Pivotal role of phosphate chain length in vasoconstrictor *versus* vasodilator actions of adenine dinucleotides in rat mesenteric arteries

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1. The isolated perfused rat mesenteric arterial bed was used to examine the activity of the adenine dinucleotides: β-nicotinamide adenine dinucleotide (NAD); β-nicotinamide adenine dinucleotide phosphate (NADP); flavin adenine dinucleotide (FAD); and of the α,ω-diadenosine polyphosphates: adenylyl adenosine (AP_1A); P^1,P^2-diadenosine pyrophosphate (AP_2A); P^1,P^3-diadenosine triphosphate (AP_3A); P^1,P^4-diadenosine tetraphosphate (AP_4A); P^1,P^5-diadenosine pentaphosphate (AP_5A); P^1,P^6-diadenosine hexaphosphate (AP_6A). Responses were compared with those of ADP, ATP, 2-methylthio-ATP (2-meSATP) and α,β-methylene ATP (α,β-meATP).

2. In basal tone preparations mono- and dinucleotides elicited *vasoconstriction* with the order of potency: α,β-meATP $\geqslant AP_5A \geqslant AP_6A \geqslant AP_4A \geqslant$ 2-meSATP $\gg$ ATP $\gg$ ADP. The dinucleotides NAD, NADP, FAD, AP_1A, AP_2A and AP_3A had no effect.

3. The P_{2X}-purinoceptor antagonist pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (30 μM) virtually abolished vasoconstrictor responses to AP_4A, AP_5A and AP_6A.

4. Auto- and cross-desensitization of vasoconstrictor responses to AP_4A, AP_5A, AP_6A, ATP and α,β-meATP were observed.

5. In raised tone preparations nucleotides elicited endothelium-dependent *vasodilatation* with the order of potency: 2-meSATP = ADP > ATP > AP_3A > AP_2A > AP_1A = NADP = FAD > NAD. The nucleotides AP_4A, AP_5A, AP_6A and α,β-meATP had no vasodilator effects.

6. It is concluded that the α,ω-adenine dinucleotides AP_4A, AP_5A and AP_6A elicit vasoconstriction, but not vasodilatation, in the rat mesenteric arterial bed via P_{2X}-purinoceptors. In contrast, the dinucleotides NADP, FAD, AP_1A, AP_2A and AP_3A elicit vasodilatation, but not vasoconstriction, via endothelial P_{2Y}-purinoceptors.

7. It is suggested that there is a crucial relationship between the structure of the α,ω-diadenosine polyphosphates and their activity at P_{2X}- and P_{2Y}-purinoceptors with a pivotal role played by the polyphosphate chain. Molecules with four or more phosphates are vasoconstrictors, while those with three or less phosphates are vasodilators.

Adenine nucleotides are naturally occurring molecules which have been attracting considerable interest recently because of increasing evidence that they can act as extracellular signal molecules (Hoyle, 1990; Ogilvie, 1992; Pintor, Porras, Mora & Miras-Portugal, 1993; Schluter *et al.* 1994). Adenine dinucleotides can be split into two broad groups. One group consists of coenzymes such as β-nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD), together with their phosphorylated and reduced forms (NADP, NADH, NADPH, FADH). These molecules consist of an adenosine moiety linked via a pyrophosphate chain to ribosylated nicotinamide (NAD) or ribotinylated flavin (FAD). The second group consists of α,ω-adenine dinucleotides or bis(5′-adenosyl)polyphosphates, which consist of two adenosine moieties linked via their 5′ position by a chain of two or more phosphates (abbreviated to AP_xA, where x represents the number of phosphates in the connecting chain; see Fig. 1). The coenzymes are constituents of all cells, whereas the α,ω-adenine dinucleotides are present

in a diverse number of biological tissues including platelets, hepatocytes, adrenal medullary chromaffin granules (Rapaport & Zamenick, 1976; Flodgaard & Klenow, 1982; Luthje & Ogilvie, 1983; Rodriguez-del-Castillo, Torres, Delicado & Miras-Portugal, 1988; Schluter *et al.* 1994) and the central nervous system (Pintor, Diaz, Torres & Miras-Portugal, 1992; Pintor *et al.* 1993).

Adenine dinucleotides have potent actions on diverse tissues; however, compared with the mononucleotides their actions have been relatively poorly characterized: receptors for mononucleotides are divided into P_1- and P_2-purinoceptor subtypes (Burnstock, 1978), with a further subdivision of the P_2-purinoceptor into P_{2X}-receptors (receptor-operated ion channels), which typically mediate constriction of the smooth muscle of blood vessels and other visceral tissues, and P_{2Y}-purinoceptors (coupled to G-proteins), which characteristically mediate relaxation in gastrointestinal smooth muscle and blood vessels (Burnstock & Kennedy, 1985; Kennedy, 1990; Hoyle & Burnstock, 1991; Abbracchio & Burnstock, 1994). α,β-Methylene ATP (α,β-meATP) is the archetypal P_{2X}-purinoceptor agonist which can also be used to desensitize this receptor (see Burnstock & Kennedy, 1985; Kennedy, 1990); arylazido-aminopropionylATP and pyridoxalphosphate-6-azo-phenyl-2′,4′-disulphonic acid (PPADS) (Lambrecht *et al.* 1992; Ziganshin *et al.* 1993; Ziganshin, Hoyle, Lambrecht, Mutschler, Bäumert & Burnstock, 1994; Windscheif, Ralevic, Bäumert, Mutschler, Lambrecht & Burnstock, 1994) are antagonists. 2-MethylthioATP (2-meSATP) is the archetypal P_{2Y}-purinoceptor agonist and Reactive Blue 2 and suramin are antagonists (Burnstock & Warland, 1987; Hoyle, Knight & Burnstock, 1990). In some instances the adenine dinucleotides elicit extracellular effects via P_1- and P_2-purinoceptors. However, the recognition of a number of cases in which their actions do not conform to P_1- or P_2-purinoceptor activation, their identification in the central nervous system and chromaffin granules, and the fact that they do have potent and diverse extracellular actions, has led to the proposal that adenine dinucleotides may represent a distinct class of signal molecules that may yet be discovered in autonomic neurones (see Hoyle, 1990, 1992; Ogilvie, 1992).

The aim of this study was to characterize the effects of adenine dinucleotides in the isolated rat mesenteric arterial bed. In this preparation P_{2X}-purinoceptors, located on the vascular smooth muscle, and P_{2Y}-purinoceptors, on the endothelium, have been shown to mediate vasoconstriction and vasodilatation, respectively, to various purine compounds (Ralevic & Burnstock, 1988). We were particularly interested in addressing the question of whether distinct receptors for adenine dinucleotides are present in rat mesenteric arteries or whether these molecules activate receptors that are part of the existing scheme for P_1- or P_2-purinoceptors. In addition, characterization of structure–activity relationships of the adenine dinucleotides was anticipated to be valuable in providing information about the nature of the active sites of the nucleotides and, hence, of the binding sites of their receptors.

METHODS

Isolated mesenteric arterial bed preparation

Male Wistar rats (300–350 g) were killed by asphyxiation with CO_2. Mesenteric beds were isolated and set up for perfusion as

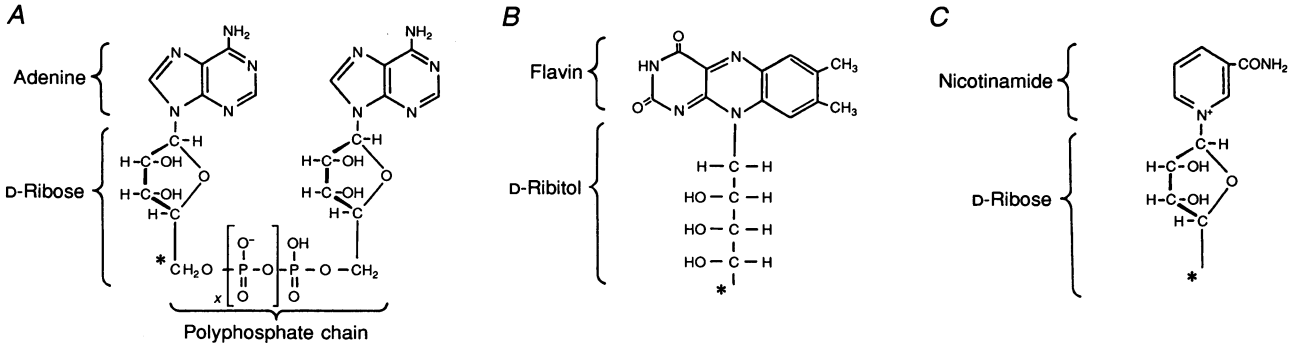

Figure 1. Basic structure of adenine dinucleotides

A, α,ω-adenine dinucleotides: for compounds with two or more phosphates, two adenosine moieties (adenine and ribose) are linked via a polyphosphate chain with a length of 2–6 phosphates ($x = 1$–5) via 5′-linkages. For adenylyl adenosine (AP_1A) the two ribose moieties are linked to a single phosphate group via a 3′- and a 5′-linkage. *B* and *C*, adenine dinucleotide coenzymes: flavin adenine dinucleotide (FAD; *B*) – flavin and D-ribitol replace one of the adenosine groups being attached to an adenosine group via a pyrophosphate chain (2 phosphates) at *; nicotinamide adenine dinucleotide (NAD; *C*) – nicotinamide replaces an adenine moiety; it is attached, together with D-ribose, to an adenosine moiety via a pyrophosphate chain at *.

described previously (Ralevic, Rubino & Burnstock, 1994). The abdomen was opened and the superior mesenteric artery exposed and cannulated with a hypodermic needle. The superior mesenteric vein was severed, the gut dissected away and the preparation mounted on a stainless-steel grid (7 × 5 cm) in a humid chamber (custom made at University College London). The preparation was perfused at a constant flow rate of 5 ml min^{-1} using a peristaltic pump (model 7554-30; Cole-Parmer Instrument Co., Chicago, IL, USA). The perfusate was Krebs solution of the following composition (mM): NaCl, 133; KCl, 4·7; NaH_2PO_4, 1·35; $NaHCO_3$, 16·3; $MgSO_4$, 0·61; $CaCl_2$, 2·52; and glucose, 7·8; gassed with 95% O_2–5% CO_2 and maintained at 37 °C. Responses were measured as changes in perfusion pressure (mmHg) with a pressure transducer (model P23XL; Viggo-Spectramed, Oxnard, CA, USA) on a side-arm of the perfusion cannula, and recorded on a polygraph (model 7D; Grass Instrument Co., Quincy, MA, USA). Preparations were allowed to equilibrate for 30 min prior to experimentation.

Basal-tone preparations

Vasoconstrictor responses of preparations to doses of the purine dinucleotides α,β-meATP, 2-meSATP and ATP were assessed at basal tone. Preliminary studies indicated a significant degree of cross-desensitization between vasoconstrictor responses. To minimize the effects of desensitization separate mesenteric arterial bed preparations were used to construct dose–response curves for each agonist, with 10–15 min being allowed to elapse between consecutive doses. Studies with the P_{2X}-purinoceptor antagonist PPADS (1, 10 and 30 μM) were also conducted on separate preparations for each agonist. PPADS was added to the perfusate 30 min before challenge with adenine mono- or dinucleotides.

Two desensitization regimes were used to examine the effects of auto- and cross-desensitization. In both regimes control responses to purine compounds were established using doses producing approximately 20% of maximal vasoconstriction for each compound to minimize the effects of desensitization at this stage. In addition, 10 min was allowed to elapse between doses. In the first desensitization regime a single injection of the most potent vasoconstrictor dinucleotide, P^1,P^5-diadenosine pentaphosphate (AP_5A), at a dose producing approximately 80% of maximal vasoconstriction (15 nmol), followed these control responses, and this was followed by a repeat of the purine compound injections at intervals of 1 min, commencing as soon as the response to AP_5A had returned to baseline (approximately 2 min). The second injection regime was similar but employed three consecutive doses of AP_5A (each 15 nmol), each applied when the response to the previous dose had returned to baseline (approximately 1·5 min). In both regimes the response to a single dose of noradrenaline (NA; 0·5 nmol) was applied after responses to the purine compounds, before and after AP_5A.

Raised-tone preparations

Vasodilator responses were examined in raised-tone mesenteric arterial bed preparations in which the tone was increased by continuous perfusion with methoxamine (5–60 μM). The resistance of vasodilator responses to desensitization and the reproducibility of responses with time allowed dose–response curves for several agonists to be constructed for the same preparation. The endothelium was removed by controlled perfusion of preparations with 2 ml of a solution of sodium deoxycholate (2 mg ml^{-1} in physiological saline) as described previously (Ralevic & Burnstock, 1988). The success of this treatment was confirmed by the fact that vasodilator responses to ATP were abolished. The integrity of the smooth muscle was confirmed by retained ability of the preparation to relax in response to application of sodium nitroprusside (SNP).

Drugs used

All adenine mono- and dinucleotides, SNP and NA were applied as 50 μl bolus injections into a rubber septum proximal to the preparation. Drug dilutions were performed daily from stock solutions of 10 or 100 mM (concentrates stored frozen) in distilled water. The following drugs were obtained from Sigma: NAD, NADP (sodium salt), FAD (disodium salt), adenylyl adenosine (AP_1A; free acid), P^1,P^2-diadenosine pyrophosphate (AP_2A; sodium salt), P^1,P^3-diadenosine triphosphate (AP_3A; ammonium salt), P^1,P^4-diadenosine tetraphosphate (AP_4A; ammonium salt), AP_5A (ammonium salt), P^1,P^6-diadenosine hexaphosphate (AP_6A; ammonium salt), α,β-meATP (lithium salt), ADP (sodium salt), ATP (disodium salt), methoxamine hydrochloride, sodium nitroprusside and sodium deoxycholate. 2-MethylthioATP (2-meSATP; tetrasodium salt) was from Research Biochemicals Inc. PPADS was a generous gift from Dr G. Lambrecht (University of Frankfurt, Germany).

Data analysis

Responses were measured as changes in perfusion pressure (mmHg) and results presented as the means ± S.E.M. Differences between means were determined by Student's unpaired t test, and were considered significant when $P < 0.05$.

RESULTS

Vasoconstrictor responses in basal-tone preparations

Basal tone of the mesenteric arterial bed preparations was 33·6 ± 0·6 mmHg ($n = 94$). At basal tone the nucleotides caused dose-dependent vasoconstriction with the following order of potency: α,β-meATP ≥ AP_5A ≥ AP_6A ≥ AP_4A ≥ 2-meSATP ≫ ATP ≫ ADP, with AP_3A, AP_2A, AP_1A, NAD, NADP and FAD having no constrictor activity at the highest doses tested (0·15–0·5 μmol). Representative traces showing vasoconstrictor actions of AP_5A are shown in Fig. 2*A*. The two highest doses of α,β-meATP and AP_5A caused smaller responses than might be expected which were probably reduced because of autodesensitization. The dose–response curves did not plateau (Fig. 3), so it was not possible to calculate ED_{50} or pD_2 (−log EC_{50}) values. Therefore, the potency of the compounds was compared by determining the dose that would cause an increase in perfusion pressure of 30 mmHg (pD_{30}, Table 1).

Following incubation with PPADS (10 μM) responses to AP_6A and AP_4A were abolished or severely attenuated across their dose range (Fig. 4*A* and *B*). AP_5A was tested against a range of concentrations of PPADS (1–30 μM). At 1 μM PPADS caused no significant inhibition, but from 3–30 μM it caused a concentration-dependent inhibition with an apparent suppression of the maximum response

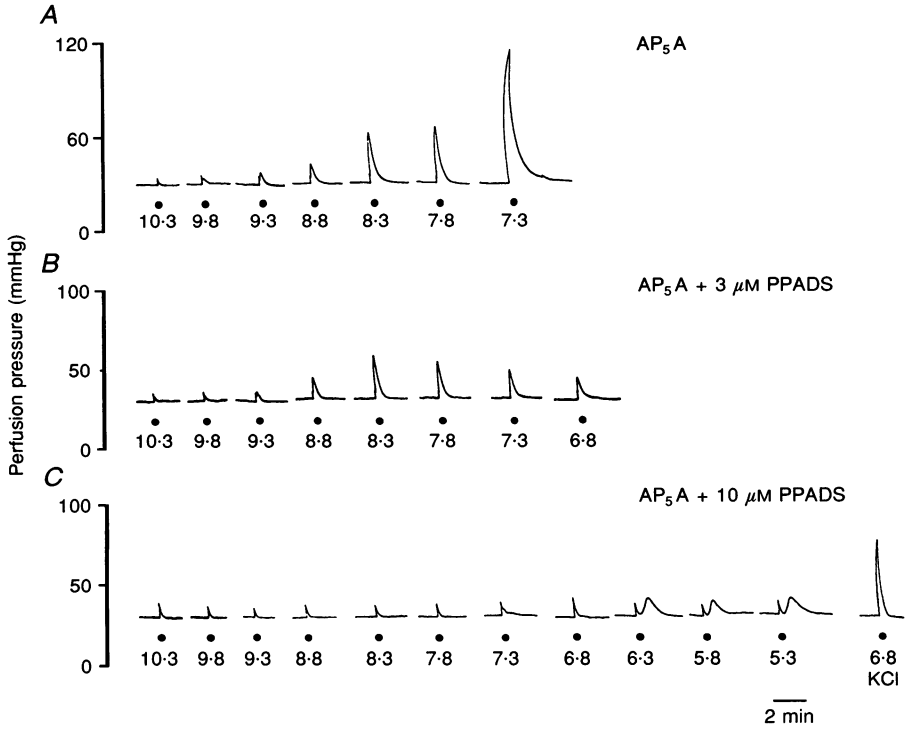

Figure 2. Effect of PPADS on vasoconstrictor responses to AP_5A

Representative traces showing vasoconstrictor responses (mmHg) of the rat mesenteric arterial bed to doses (at the doses indicated beneath traces in −log mol) of AP_5A at basal tone and inhibition of these responses by the P_{2x}-purinoceptor antagonist PPADS. *A*, AP_5A alone; *B*, AP_5A in the presence of 3 μM PPADS; *C*, AP_5A in the presence of 10 μM PPADS.

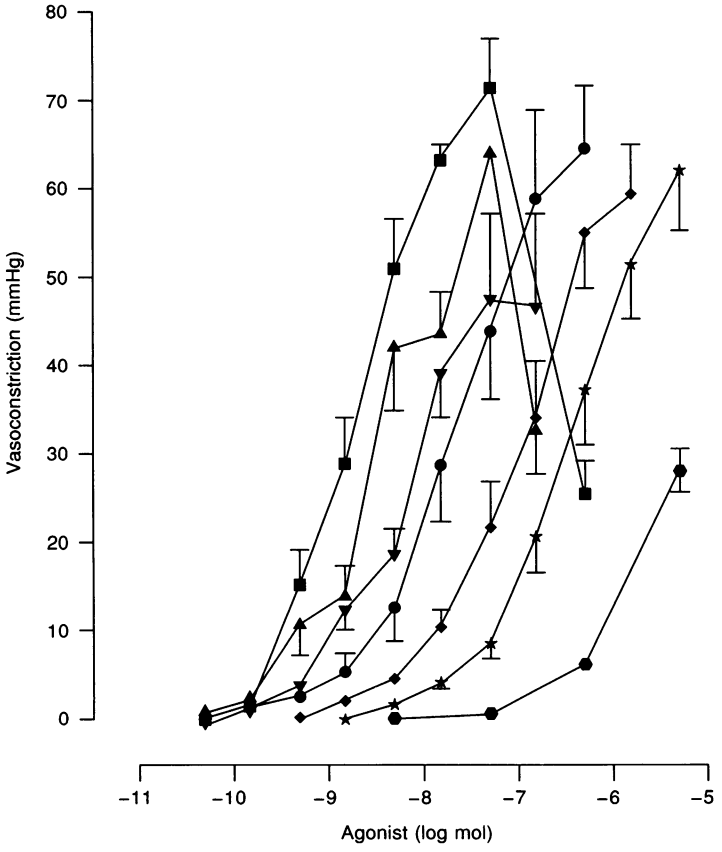

Figure 3. Vasoconstrictor dose–response relationships of adenine mono- and dinucleotides in basal-tone preparations

Points show means ± S.E.M. ($n = 4$–7).
■, α,β-meATP; ▲, AP_5A; ▼, AP_6A; ●, AP_4A; ◆, 2-meSATP; ★, ATP; ⬣, ADP.

Table 1. Vasoconstrictor pD_{30} values and relative potencies for adenine mono- and dinucleotides in basal-tone preparations

Compound	pD_{30} (−log mol)	Relative potency
α,β-meATP	8·8 ± 0·14 (6)	251
AP_5A	8·5 ± 0·15 (8)	126
AP_6A	8·2 ± 0·09 (7)	63
AP_4A	7·9 ± 0·16 (6)	32
2-meSATP	7·0 ± 0·17 (4)	4
ATP	6·4 ± 0·17 (6)	1
ADP	5·2 ± 0·12 (4)	0·063

Values are given as means ± S.E.M., n values are given in parentheses. Potency is expressed relative to ATP.

(Figs 2*B* and *C*, and 4*C*). The nature of this antagonism cannot be determined from these dose–response relationships, but the pA_2 (−log K_d) determined for 3 μM PPADS at the level of 20 mmHg was 5·72. PPADS at 10 and 30 μM shifted the dose–response relationship for ATP successively to the right (Fig. 4*D*). The pA_2 values calculated for these two concentrations of PPADS were 5·36 and 5·39, respectively.

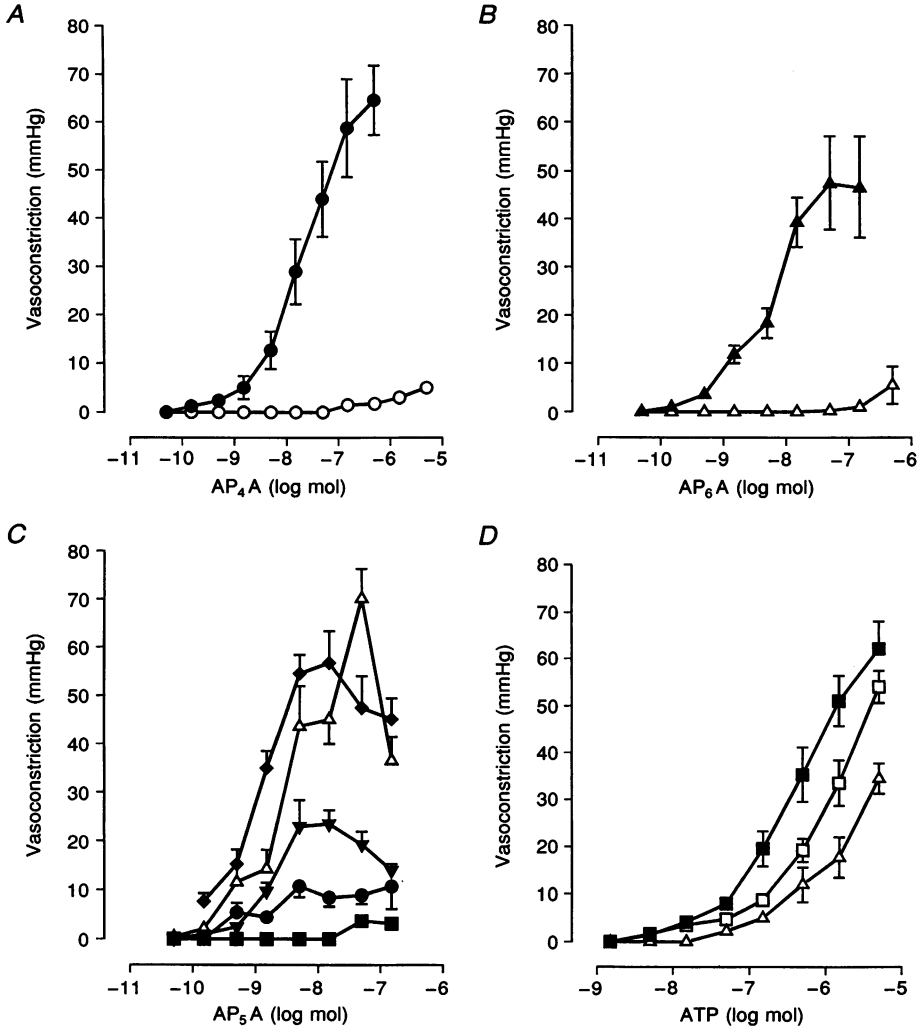

Figure 4. Effect of PPADS on vasoconstrictor responses to adenine mono- and dinucleotides in basal-tone preparations

Vasoconstrictor responses to: *A*, AP_4A alone (●, $n = 4$) and in the presence of 30 μM PPADS (○, $n = 4$); *B*, AP_6A alone (▲, $n = 6$) and in the presence of 30 μM PPADS (△, $n = 4$); *C*, AP_5A alone (△, $n = 7$) and in the presence of PPADS at 1 (◆, $n = 4$), 3 (▼, $n = 8$), 10 (●, $n = 4$) and 30 μM (■, $n = 4$); *D*, ATP alone (■, $n = 6$) and in the presence of PPADS at concentrations of 10 (□, $n = 4$) and 30 μM (△, $n = 4$).

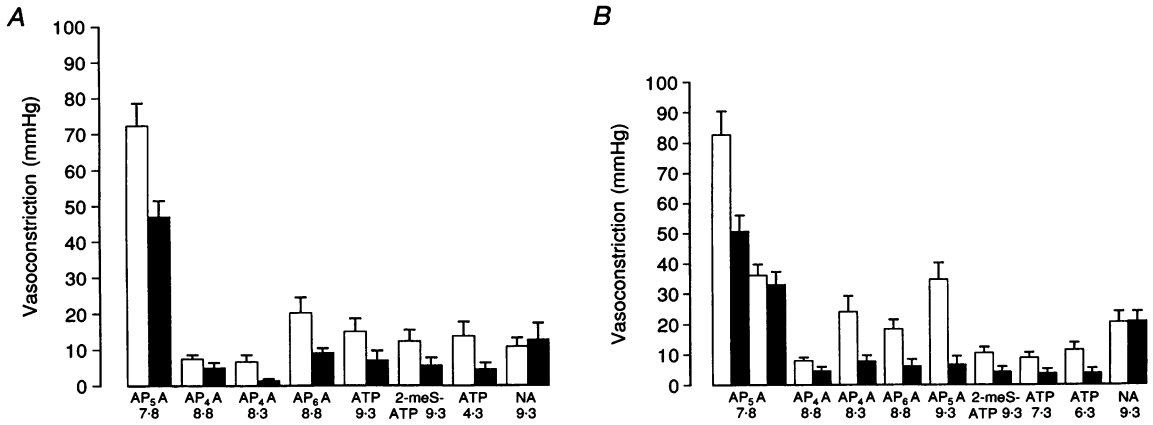

Figure 5. Effect of desensitization with AP_5A on vasoconstrictor responses to adenine mono- and dinucleotides in basal-tone preparations

Two regimes of desensitization with AP_5A on vasoconstrictor responses to ATP, AP_4A, AP_5A, AP_6A, 2-meSATP and NA. Open columns represent control responses, filled columns represent responses obtained following application of AP_5A (15 nmol) according to one of the following regimes: *A*, regime 1 – desensitization with a single dose of AP_5A; *B*, regime 2 – desensitization with three consecutive doses of AP_5A applied in rapid succession. Note that regimes 1 and 2 desensitized responses to adenine nucleotides and dinucleotides (at the indicated concentrations in −log mol) by approximately 65 and 75%, respectively, while responses to NA were unaffected.

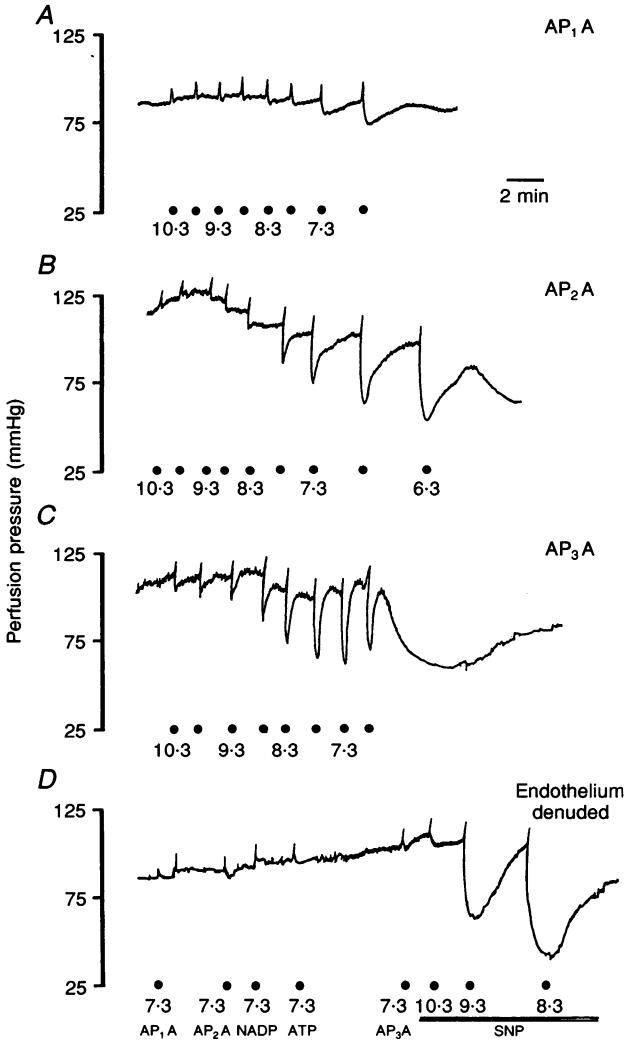

Figure 6. Vasodilator responses of adenine mono- and dinucleotides in raised-tone preparations

Representative traces showing comparative vasodilator responses (mmHg) of a single mesenteric arterial bed preparation to the indicated doses (in −log mol) of: *A*, AP_1A; *B*, AP_2A; and *C*, AP_3A. The tone of the preparation was raised with 10 μM methoxamine. Note that, at the highest dose tested, the response to AP_3A was biphasic, with vasoconstriction preceding vasodilatation. In some preparations a second vasoconstriction followed the vasodilatation. In the endothelium-denuded preparation (*D*) vasodilator responses to high doses of AP_1A, AP_2A, AP_3A, ATP and NADP were abolished (the preparation was still able to relax in response to SNP application).

Table 2. Vasodilator pD_{20} values for adenine mono- and dinucleotides in raised-tone preparations

Compound	pD_{20} (−log mol)	Relative potency
ADP	10·5 ± 0·16 (4)	4
2-meSATP	10·5 ± 0·21 (8)	4
ATP	9·9 ± 0·20 (8)	1
AP_3A	8·5 ± 0·18 (10)	0·040
AP_2A	7·5 ± 0·21 (10)	0·004
AP_1A	7·1 ± 0·23 (8)	0·002
NADP	7·1 ± 0·21 (9)	0·002
FAD	6·8 ± 0·21 (7)	0·0008
NAD	5·9 ± 0·26 (7)	0·0001

Values are given as means ± S.E.M., n values are given in parentheses. pD_{20} is defined as −log(dose) that evoked a decrease in perfusion pressure of 20 mmHg. Potency is expressed relative to ATP.

Auto- and cross-desensitization

In the first series of experiments, in which the preparation was given a conditioning dose of AP_5A (15 nmol), responses to ATP, AP_4A, AP_5A, AP_6A and α,β-meATP, at doses that were approximately equipotent, were all attenuated by approximately 65% (Fig. 5*A*). Responses to NA were unaffected, with values of 10·8 ± 1·67 and 12·5 ± 3·33 mmHg ($n = 4$) before and after AP_5A application, respectively. In the second series of experiments, in which three consecutive doses of AP_5A (15 nmol) were applied, the responses to ATP, AP_4A, AP_5A, AP_6A and α,β-meATP, but not NA (0·5 nmol), were attenuated by approximately 75% (Fig. 5*B*). Responses to NA were 17·7 ± 2·5 and 20·4 ± 3·77 mmHg ($n = 7$) before and after AP_5A application, respectively. The desensitization to AP_5A was maintained, because the response to the fourth dose of AP_5A, after testing all other compounds, was not significantly different from the response to the third dose.

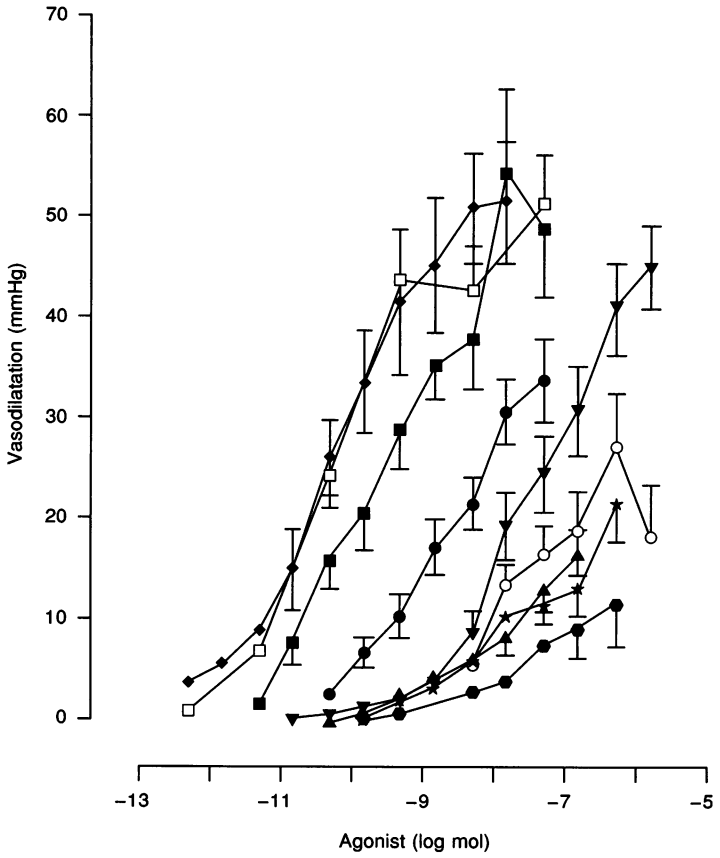

Figure 7. Vasodilator dose–response relationships of adenine mono- and dinucleotides in raised-tone preparations

Points show means ± S.E.M. ($n = 4$–10). ◆, 2-meSATP; □, ADP; ■, ATP; ●, AP_3A; ▼, AP_2A; ▲, AP_1A; ★, NADP; ○, FAD; ⬢, NAD.

Vasodilator responses in raised-tone preparations

When the tone of the mesenteric arterial bed was raised, by including methoxamine (5–60 μM) in the perfusate, the perfusion pressure increased by $63{\cdot}3 \pm 3{\cdot}1$ mmHg ($n = 21$). The nucleotides evoked vasodilatation with a rank order of potency of: 2-meSATP = ADP > ATP > $AP_3A > AP_2A \geqslant AP_1A$ = NADP = FAD > NAD (Figs 6 and 7, Table 2). The remaining compounds, α,β-meATP, AP_6A, AP_5A and AP_4A, did not evoke dilatation. Both AP_3A (Fig. 2) and NAD, at doses above 50 nmol, caused biphasic responses with a phase of constriction preceding the vasodilatation. In some preparations a pronounced second vasoconstrictor response was observed, occurring after the relaxation.

Endothelium removal in raised-tone preparations

Removal of the endothelium abolished vasodilator responses to 2-meSATP, ADP, ATP, AP_1A, AP_2A, AP_3A, FAD, NAD and NADP. Figure 6*D* illustrates the lack of vasodilator responses of the endothelium-denuded mesenteric arterial bed preparation to maximal doses of some of these agents. The integrity of the smooth muscle after endothelial removal was unimpaired as evidenced by retained responses to SNP.

DISCUSSION

The results of the present study indicate that the adenine dinucleotides comprise a family with diverse extracellular activity in terms of both their response (vasoconstrictor or vasodilator) and relative potency. In addition, a crucial relationship between activity and the structure of the molecule has been described, with a pivotal role played by the polyphosphate chain.

Vasoconstrictor responses, elicited by AP_4A, AP_5A and AP_6A, but not by the remaining adenine dinucleotides, were blocked by the P_{2X}-purinoceptor antagonist PPADS (Lambrecht *et al.* 1992; Ziganshin *et al.* 1993, 1994; Windscheif *et al.* 1994) indicating activation of the P_{2X}-purinoceptor. The relative resistance of vasoconstriction, in response to ATP, to antagonism by PPADS, at a concentration (30 μM) which abolished responses to the adenine dinucleotides, might suggest that a component of the response to ATP is mediated via receptors other than P_{2X}-purinoceptors. A likely candidate is the P_{2U}-purinoceptor which is activated by both UTP and ATP, causes vasoconstriction of the mesenteric arterial bed at basal tone (Ralevic & Burnstock, 1991*a*), and is resistant to antagonism by PPADS (Windscheif *et al.* 1994). P_{2X}- and P_{2U}-receptors are located on the vascular smooth muscle in rat mesenteric arteries as in many other vessels (Ralevic & Burnstock, 1988, 1991*a*, *b*). On the other hand, the similar pA_2 values for antagonism by PPADS of vasoconstriction in response to ATP and AP_5A would suggest that these are acting on a homogeneous population of receptors, namely P_{2X}-purinoceptors. The extreme susceptibility of the vasoconstrictor adenine dinucleotides, the archetypal P_{2X}-agonist α,β-meATP and ATP to auto- and cross-desensitization is consistent with actions mediated via the P_{2X}-purinoceptor, since desensitization is a characteristic property of this receptor. Indeed, this forms the basis for use of α,β-meATP as a desensitizing agent to inhibit P_{2X}-mediated responses (see Hoyle, 1992). A similar degree of desensitization of AP_4A, AP_5A, AP_6A, α,β-meATP and ATP supports the concept that these agents activate a homogeneous receptor population, at least at those doses tested for desensitization.

Vasodilator responses, elicited by $AP_3A > AP_2A > AP_1A \geqslant$ NADP $\geqslant$ FAD > NAD, but not by the remaining adenine dinucleotides, were endothelium dependent since these were abolished following sodium deoxycholate treatment. It is possible that these are mediated via the P_{2Y}-purinoceptor, since in rat mesenteric arteries the P_{2Y}-purinoceptor is present on endothelial cells and mediates relaxation to ATP and 2-meSATP via nitric oxide (Ralevic & Burnstock, 1988, 1991*a*). In contrast, relaxations in response to AP_3A in the rabbit mesenteric artery are endothelium independent and are not antagonized by the P_{2Y}-purinoceptor antagonist Reactive Blue 2 (Busse, Ogilvie & Pohl, 1988). Unfortunately, we were unable to use Reactive Blue 2 in the present study since this agent caused a significant fall in tone of the raised-tone preparation at operant concentrations, and an action of the adenine dinucleotides at endothelial receptors other than the P_{2Y}-purinoceptor remains a possibility. It is unlikely that P_1-purinoceptors mediate the vasodilator actions of the adenine dinucleotides in the rat mesenteric arterial bed, since the adenine dinucleotides are largely resistant to degradation (Blackburn, Taylor, Thatcher, Prescott & McLennan, 1987; Busse *et al.* 1988), and because adenosine and 2-chloroadenosine have only very weak vasodilator properties in this tissue (authors' unpublished observations). This contrasts with relaxations elicited by NAD in the guinea-pig taenia coli, which are mediated via P_1-purinoceptors, following breakdown to adenosine (Burnstock & Hoyle, 1985).

The present study identifies a critical relationship between activity of the adenine dinucleotides and their structure, with a pivotal role played by the polyphosphate chain. This influences both the direction of the response, namely vasoconstriction or vasodilatation, and its magnitude, and is likely to be a reflection of selective activation of P_{2X}- and possibly P_{2Y}-purinoceptors, respectively. A critical number of phosphates, three or less, is required for vasodilator action, while a chain length of three or more phosphates produces a dramatic conversion to vasoconstrictor action at the P_{2X}-purinoceptor. The ability of ATP, ADP and AP_3A to

act on both P_{2X}- and P_{2Y}-purinoceptors to cause vasoconstriction and vasodilatation, respectively, therefore is appropriate – with the exception of these nucleotides, vasoconstrictors did not also elicit vasodilatation, while vasodilators did not elicit vasoconstriction. Interestingly, the nucleotides that had dual activity were also the most potent vasodilators and least potent vasoconstrictors; however, it is unlikely that their responses at basal or raised tone were a compromise between opposing vasoconstrictor and vasodilator actions since vasodilatation occurred at doses which were subthreshold for P_{2X}-purinoceptor activation. Furthermore, no underlying vasoconstrictor activity by low doses of these agents was revealed at raised tone following removal of the endothelium.

Comprehensive structure–activity relationships for the adenine dinucleotides in blood vessels have not been carried out previously, but where responses have been tested these conform to the patterns of activity observed in the present study. Limited studies in vascular preparations include those showing that AP_4A causes an increase in perfusion pressure in the rat hepatic portal vein with a pharmacological profile similar to that of ATP (Busshardt, Gerok & Haussinger, 1989), while in the rabbit mesenteric artery AP_4A elicits endothelium-independent contraction and AP_3A elicits only vasodilatation (Busse *et al.* 1988). More recently, AP_5A and AP_6A have been shown to elicit vasoconstriction in the aorta and isolated perfused kidney of the rat (Schluter *et al.* 1994). It is interesting to note that in the guinea-pig taenia coli NADP (and adenosine 5′-(2-fluorodiphoshate) (ADPβF) and ADP–ribose) causes P_{2Y}-mediated relaxation of similar potency to ATP (Burnstock & Hoyle, 1985; Hourani, Welford, Loizou & Cusack, 1988; Hoyle & Edwards, 1992). In the present study NADP was 800–10 000 times less potent than ATP, indicating that the P_{2Y}-purinoceptor in the guinea-pig taenia coli is not the same as the endothelial P_{2Y}-purinoceptor in the rat mesenteric arterial bed, confirming the findings of previous studies (Fischer *et al.* 1993; Burnstock *et al.* 1994). At the P_{2Y}-purinoceptor in the human colon circular muscle (Hoyle & Burnstock, 1992) a rank order of potency of purine dinucleotides, in which AP_5A is the most potent agonist (Hoyle & Burnstock, 1992), also indicates that this intestinal P_{2Y}-purinoceptor is different from the endothelial P_{2Y}-purinoceptor.

An important determinant of activity of the adenine dinucleotides, directly related to the phosphate chain length, may be the total negative charge presented by the molecule since each phosphate contributes a single negative charge. Charge has previously been shown to be important in activation of the P_{2X}-purinoceptor (Fedan, Dagirmanjian, Attfield & Chideckel, 1990). The present study indicates a minimum requirement of a triple negative charge, which would explain the inability of AP_1A, AP_2A, FAD, NAD and NADP to elicit vasoconstriction. While three negative charges appear to be the minimum required for P_{2X} receptor activation, the rank order of nucleotide potency suggests that four or more negative charges is even better. Since ATP^{4-} forms only approximately 0·1 % of the total ATP concentration in the type of Krebs solution used (Fedan *et al.* 1990) this may explain why relatively high doses of ATP are required to elicit vasoconstriction. According to this hypothesis, ADP qualifies as a vasoconstrictor by virtue of its ability to carry three negative charges; appropriately, ADPβF and ADP–ribose, possessing the same phosphate chain length as ADP but differing in that they are able to bear a maximum of only two negative charges, do not activate the P_{2X}-purinoceptor (Hourani *et al.* 1988; Hoyle & Edwards, 1992). Activation of the P_{2Y}-purinoceptor appears to occur with adenine compounds with a negative charge of three or less; while 2-meSATP and ATP can carry a possible maximum of four negative charges, this is not neccessarily the charge carried by them in Krebs solution or *in vivo* under normal physiological conditions. Structurally, AP_2A is similar to FAD and NAD except that the second adenosine is replaced by flavin or ribosylated nicotinamide; however, AP_2A is approximately 2–40 times more potent. This suggests that the possible contribution of a positive influence of the adenosine moiety, or a negative influence by any group replacing an adenosine moiety, on binding to or activation of the receptor by the polyphosphate chain, should be considered.

Polyphosphate chain length and its associated negative charge may not be the only determinant of agonist potency, as revealed by a comparison of relative potencies of the adenine mono- and dinucleotides. The fact that vasoconstrictor responses to AP_3A were so weak that they were apparent only at high doses in the raised-tone preparation suggests that steric hindrance presented by the additional adenosine moiety comprising this dinucleotide, which is the only respect in which it differs from ATP, restricts access of the triphosphate chain to the P_{2X}-purinoceptor binding site. For the P_{2X}-purinoceptor the limits of this hindrance can be estimated to be the length of a single phosphate since steric hindrance did not occur for AP_4A, AP_5A and AP_6A. Flexibility of the molecules does not appear to be critically important to potency since AP_4A and AP_6A were equipotent, and since AP_2A, AP_3A, AP_4A and AP_5A have been shown to adopt stable intramolecularly stacked conformations in solution at around physiological pH and temperature (Kolodny, Kisteneff, Redfield & Rapaport, 1979). Interestingly, AP_4A and AP_5A, but not AP_2A and AP_3A, have been shown to prefer folded unstacked conformations at pH 4–5 (Kolodny *et al.* 1979), raising the possibility of a flexible dinucleotide conformation influenced by the charge presented locally at the receptor binding site.

Platelets, hepatocytes, adrenal medullary chromaffin granules (Rapaport & Zamenick, 1976; Flodgaard & Klenow, 1982; Luthje & Ogilvie, 1983; Rodriguez-del-Castillo *et al.* 1988; Schluter *et al.* 1994) are all sources of locally released or circulating adenine dinucleotides which might act on the mesenteric vasculature as decribed in this study. The physiological relevance of the differential activities of the adenine dinucleotides according to their chain length is not clear, but may be related to the relative proportions that are released from different sources under different conditions such as altered pH or P_{O_2}. Differential release of these compounds provides greater potential for fine tuning of vascular tone according to the balance of vasoconstrictor *versus* vasodilator activity. However, more comprehensive data are needed to establish the physiological significance of the pivotal role of phosphate chain length of these compounds.

In conclusion, this study, by characterizing the actions of adenine dinucleotides in the rat mesenteric arterial bed, has identified a critical relationship between their structure and activity, with a pivotal role played by the polyphosphate chain and its charge; adenine dinucleotides with four or more phosphates are vasoconstrictors via P_{2X}-purinoceptors on the smooth muscle, while those with three or less phosphates are vasodilators at endothelial receptors, possibly P_{2Y}-purinoceptors. The order of agonist potency of the mono- and dinucleotides has allowed speculation on the stereochemical requirements of the active sites of P_{2X}- and P_{2Y}-purinoceptors and may prove valuable in the design of more potent agonists for these receptors. Furthermore, it is suggested that the subtle differences in structure and/or charge of the adenine dinucleotides may have profound influences on the potent biological activity of these naturally occurring molecules.

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