British Journal of Pharmacology (2001) 134, 1073–1083

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Structure-activity relationships of diadenosine polyphosphates (Ap_nAs), adenosine polyphospho guanosines (Ap_nGs) and guanosine polyphospho guanosines (Gp_nGs) at P2 receptors in the rat mesenteric arterial bed

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1 Vascular effects of diadenosine polyphosphates (Ap_nAs), adenosine polyphospho guanosines (Ap_nGs) and guanosine polyphospho guanosines (Gp_nGs), novel families of naturally-occurring signalling molecules, were investigated in methoxamine preconstricted rat isolated perfused mesenteric arterial beds.

2 Three different types of response were elicited by Ap_nAs and Ap_nGs. Those with a short polyphosphate chain ($n=2-3$) elicited vasorelaxation. Ap_3A was more potent than Ap_2A, and both were more potent than the corresponding Ap_nG. Relaxations to Ap_3A and Ap_3G, but not to Ap_2A and Ap_2G, were blocked by endothelium removal and pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), a P2 receptor antagonist.

3 Longer polyphosphate chain Ap_nAs and Ap_nGs ($n=4-6$) elicited dose-dependent vasoconstriction followed by prolonged vasorelaxation, with a potency order for both types of response of $Ap_5A \geqslant Ap_6A > Ap_4A$. A similar order and potency was observed for Ap_nGs. Contractions and prolonged relaxations were blocked by PPADS and $P2X_1$ receptor desensitization with α,β-methylene ATP (α,β-meATP), and were largely endothelium-independent.

4 In the presence of α,β-meATP rapid relaxations to contractile Ap_nAs and Ap_nGs ($n=4-6$) were revealed.

5 Gp_nGs were virtually inactive, except for Gp_2G which elicited vasoconstriction *via* PPADS- and α,β-meATP-sensitive smooth muscle $P2X_1$-like receptors.

6 These data show that, as with Ap_nAs, the length of the polyphosphate chain (n) is an important determinant of the activity of Ap_nGs at P2 receptors in the rat mesenteric arterial bed. When the chain is short ($n=2-3$) the purines elicit rapid vasorelaxation, which for Ap_3A and Ap_3G is mediated *via* endothelial $P2Y_1$-like receptors. When the chain is long ($n=4-6$) Ap_nAs and Ap_nGs elicit vasoconstriction *via* $P2X_1$-like receptors, followed by prolonged endothelium-independent vasorelaxation. Rapid relaxation to contractile dinucleotides ($n=4-6$) is revealed by block of vasoconstriction. Regarding the purine moiety, one adenine is crucial and sufficient for vasoactivity as Gp_nGs were largely inactive, and Ap_nAs and Ap_nGs approximately equipotent.
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Keywords: P2 purine receptors; diadenosine polyphosphates; adenosine polyphospho guanosines; guanosine polyphospho guanosines; rat mesenteric arterial bed

Abbreviations: Ap_nAs, diadenosine polyphosphates; Ap_nGs, adenosine polyphospho guanosines; Gp_nGs, guanosine polyphospho guanosines; α,β-me ATP, α,β-methylene ATP; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid

Introduction

The diadenosine polyphosphates (Ap_nAs), adenosine polyphospho guanosines (Ap_nGs) and guanosine polyphospho guanosines (Gp_nGs) have attracted considerable recent interest as novel families of extracellular signalling molecules (Hoyle, 1990; Schlüter *et al*., 1994; Ralevic *et al*., 1995; van der Giet *et al*., 1997; Lewis *et al*., 2000; Hoyle *et al*., 2001). They are released from a number of different cell types, but are found in particularly high concentrations in platelets (Flodgaard & Klenow, 1982; Lüthje & Ogilvie, 1983; Schlüter *et al*., 1994; 1998; Jankowski *et al*., 1999; 2001; Luo *et al*., 1999a,b). Ap_nAs, Ap_nGs and Gp_nGs are endogenous ligands at P1 and P2 purine receptors (Ralevic & Burnstock, 1998), although in the central nervous system distinct receptors for these compounds have been proposed (Pintor & Miras-Portugal, 1995).

Interestingly, the potency and type of response of the Ap_nAs has been shown to be largely determined by the number of phosphates (n) in the polyphosphate chain (Ralevic *et al*., 1995; van der Giet *et al*., 1997; Lewis *et al*., 2000). In general, Ap_nAs with a phosphate chain length of

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$n=2-3$ are vasodilators, whilst Ap_nAs with a phosphate chain length of $n=4-6$ are vasoconstrictors. Ap_3A is typically the most potent vasorelaxant, mediating its effect *via* P2Y receptors on the endothelium and smooth muscle (for example in rat and rabbit mesenteric arteries respectively) (Busse *et al*., 1988; Ralevic *et al*., 1995), or *via* A_2 receptors (in rat kidney) (van der Giet *et al*., 1997). However, smooth muscle vasorelaxation mediated by Ap_4A and Ap_5A in porcine coronary arteries (Sumiyoshi *et al*., 1997), and endothelium-dependent vasorelaxation mediated by Ap_4A in rabbit mesenteric arteries (Busse *et al*., 1988) has also been described. Vasocontractile actions of Ap_nAs ($n=4-6$) in rat mesenteric arteries are mediated by ionotropic $P2X_1$-like receptors on the smooth muscle (Ralevic *et al*., 1995; Lewis *et al*., 2000). A recent study has show that longer chain length Ap_nGs ($n=4-6$) can also elicit vasoconstriction *via* smooth muscle $P2X_1$-like receptors (Lewis *et al*., 2000). However, whether Ap_nGs can mediate vasorelaxation is unclear. Most recently, Gp_nGs were isolated (Schlüter *et al*., 2000), but their cardiovascular actions have been investigated only in the rat isolated kidney and dissociated smooth muscle cells (Schlüter *et al*., 1998).

The aim of this study was to investigate the vascular effects of Ap_nGs and Gp_nGs, compared to responses mediated by the Ap_nAs, in the rat isolated mesenteric arterial bed. The complement of purine receptors in rat mesenteric arteries includes vasorelaxant $P2Y_1$- and $P2Y_2$-like receptors on the endothelium (Ralevic *et al*., 1995; Ralevic & Burnstock, 1988; 1996), vasorelaxant A_{2B} receptors on the smooth muscle (Rubino *et al*., 1995), and vasocontractile $P2X_1$ receptors on the smooth muscle (Lewis & Evans, 2000). The actions of Ap_nAs, Ap_nGs and Gp_nGs were characterized using pyridoxalphosphate-6-azophenyl 2′,4′-disulphonic acid (PPADS), a selective P2 receptor antagonist (Lambrecht *et al*., 1992; Ziganshin *et al*., 1994; Windscheif *et al*., 1994), and by desensitization of smooth muscle $P2X_1$ receptors with α,β-methylene ATP (α,β-meATP).

Methods

Male Wistar rats (250–300 g) were killed by exposure to CO_2 and decapitation. Mesenteric beds were isolated and perfused, *via* the superior mesenteric artery, as described previously (Ralevic *et al*., 1995). In brief, the abdomen was opened and the superior mesenteric artery exposed and cannulated with a hypodermic needle. The superior mesenteric vein was cut, blood flushed from the preparation with 0.5 ml of Krebs' solution and the gut dissected carefully away from the mesenteric vasculature. The preparation was mounted on a stainless steel grid (7×5 cm) in a humid chamber and perfused at a constant flow rate of 5 ml min^{-1} using a peristaltic pump (model 7554-30, Cole-Parmer Instrument Co., Chicago, IL, U.S.A.). The perfusate was Krebs'-Bülbring 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. Preparations were allowed to equilibrate for 30 min prior to experimentation. Responses were measured as changes in perfusion pressure (mmHg) with a pressure transducer (model P23XL, Viggo-Spectramed, Oxnard, CA, U.S.A.) on a side arm of the perfusion cannula, and recorded on a polygraph (model 7D, Grass Instrument Co., Quincy, MA, U.S.A.).

Experimental protocol

After 30 min equilibration, a submaximal concentration of methoxamine (10–50 μM) was added in order to increase the perfusion pressure of the preparations (by 40–80 mmHg) above baseline, and responses to doses of the Ap_nAs, Ap_nGs and Gp_nGs (50 μl bolus injections; 0.05–50 nmol) were investigated. Preliminary experiments showed that the Gp_nGs were relatively weak or inactive and thereafter this family was always tested first. One or both of the families of Ap_nGs and Ap_nAs were tested in the same preparations. When both were tested the order in which they were applied was alternated. The smaller polyphosphate chain length Ap_nAs and Ap_nGs ($n=2$ and 3) were typically tested first, but the greater chain length members ($n=4-6$) were applied in a randomized order. Antagonists/inhibitors were added to the perfusate during equilibration, 30 min before construction of the dose-response curves. In a group of mesenteric beds the endothelium was removed by perfusing the preparations with water (for 7–8 min). Endothelial damage was confirmed by testing with acetylcholine (ACh; 50 nmol), which produced a relaxation of $12.84\pm2.2\%$ ($n=10$). This is less than 20% of the normal relaxation response (80–90% relaxation) to 50 nmol ACh in endothelially-intact preparations.

Drugs

α,β-methylene ATP (lithium salt), acetylcholine (chloride), methoxamine (hydrochloride) and suramin (sodium salt) were from Sigma. Pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) was from TOCRIS Cookson Ltd. 8-(p-sulphophenyl)theophylline (8-PSPT) was from Research Biochemicals International. Ap_nAs, Ap_nGs and Gp_nGs were synthesized and HPLC purified by H Schlüter. All drugs were dissolved in distilled water.

Data analysis

Vasocontractile and vasorelaxant responses of the mesenteric arterial beds were measured as changes in perfusion pressure (mmHg) and vasorelaxant responses expressed as percentage relaxation of the methoxamine-induced increase in tone above baseline. Data are expressed as mean ± s.e.mean. Data were analysed by analysis of variance followed by Tukey's multiple comparison or Student's *t*-test. pD_{30} is the dose that would cause an increase in perfusion pressure of 30 mmHg, and was calculated as the mean of individual pD_{30} values obtained from graphs of dose-response relationships for each experiment. A value of $P<0.05$ was taken to indicate a statistically significant difference.

Results

Effects of Ap_nGs and Ap_nAs in the rat isolated mesenteric arterial bed

Three different types of response to the Ap_nGs and Ap_nAs were observed (Figures 1 and 2). Short chain purine

compounds ($n=2-3$) elicited dose-dependent vasorelaxation and longer chain purine compounds ($n=4-6$) elicited dose-dependent vasoconstriction followed by prolonged vasorelaxation.

The adenosine polyphospho guanosines Ap_4G, Ap_5G and Ap_6G elicited dose-dependent vasoconstriction with an order of potency of: $Ap_5G \geqslant Ap_6G > Ap_4G$ (Figures 1 and 2a). pD_{30} values for Ap_4G, Ap_5G and Ap_6G were 7.78 ± 0.1, 8.93 ± 0.18 and 8.71 ± 0.23, respectively ($n=6$). A similar contractile potency order was observed for the diadenosine polyphosphates, where $Ap_5A \geqslant Ap_6A > Ap_4A$ (Figure 2b). pD_{30} values for Ap_4A, Ap_5A and Ap_6A were 7.85 ± 0.14, 8.5 ± 0.15 and 8.2 ± 0.09, respectively ($n=5-6$). pD_{30} values for the Ap_nAs were not significantly different to pD_{30} values that we have reported previously for these compounds in the rat isolated mesenteric arterial bed (Ralevic *et al*., 1995). There was no significant difference between the contractile responses elicited by any Ap_nG and its corresponding Ap_nA.

Vasocontractile responses to Ap_nAs and Ap_nGs were followed by slow and prolonged vasorelaxations (Figures 1 and 2). The rank order of potency of the Ap_nAs and Ap_nGs in eliciting this response was similar to that of the contractile potency order of the compounds. There was no significant difference between prolonged vasorelaxant responses to Ap_5G and Ap_5A, and between Ap_6G and Ap_6A, but Ap_4G was significantly more potent than Ap_4A ($P<0.05$) (Figure 2).

Ap_2G and Ap_3G elicited only modest vasorelaxation and Ap_3G was more potent than Ap_2G (Figure 1 and 2a). Ap_3A was a more potent vasorelaxant than Ap_2A and both were significantly more potent as vasorelaxants than the corresponding Ap_nG ($P<0.05$ and $P<0.001$, respectively; $n=6$) (Figure 2).

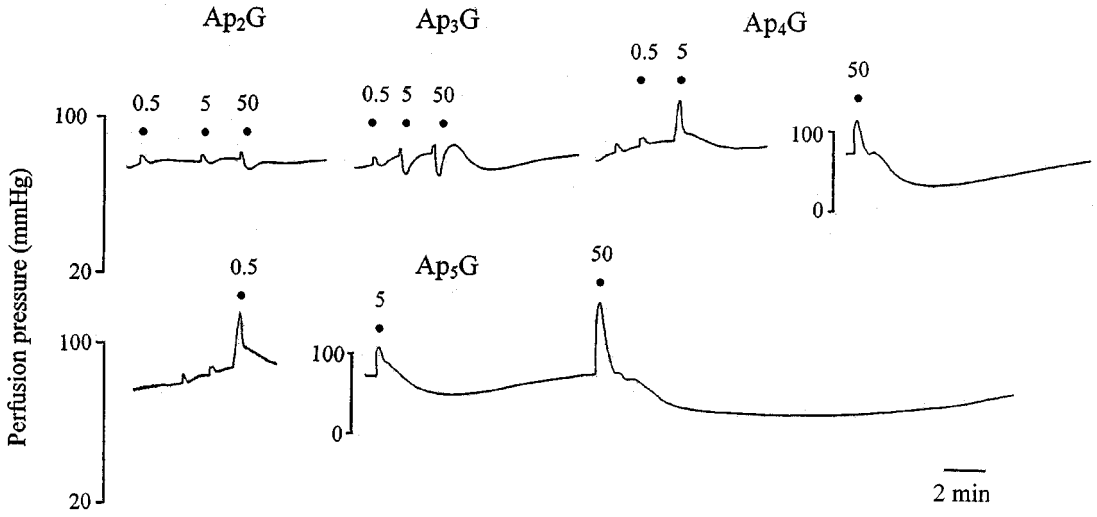

Figure 1 Representative trace showing responses to Ap_nGs ($n=2-5$) in the rat isolated perfused methoxamine-preconstricted mesenteric arterial bed. Ap_2G and Ap_3G elicited only vasorelaxation, whereas Ap_4G and Ap_5G elicited vasoconstriction followed by slow and prolonged vasorelaxation. Responses to Ap_6G (not shown) were similar to those to Ap_5G. Ap_nGs were applied at the doses (nmol) indicated. The very small increases in perfusion pressure observed at low doses of all agonists are injection artefacts. Note the different vertical scale for responses to 50 nmol Ap_4G and 5 and 50 nmol Ap_5G.

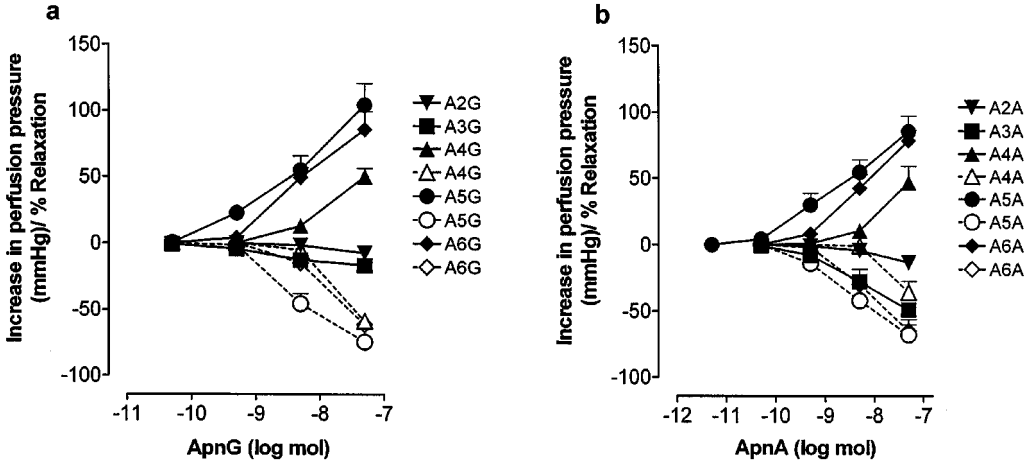

Figure 2 Dose response curves to (a) Ap_nGs and (b) Ap_nAs in the rat isolated perfused methoxamine-preconstricted mesenteric arterial bed. Solid lines indicate constrictions (increase in perfusion pressure, mmHg) or rapid relaxations (% of tone) and dashed lines indicate prolonged relaxations (% of tone). $n=5-6$ for each point. Data are shown as mean + s.e.mean.

Effect of PPADS, endothelium removal and α,β-meATP on contractile responses to Ap_nGs

Contractile responses to Ap_4G and Ap_6G were virtually abolished by PPADS (10 μM), and contractions to Ap_5G were significantly attenuated ($P<0.01$; $n=4$) (Figure 3a). Endothelium removal had no significant effect on contractions to Ap_4G, but attenuated slightly contractions to Ap_5G ($P<0.01$) and Ap_6G ($P<0.05$) ($n=6$) (Figures 3b and 4). In the presence of α,β-meATP (10 μM), contractions to Ap_4G, Ap_5G and Ap_6G were abolished, and rapid vasorelaxations were revealed ($n=4$) (Figures 3c and 4).

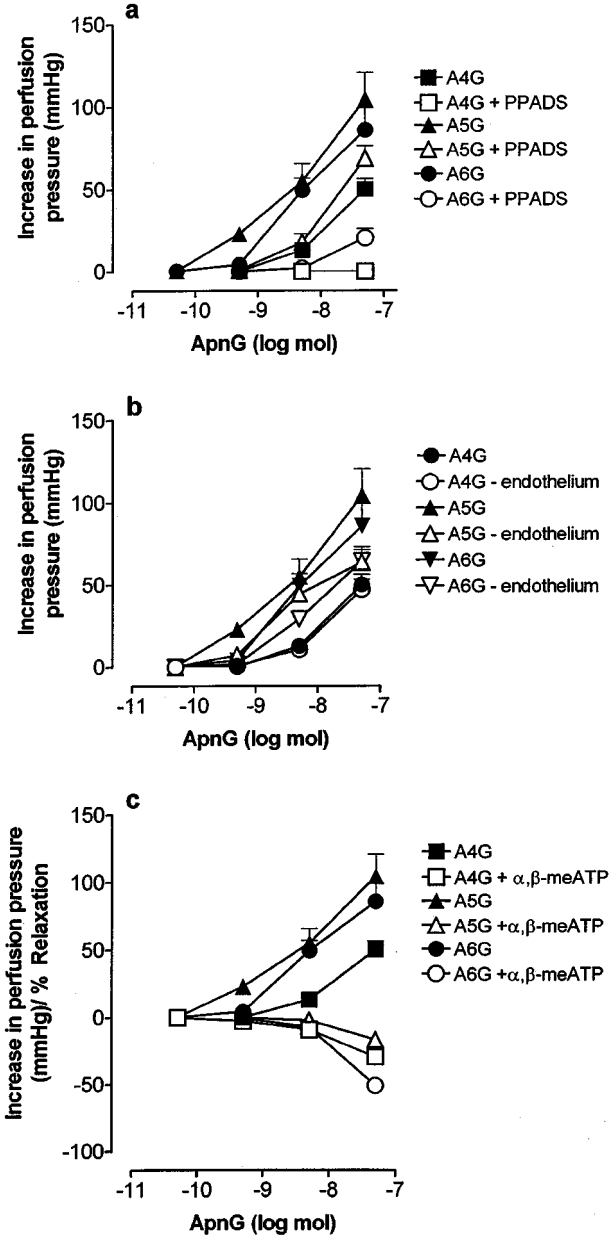

Figure 3 Dose response curves to Ap_nGs under control conditions (solid symbols; $n=6$) and (a) in the presence of pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS; 10 μM) ($n=4$), (b) after endothelium removal ($n=6$), and (c) in the presence of α,β-methylene ATP (α,β-meATP; 10 μM) ($n=4$), in the rat isolated perfused methoxamine-preconstricted mesenteric arterial bed. Data are shown as mean + s.e.mean.

Effect of PPADS, endothelium removal and α,β-meATP on contractile responses to Ap_nAs

Figure 5 (top panel) shows the pivotal role played by the phosphate chain length in determining vasodilator ($n=3$; Ap_3A) and vasoconstrictor ($n=4$; Ap_4A) activity of the purines. Contractile responses to Ap_4A were virtually abolished by PPADS (10 μM), and contractions to Ap_5A ($P<0.05$) and Ap_6A ($P<0.001$) were significantly attenuated ($n=6$) (Figures 5 and 6a). Endothelium removal reduced contractions to Ap_4A ($P<0.001$) but had no significant effect on responses to Ap_5A and Ap_6A ($n=6$) (Figure 6b). In the presence of α,β-meATP (10 μM), contractions to Ap_4A, Ap_5A and Ap_6A were abolished, and rapid vasorelaxations revealed ($n=6$) (Figures 5 and 6c). Rapid vasorelaxations to Ap_4A and Ap_6A in the presence of α,β-meATP were significantly less potent than rapid relaxations to the corresponding Ap_nGs under the same conditions ($P<0.001$) (Figures 3c and 6c). Rapid relaxations to Ap_5A and Ap_5G in the presence of α,β-meATP were not significantly different (Figures 3c and 6c).

Characterization of rapid relaxations to Ap_nAs and Ap_nGs ($n=4-6$) revealed in the presence of α,β-meATP

In order to characterize the rapid relaxations to Ap_nAs and Ap_nGs ($n=4-6$) revealed when contractions were blocked by P2X receptor desensitization with α,β-meATP (10 μM), these were investigated in the additional presence of suramin (100 μM), PPADS (10 μM), 8-PSPT (1 μM), or after endothelium removal. No clear pattern of effect of these treatments was observed. In the presence of α,β-meATP rapid relaxations to Ap_nAs were unaffected by 8-PSPT, but those to Ap_4G and Ap_6G were attenuated ($n=6$). With the exception of Ap_4G, relaxations to Ap_nAs and Ap_nGs were unaffected by PPADS ($n=6$). Relaxations to Ap_4A, Ap_4G and Ap_6G were attenuated by endothelium removal ($n=6$). Suramin attenuated relaxations to Ap_4A, Ap_4G and Ap_6G, but appeared to prevent full desensitization of the P2X receptor, as Ap_5A was still able to elicit vasoconstriction (18.8 ± 4.7 mmHg, at 50 nmol; $n=6$).

Effect of PPADS on rapid and prolonged relaxations to Ap_nGs and Ap_nAs

PPADS (10 μM) significantly attenuated rapid relaxations to Ap_3G ($P<0.05$) and Ap_3A ($P<0.001$) ($n=4-6$), but had no effect on rapid relaxations to Ap_2G and Ap_2A (Figures 5 and 7a,b). PPADS virtually abolished prolonged relaxations to Ap_4G, Ap_6G and Ap_4A, and significantly attenuated prolonged relaxations to Ap_5G ($P<0.001$), Ap_5A ($P<0.001$) and Ap_6A ($P<0.01$) ($n=4-6$) (Figures 5 and 7c,d).

Effect of α,β-meATP on rapid and prolonged relaxations to Ap_nGs and Ap_nAs

α,β-meATP (10 μM) had no significant effect on rapid relaxations to Ap_2G, Ap_3G and Ap_2A, but rapid

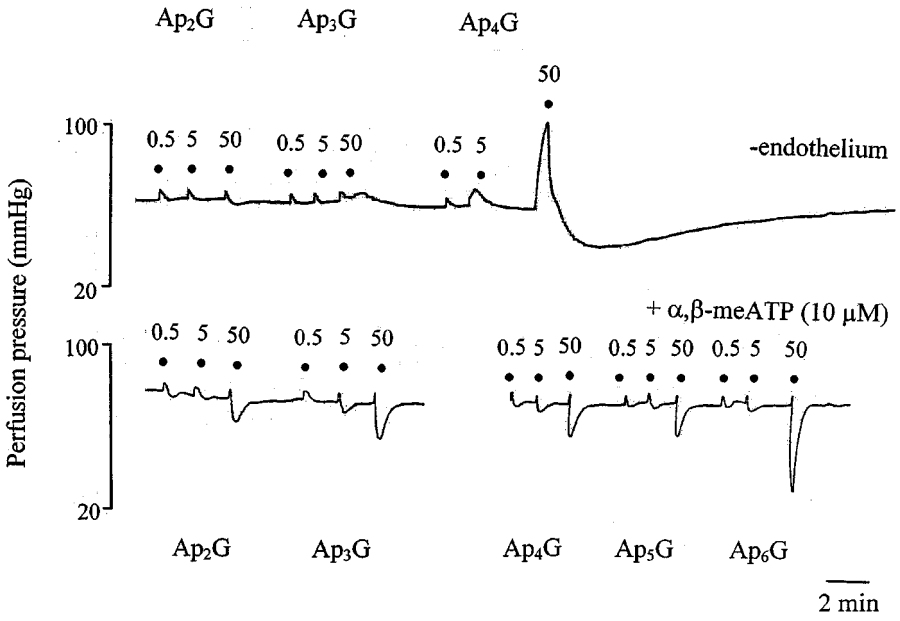

Figure 4 Representative trace showing responses to Ap_nGs ($n=2-6$) in the rat isolated perfused methoxamine-preconstricted mesenteric arterial bed without endothelium (*upper trace*) or in the presence of α,β-methylene ATP (α,β-meATP; 10 μM) (*lower trace*). Rapid vasorelaxations to Ap_3G were abolished by endothelium removal, but contraction and prolonged relaxation to Ap_4G was unaffected. α,β-meATP blocked contraction and prolonged relaxation to Ap_4G, Ap_5G and Ap_6G and revealed rapid relaxations. Ap_nGs were applied at the doses (nmol) indicated. The very small increases in perfusion pressure observed at low doses of all agonists are injection artefacts.

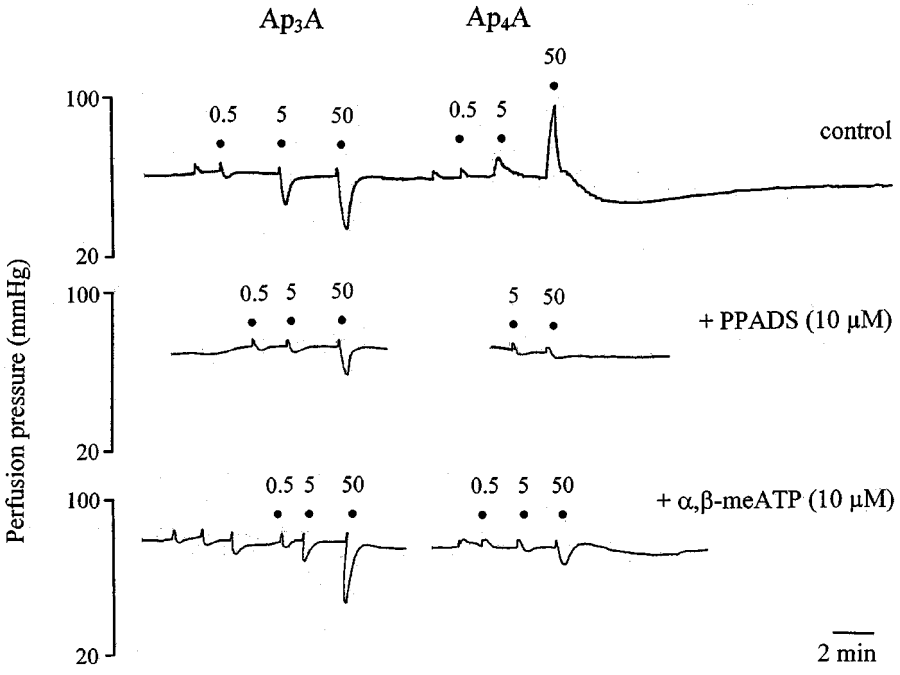

Figure 5 Representative trace showing responses to Ap_nAs ($n=3$ and 4) in the rat isolated perfused methoxamine-preconstricted mesenteric arterial bed under control conditions (*top trace*), in the presence of pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS; 10 μM) (*middle trace*), and in the presence of α,β-methylene ATP (α,β-meATP; 10 μM) (*bottom trace*). Note that an increase by a single phosphate of the polyphosphate chain length of Ap_3A to produce Ap_4A leads to a loss of rapid relaxation and the response is contraction followed by slow relaxation. Rapid vasorelaxations to Ap_3A, and contractions and prolonged relaxation to Ap_4A were attenuated by PPADS. Rapid relaxation to Ap_3A was unaffected by α,β-meATP but contractions and prolonged relaxation to Ap_4A was attenuated. Ap_nAs were applied at the doses (nmol) indicated. The very small increases in perfusion pressure observed at low doses of all agonists are injection artefacts.

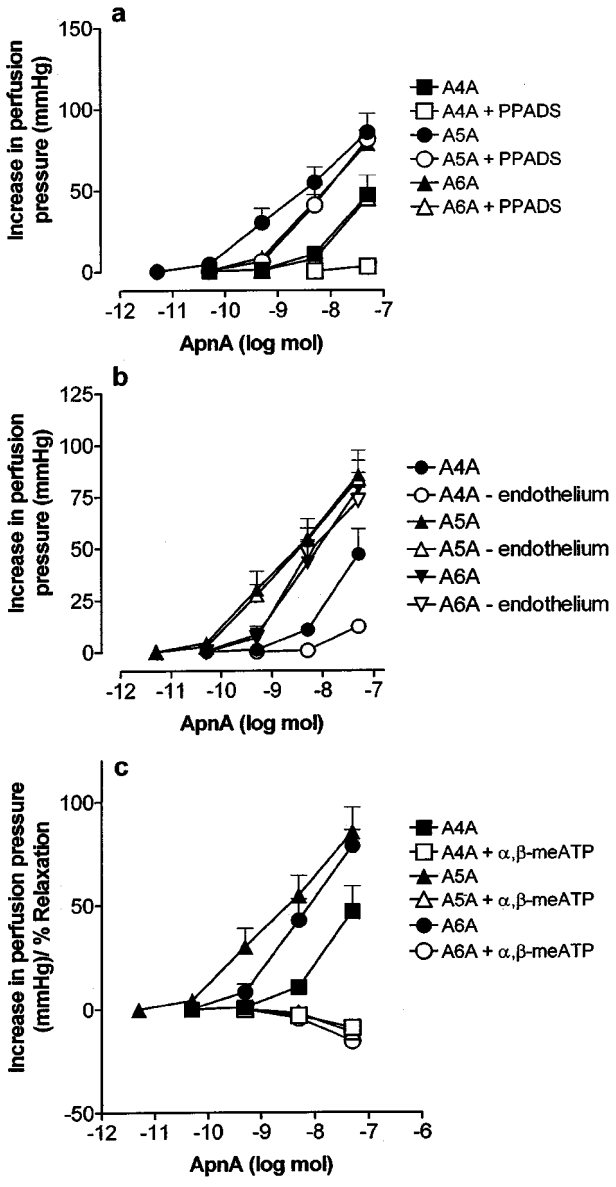

Figure 6 Dose response curves to Ap_nAs under control conditions (solid symbols) and (a) in the presence of pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS; 10 μM), (b) after endothelium removal, and (c) in the presence of α,β-methylene ATP (α,β-meATP; 10 μM), in the rat isolated perfused methoxamine-preconstricted mesenteric arterial bed. $n=5-6$. Data are shown as mean + s.e.mean.

relaxations to Ap_3A were slightly attenuated ($n=4-6$) (Figures 4, 5 and 8a,b). In contrast, α,β-meATP abolished prolonged relaxations to Ap_4G, Ap_5G, Ap_6G, Ap_4A, Ap_5A and Ap_6A ($n=4-6$) (Figures 4, 5 and 8c,d).

Effect of endothelium removal on rapid and prolonged relaxations to Ap_nGs and $ApnAs$

Endothelial denudation attenuated rapid relaxations to Ap_3G and Ap_3A ($P<0.001$), but had no effect on rapid relaxations to Ap_2G and Ap_2A ($n=6$) (Figures 4 and 9a,b). Endothelium removal had no significant effect on prolonged relaxations to Ap_4G, Ap_5G and Ap_6G. Prolonged relaxations to Ap_4A were blocked and those to Ap_5A ($P<0.01$) and Ap_6A ($P<0.05$) were augmented ($n=6$) (Figures 4 and 9c,d).

Effect of Gp_nGs in the rat isolated mesenteric arterial bed

The Gp_nGs were inactive or elicited very weak vasorelaxation, with the exception of Gp_2G, which was a vasoconstrictor ($n=4-6$) (Figure 10a). The effect of Gp_2G was blocked by PPADS (10 μM; $n=4$) and α,β-meATP (10 μM; $n=6$), but was not significantly affected by removal of the endothelium ($n=6$) (Figure 10b,c). In the presence of α,β-meATP a small vasorelaxation to Gp_2G was observed (Figure 10b).

Discussion

This study has shown that for Ap_nGs as well as for Ap_nAs, the length of the polyphosphate chain is a crucial determinant of vasomotor activity *via* P2 receptors in the rat isolated mesenteric arterial bed. Hence, short chain Ap_nGs and Ap_nAs ($n=2-3$) are vasorelaxants (*via* endothelial $P2Y_1$-like receptors when $n=3$), whilst longer chain Ap_nGs and Ap_nAs ($n=4-6$) are vasoconstrictors, *via* smooth muscle $P2X_1$-like receptors. In addition, this study shows that contractile Ap_nAs and Ap_nGs ($n=4-6$) can additionally mediate vasorelaxation; prolonged vasorelaxation following vasoconstriction, and rapid vasorelaxation after blockade of $P2X_1$ receptor-mediated vasoconstriction. Regarding the purine moiety, one adenine is crucial and sufficient for vasoactivity as Gp_nGs were largely inactive, and Ap_nAs and Ap_nGs approximately equipotent.

Ap_4A, Ap_5A and Ap_6A were vasoconstrictors, with Ap_4A being the least potent, as reported previously in the rat isolated mesenteric arterial bed (Ralevic *et al.*, 1995). The pD_{30} values were not significantly different from those we reported previously for Ap_nAs, indicating consistency between the commercially available (Ralevic *et al.*, 1995) and synthesized compounds (present study) and reproducibility of the assay. A similar potency order of Ap_nAs, as activators of $P2X_1$-like receptor inward currents and mediators of vasoconstriction, has recently been reported in rat mesenteric artery rings and acutely dissociated smooth muscle cells (Lewis *et al.*, 2000). In the present study, Ap_4G, Ap_5G and Ap_6G elicited vasoconstriction with a similar potency, and order of potency, as the corresponding Ap_nAs. Similar activities of contractile Ap_nAs and their corresponding Ap_nGs have also been reported in the rat isolated perfused kidney (van der Giet *et al.*, 1997; Schlüter *et al.*, 1998). In contrast, Lewis *et al.* (2000) found that Ap_nGs were significantly less potent than the corresponding Ap_nAs at mediating P2X responses in rat mesenteric arteries and dissociated smooth muscle cells. The reason for this difference is not entirely clear. However, in the present study dinucleotides were applied luminally as bolus doses, and there was a small but significant decrease in the contractile action of Ap_5G and Ap_6G after removing the endothelium, which was not observed for the corresponding Ap_nAs, which may indicate a partial degradation of the ApnGs by

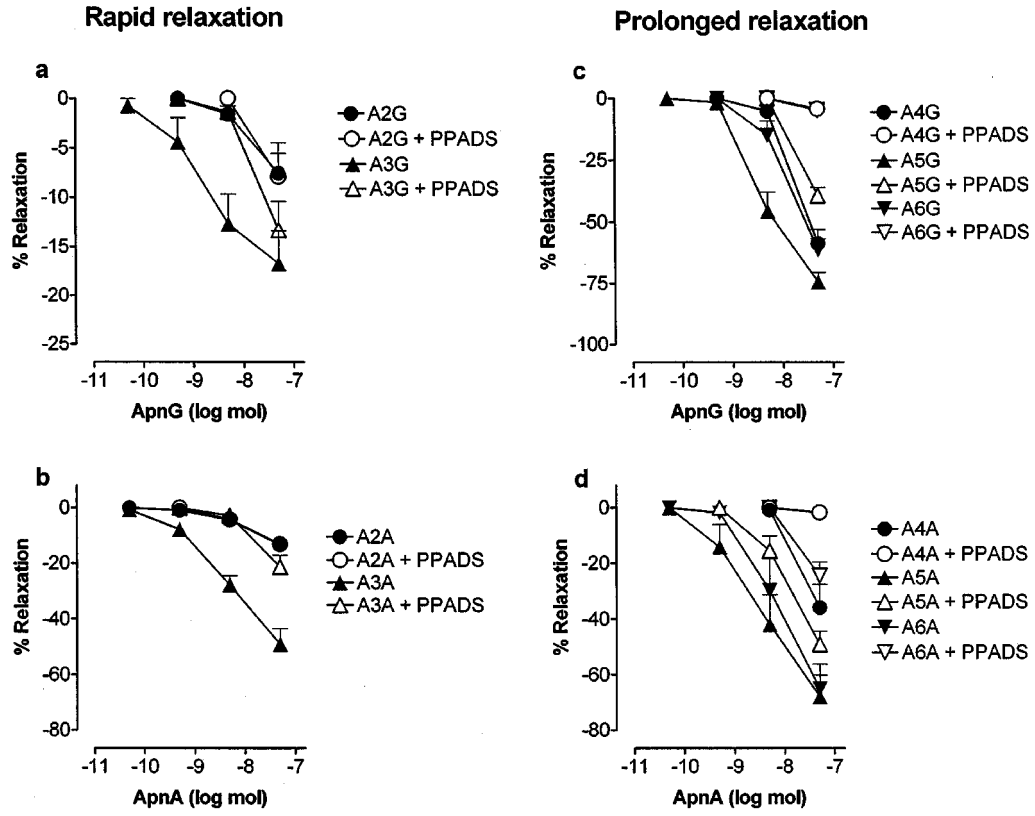

Figure 7 Effect of pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS; 10 μM) on relaxation dose-response curves to Ap_nGs and Ap_nAs ($n=2-6$) in the rat isolated perfused methoxamine-preconstricted mesenteric arterial bed. Rapid relaxations to: (a) Ap_2G and Ap_3G ($n=4-6$); (b) Ap_2A and Ap_3A ($n=4-6$). Prolonged relaxations to: (c) Ap_4G, Ap_5G and Ap_6G ($n=6$); (d) Ap_4A, Ap_5A and Ap_6A ($n=5-6$). Data are shown as mean + s.e.mean.

endothelial ectohydrolases. Hence, in the presence of the endothelium ATP may be produced, which masks the lower efficacy of Ap_5G and Ap_6G at the $P2X_1$ receptor.

Ap_nAs and Ap_nGs are degraded by asymmetrical and symmetrical hydrolases and phosphorylases to yield ATP, ADP, AMP, adenosine, GTP, GDP, GMP and guanosine. However, the very much higher contractile activity of the dinucleotides compared with their metabolites (e.g. Ap_5A, Ap_6A and Ap_4A are 126 fold, 63 fold and 32 fold more potent, respectively than ATP at mediating contractions in the rat mesenteric arterial bed (Ralevic *et al*., 1995)) indicates that it is unlikely that their actions are mediated principally by mononucleotide breakdown products. Similar conclusions were drawn by Schlüter *et al*. (1998), who showed that all potential degradation products of the Ap_nGs were considerably less active than the diguanosine polyphosphates at mediating vasoconstriction in the rat isolated perfused kidney. Further support for a direct action of Ap_nAs and Ap_nGs was provided with the finding that recovery of these compounds in the effluent of the perfused kidney was similar to that of the nonhydrolyzable compound α,β-meATP, and that their half lives when incubated with smooth muscle cells in culture were of the order of 1 h (Schlüter *et al*., 1998). Moreover, there is direct evidence for an action of Ap_nAs ($n=4-6$) at recombinant $P2X_1$ receptors (Wildman *et al*., 1999) and for Ap_nAs and Ap_nGs ($n=4-6$) in evoking contraction and P2X receptor currents (Lewis *et al*., 2000). However, Lewis *et al*. (2000) did reveal a mismatch between the activities of longer chain length polyphosphates at mediating contraction in mesenteric arteries and inward currents in dissociated smooth muscle cells, the latter carried out under concentration clamp conditions where agonist breakdown is minimized, which indicates that the activity of certain dinucleotides at P2X receptors may be overestimated due to metabolic breakdown.

Contractions mediated by Ap_nAs and Ap_nGs were blocked by PPADS, a P2 receptor antagonist (Lambrecht *et al*., 1992; Ziganshin *et al*., 1994; Windscheif *et al*., 1994) and by α,β-meATP, indicating actions at $P2X_1$ receptors, the principal isoform of P2X receptor in rat mesenteric arteries (Lewis & Evans, 2000). Contractions were largely unaffected by endothelium removal, consistent with actions mediated *via* receptors on the smooth muscle. Lewis *et al*. (2000) also found that responses to Ap_nAs and Ap_nGs were mediated *via* $P2X_1$ receptors in rat mesenteric artery dissociated smooth muscle cells. In contrast, a suramin- and PPADS-resistant component of vasoconstriction to Ap_4A and Ap_6A in rat kidney may be mediated independently of P2X receptors (van der Giet *et al*., 1997).

A large and prolonged vasorelaxation following vasoconstriction by the longer chain purines ($n=4-6$) was observed for both Ap_nAs and Ap_nGs. Prolonged vasorelaxation following P2X receptor-mediated vasoconstriction

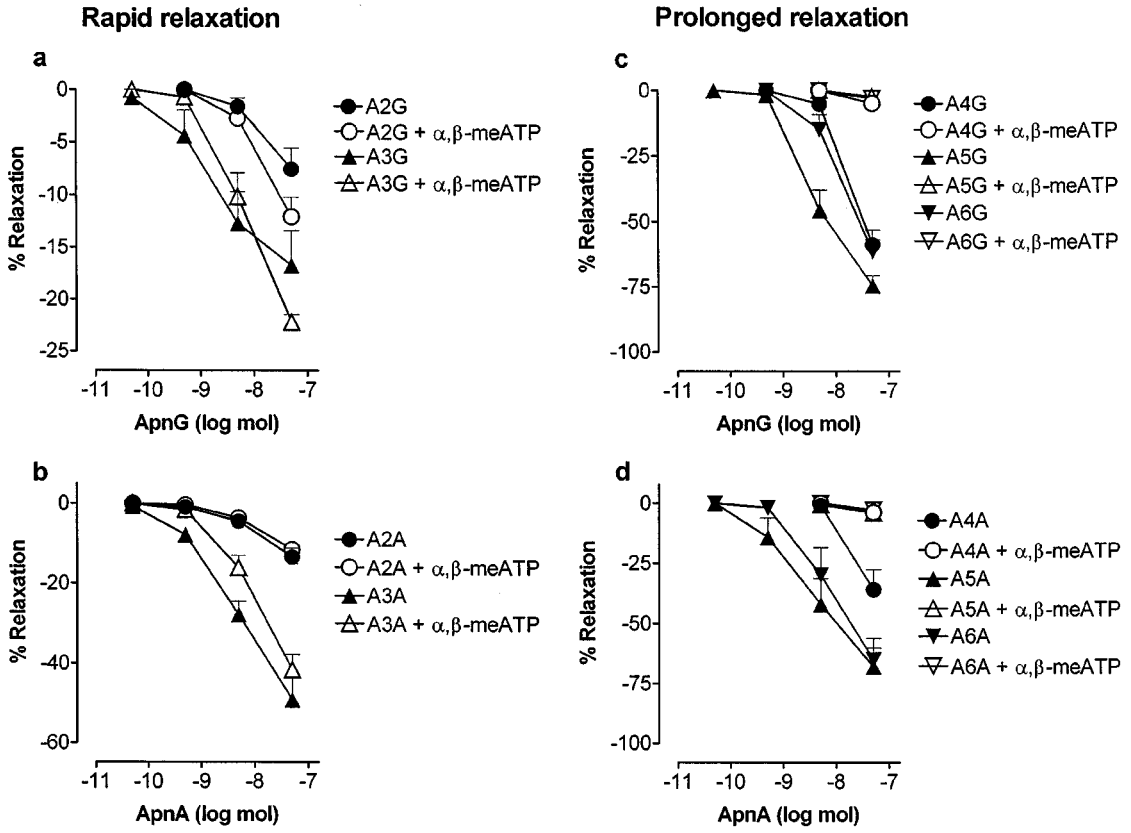

Figure 8 Effect of α,β-methylene ATP (α,β-meATP; 10 μM) on relaxation dose-response curves to Ap_nGs and Ap_nAs ($n=2-6$) in the rat isolated perfused methoxamine-preconstricted mesenteric arterial bed. Rapid relaxations to: (a) Ap_2G and Ap_3G ($n=4-6$); (b) Ap_2A and Ap_3A ($n=4-6$). Prolonged relaxations to: (c) Ap_4G, Ap_5G and Ap_6G ($n=6$); (d) Ap_4A, Ap_5A and Ap_6A ($n=5-6$). Data are shown as mean + s.e.mean.

has previously been shown for ATP in the rat isolated mesenteric arterial bed, and is endothelium-independent and blocked by PPADS and by α,β-meATP desensitization (Stanford & Mitchell, 1998; Ralevic, 2001). The present study shows that prolonged vasorelaxations mediated by Ap_nAs and Ap_nGs ($n=4-6$) are also endothelium-independent and blocked by α,β-meATP and PPADS. Interestingly, the rank order of potency of the Ap_nAs and Ap_nGs in eliciting prolonged vasorelaxation was similar to that of the contractile potency order of the compounds, suggesting a possible involvement of P2X receptor activation in initiation of the response. PPADS-sensitive, endothelium-independent prolonged relaxations following vasoconstriction to Ap_nAs have also recently been described in rat isolated mesenteric resistance arteries (Steinmetz *et al*., 2000).

Interestingly, in the presence of α,β-meATP rapid vasorelaxations to Ap_nAs and Ap_nGs ($n=4-6$) were observed, indicating that actions of these compounds at contractile $P2X_1$-like receptors normally opposes rapid vasorelaxation. Similarly, in the rat raised-tone isolated perfused kidney, suramin block of Ap_4A-mediated vasoconstriction revealed vasorelaxation (van der Giet *et al*., 1997). The present study is the first full report of vasorelaxant actions of Ap_4G, Ap_5G and Ap_6G, masked under normal conditions by the contractile actions of these compounds. Rapid relaxations preceding vasoconstriction to Ap_nAs have recently been reported in rat isolated preconstricted mesenteric resistance arteries (Steinmetz *et al*., 2000). Dual and opposing actions of dinucleotides were also reported by Busse *et al*. (1988) who described an Ap_4A-mediated endothelium-dependent vasorelaxation, which was reversed to a pronounced contraction after endothelium removal in rabbit mesenteric arteries. Collectively, these data indicate that contractile Ap_nAs and Ap_nGs can have complex effects at multiple purine receptors in the vasculature.

Experiments carried out in order to characterize the receptor mediating rapid relaxations to contractile Ap_nAs and Ap_nGs ($n=4-6$) after P2X receptor blockade produced mixed results. In the presence of α,β-meATP rapid relaxations to Ap_nAs were not blocked by 8-PSPT, suggesting no involvement of an adenosine P1 receptor, but neither were they all blocked by PPADS or endothelium removal, appearing to rule out an involvement of $P2Y_1$ and $P2Y_2$ receptors. Consistent with this is the report that Ap_4A, Ap_5A and Ap_6A are weak or inactive at recombinant $P2Y_1$ receptors (Pintor *et al*., 1996). In contrast, rapid relaxations to Ap_nGs were variously blocked by endothelium removal, PPADS and 8-PSPT, suggesting possible actions at both P1 and P2 receptors. Although suramin was able to block responses to both Ap_nAs and Ap_nGs these data may have to be viewed with caution as Ap_5A was still able to elicit

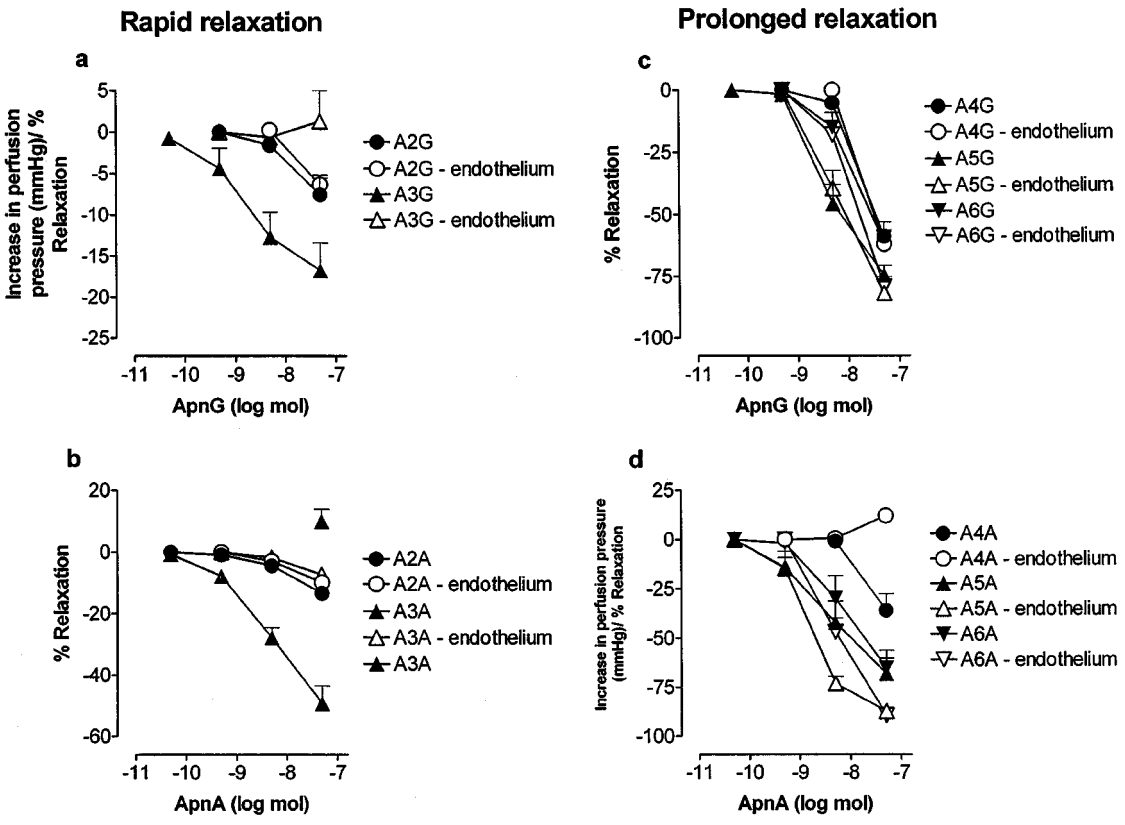

Figure 9 Effect of endothelium removal on relaxation dose-response curves to Ap_nGs and Ap_nAs ($n=2-6$) in the rat isolated perfused methoxamine-preconstricted mesenteric arterial bed. Rapid relaxations to: (a) Ap_2G and Ap_3G ($n=6$); (b) Ap_2A and Ap_3A ($n=6$). Prolonged relaxations to: (c) Ap_4G, Ap_5G and Ap_6G ($n=6$); (d) Ap_4A, Ap_5A and Ap_6A ($n=5-6$). Data are shown as mean + s.e.mean. In the absence of endothelium the attenuated rapid relaxation response to Ap_3A was followed by a small constriction (isolated symbol in panel b).

vasoconstriction, suggesting that there was no longer full desensitization of P2X receptors. Whether the relaxations are due to direct actions of the compounds, or due to vasorelaxant fragments generated by ectoenzymatic hydrolysis, remains to be determined.

Vasorelaxations to Ap_2A and Ap_3A were observed and, as reported previously, Ap_3A was more potent than Ap_2A (Ralevic *et al*., 1995). In the present study we additionally show for the first time that the shorter phosphate chain Ap_nGs, Ap_2G and Ap_3G, are vasorelaxants in the rat isolated mesenteric arterial bed. Both were less potent than the corresponding Ap_nA, and Ap_3G was more potent than Ap_2G. Interestingly, responses to both Ap_3A and Ap_3G were endothelium-dependent, and were blocked by PPADS, but those to Ap_2A and Ap_2G were independent of the endothelium and were unaffected by PPADS. It seems likely, therefore, that relaxations to Ap_3A and Ap_3G are mediated by PPADS-sensitive $P2Y_1$ receptors and not by PPADS-insensitive $P2Y_2$ receptors coexpressed on the endothelium (Ralevic & Burnstock, 1996). In line with this suggestion, Ap_3A, but not Ap_2A, is an agonist at recombinant $P2Y_1$ receptors (Pintor *et al*., 1996). In contrast, in the rat isolated perfused kidney vasorelaxant responses to Ap_3A and Ap_3G are mediated *via* A_2 receptors, and these compounds also elicit vasoconstriction *via* A_1 receptors (van der Giet *et al*., 1997). In the present study, relaxation by Ap_2A and Ap_2G may be mediated *via* adenosine A_2 (A_{2B} subtype) receptors on the smooth muscle of rat mesenteric arteries (Rubino *et al*., 1995). Thus, the length of the polyphosphate chain can determine not only the selectivity and potency of these purine compounds at different subtypes of P2 receptors, but also their selectivity for P2 versus P1 receptors.

The Gp_nGs were largely inactive, except for Gp_2G (which elicited α,β-meATP- and PPADS-sensitive $P2X_1$-like receptor-mediated vasoconstriction), indicating that expression of an adenine moiety is crucial for vasoactivity of dipurine polyphosphates. Moreover, a single adenine was sufficient for full activity of the molecule, as potency was generally not greater with two adenines (Ap_nAs) compared to one adenine (Ap_nGs). Gp_nGs have also been shown to be inactive as modulators of vascular tone in the rat isolated perfused kidney, but are potent modulators of growth in vascular smooth muscle cells (Schlüter *et al*., 1998).

Ap_nAs, Ap_nGs and Gp_nGs are found in a number of different cell types, including hepatocytes, adrenal medullary chromaffin and myocardial granules, erythrocytes and platelets (Rapaport & Zamenick, 1976; Flodgaard & Klenow, 1982; Lüthje & Ogilvie, 1983; Rodriguez-del-Castillo *et al*., 1988; Schlüter *et al*., 1994; Luo *et al*., 1999a,b). Platelets and erythrocytes may be particularly significant sources of these compounds relevant to the

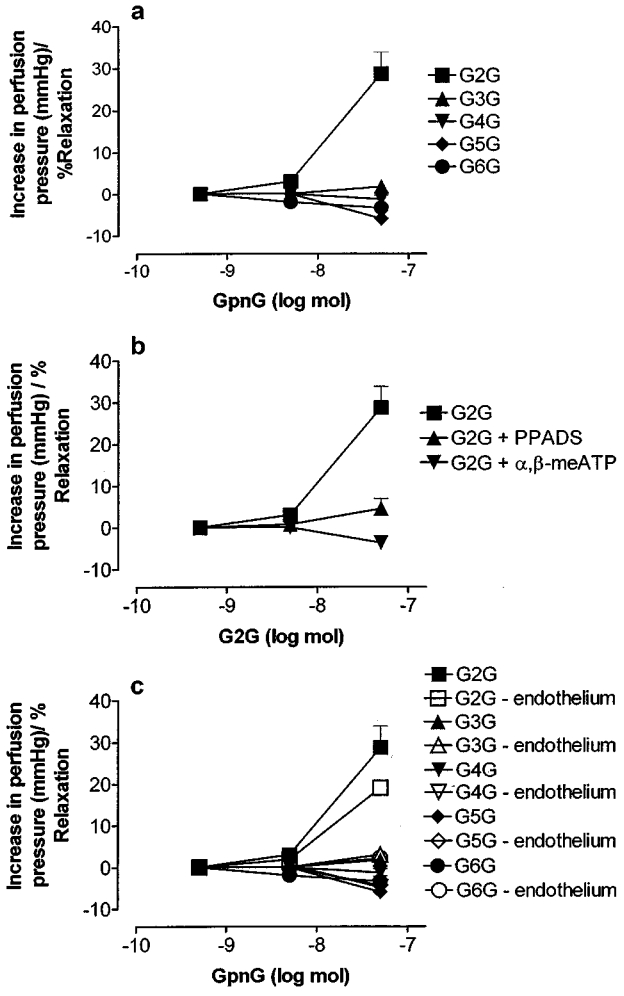

Figure 10 Dose-response curves to Gp_nG ($n=2-6$) in the rat isolated perfused methoxamine-preconstricted mesenteric arterial bed. (a) Control conditions; (b) responses to Gp_nG in the presence of pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS; 10 μM) and α,β-methylene ATP (α,β-meATP; 10 μM); (c) responses to Gp_nGs with and without endothelium removal ($n=4-6$). Data are shown as mean + s.e.mean. Where error bars do not appear, these fall within the symbol.

regulation of vascular tone. Endothelial cell damage is a trigger for platelet aggregation, which may lead to the release of Ap_nAs, Ap_nGs and Gp_nGs to vasoactive concentrations extracellularly, and vasoconstriction. However, evidence of a prolonged relaxation that follows contraction to Ap_nAs and Ap_nGs ($n=4-6$) indicates that the vasospasmic action of the purines may be self-limiting.

In conclusion, we have shown that for Ap_nGs as well as for Ap_nAs, the length of the phosphate chain has a pivotal role in determining activity at P2 receptors in the rat isolated mesenteric arterial bed. Hence, when the phosphate chain is short ($n=2-3$) the compounds are vasodilators, whilst when the chain is long ($n=4-6$) the compounds are vasoconstrictors *via* $P2X_1$-like receptors. Moreover, we have shown for the first time that contractile Ap_nAs and Ap_nGs ($n=4-6$) can mediate vasorelaxation; prolonged vasorelaxation following vasoconstriction, and rapid relaxation after block of $P2X_1$ receptor-mediated constriction. Regarding the purine moiety, one adenine is crucial and sufficient for vasoactivity as Gp_nGs are largely inactive, and Ap_nA and Ap_nGs approximately equipotent.

We are grateful to the Royal Society for financial support.

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(Received June 25, 2001
Revised August 6, 2001
Accepted August 14, 2001)