P < 0.05$) 562 fold rightward shift of the dose-response curve to ATP (Figure 4). There was a significant ($P < 0.05$) 102 fold rightward shift in the concentration-response curve to NECA in the presence of $10 \mu\text{M}$ haemoglobin (Figure 5). Pretreatment with haemoglobin ($10 \mu\text{M}$) does not cause a significant shift ($P > 0.05$) in concentration-response curves to sodium nitroprusside (data not shown).

The effect of indomethacin

Prior treatment of intact ring segments with indomethacin ($1 \mu\text{M}$) did not significantly alter concentration-response curves to either ATP or NECA (data not shown).

The effects of 8-phenyltheophylline

Prior treatment of intact rings with $10 \mu\text{M}$ 8-PT significantly ($P < 0.05$) inhibited the relaxant response to $10 \mu\text{M}$ NECA. The relaxant responses of intact aortic rings to $30 \mu\text{M}$ ATP

Figure 2 Trace showing the effect of adenosine 5'-triphosphate (ATP, upper panel) and 5'-N-ethyl carboxamidoadenosine (NECA, lower panel) on noradrenaline (NA, 30 nM)-contracted rat thoracic aorta in endothelium intact (+E) and denuded (–E) tissues.

Figure 3 The effect of purine analogues on the noradrenaline (30 nM)-contracted thoracic aorta in endothelium intact (a) and denuded (b) tissues. ($n = 5-7$). (◆) Adenosine; (◇) adenosine 5'-phosphate; (△) 5'-N-ethyl carboxamidoadenosine; (▲) L-N⁶-phenylisopropyladenosine. Data are expressed as means with s.e.mean shown by vertical bars.

were enhanced by 10 μ M 8-PT (Figure 6) but the change did not reach the level of significance ($P > 0.05$). The addition of 10 μ M 8-PT to endothelium-denuded tissues significantly reduced the relaxant response of the thoracic aorta to 10 μ M NECA ($P < 0.05$). Any residual effect of ATP on the endothelium-denuded tissue was completely blocked by 8-PT (10 μ M, data not shown). The vehicle used for 8-PT stock solutions did not affect relaxant responses to the purines in this tissue.

Figure 4 The effect of adenosine 5'-triphosphate (ATP) on noradrenaline (30 nM)-contracted rat thoracic aorta in the presence (■) and absence (●) of haemoglobin 10 μ M, $n = 6$ per group. Each point represents mean with s.e.mean shown by vertical bars.

Figure 5 The effect of 5'-N-ethyl carboxamidoadenosine (NECA) on noradrenaline (30 nM)-contracted rat thoracic aorta in the presence (■) and absence (●) of haemoglobin 10 μ M, $n = 6$ per group. Each point represents mean with s.e.mean shown by vertical bars.

Figure 6 Histogram showing the effects of 8-phenyltheophylline (8-PT, 10 μ M) on relaxant responses to 5'-N-ethyl carboxamidoadenosine (NECA, 10 μ M) and adenosine 5'-triphosphate (ATP, 30 μ M) in noradrenaline-contracted rat thoracic aorta ($n = 5-6$). *Indicates a statistically significant change from control response ($P < 0.05$, two tailed Student's t test).

Discussion

Our results show that the P_2 purinoceptor which mediates relaxation in the thoracic aorta is located on the endothelium since removal of endothelium markedly attenuated relaxation. As pretreatment with haemoglobin, which inhibits the action of endothelium-derived relaxing factor (EDRF) (Martin *et al.*, 1985) also abolished ATP-induced relaxation in this preparation, P_2 receptor-mediated relaxation is due to the release of EDRF. These results are similar to those obtained by Houston *et al.* (1987) for canine blood vessels. Although purine nucleotides have been reported to stimulate prostaglandin synthesis in endothelial cells (Van Coevorden & Boeynaems, 1984) the release of these mediators does not appear to play a role in nucleotide-induced relaxation in this tissue since pretreatment with the cyclo-oxygenase inhibitor indomethacin did not alter the response.

P_1 -purinoceptor agonists also relaxed the NA-contracted rat thoracic aorta. The stable analogue NECA is a non-selective P_1 purine receptor agonist but is considered more potent at the A_2 receptor while L-PIA is a selective A_1 receptor agonist (Williams, 1987). NECA was more potent at inducing relaxation of the rat aorta than L-PIA and both adenosine and AMP. This order of potency is consistent with the presence of an A_2 receptor (Van Calcar *et al.*, 1979; Londos *et al.*, 1980) and is in agreement with the findings of

other workers (see Introduction for references). Removal of the endothelium attenuated but did not abolish the relaxant responses to all four agonists. This indicates that a component of the response is endothelium-dependent and we suggest that A₂ receptors are present on both endothelial cells and smooth muscle. The endothelial receptors appear, like the P_{2Y} receptors, to mediate the release of EDRF, since haemoglobin caused a significant rightward shift in the concentration-response curve to NECA. Relaxant responses to a single concentration of NECA, once established, were reversed by the addition of haemoglobin to the bath. Cyclo-oxygenase products do not appear to contribute to NECA-induced relaxation in this preparation as indicated by the lack of effect of indomethacin.

The possibility that the endothelium-dependent component of relaxation in responses to NECA was due to an action on P_{2Y} receptors was excluded by the use of the adenosine receptor antagonist 8-PT. 8-PT significantly reduced responses to NECA in both intact and endothelium-denuded tissues, whilst relaxant responses to ATP were enhanced in the intact tissues. The reason for this enhancement is not clear. The very small residual response to ATP in endothelium-denuded tissues was abolished by prior treatment with 8-PT, which suggests that the response was due to adenosine formed by the breakdown of ATP.

The presence of adenosine A₂ receptors on endothelial cells may be of physiological importance in controlling blood flow. It has been suggested that adenosine is a local regulator of

blood flow in several vascular systems (see Berne *et al.*, 1979; Burnstock, 1987). Since intravenous administration of adenosine or its stable analogues causes increases in coronary flow in the dog (Raberger *et al.*, 1977) these agonists are either able to cross the endothelium to activate the smooth muscle receptors directly or the vasodilatation is produced by an action on the luminal surface of the vessel. Since the endothelium provides a metabolic barrier to the penetration of a number of vasoactive substances such as 5-hydroxytryptamine and NA (Verbeuren *et al.*, 1988), and adenosine itself in a concentration below the micromolar range is subject to very comprehensive metabolism by the endothelium cells (Nees *et al.*, 1985), it is unlikely that under normal conditions circulating adenosine can cross the endothelium. Von Borstel *et al.* (1983) found that the basal plasma levels of adenosine in the rat were in the order of 1–4 µM and that decreases in blood pressure following intravenous infusion of adenosine occurred at plasma adenosine concentration of 5–6 µM.

The results described here suggest that A₂ receptors are located on both the endothelium and smooth muscle. We propose that the endothelial cell A₂ purine receptor may have a role in rapidly maintaining blood flow in conditions of increased adenosine release such as hypoxia. Increased levels of adenosine may then be able to cross the endothelial barrier to activate smooth muscle receptors directly and thus provide a longer lasting component of vasodilatation as suggested previously by Burnstock (1987).

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