British Journal of Pharmacology (2000) 129, 124–130

www.nature.com/bjp

Effects of diadenosine polyphosphates (Ap_nAs) and adenosine polyphospho guanosines (Ap_nGs) on rat mesenteric artery P2X receptor ion channels

[1]C.J. Lewis, [1]D.P. Gitterman, [2]H. Schlüter & *,[1]R.J. Evans

[1]Department of Cell Physiology and Pharmacology, Medical Sciences Building, University of Leicester, Leicester LE1 9HN and [2]Ruhr-Universität Bochum, Marienhospital Herne, Med. Klinik 1, Hölkeskampring 40, 44625 Herne, Germany

1 Diadenosine polyphosphates (Ap_nAs, $n=3-7$) and adenosine polyphospho guanosines (Ap_nGs, $n=3-6$) are naturally occurring vasoconstrictor substances found in platelets. These vasoconstrictor actions are thought to be mediated through the activation of P2X receptors for ATP. The effects of Ap_nAs and Ap_nGs at P2X receptors on rat mesenteric arteries were determined in contraction studies and using the patch clamp technique on acutely dissociated artery smooth muscle cells.

2 $P2X_1$ receptor immunoreactivity was detected in the smooth muscle layer of artery rings. The sensitivity to α,β-methylene ATP and desensitizing nature of rat mesenteric artery P2X receptors correspond closely to those of recombinant $P2X_1$ receptors.

3 Ap_4A, Ap_5A and Ap_6A evoked concentration dependent P2X receptor inward currents which desensitized during the application of higher concentrations of agonist. The agonist order of potency was $Ap_5A \geqslant Ap_6A \geqslant Ap_4A > > Ap_3A$. Ap_2A and Ap_7A were ineffective. Similar results were obtained in contraction studies except for Ap_7A which evoked a substantial contraction.

4 Ap_nGs ($n=2-6$)(30 μM) evoked P2X receptor inward currents in mesenteric artery smooth muscle cells. Ap_nGs ($n=4-6$) were less effective than the corresponding Ap_nA.

5 This study shows that at physiologically relevant concentrations Ap_nAs and Ap_nGs can mediate contraction of rat mesenteric arteries through the activation of $P2X_1$-like receptors. However the activity of the longer chain polyphosphates ($n=6-7$) may be overestimated in whole tissue studies due to metabolic breakdown to yield the P2X receptor agonists ATP and adenosine tetraphosphate. *British Journal of Pharmacology (2000)* **129,** 124–130

Keywords: P2X receptors; artery; diadenosine polyphosphates; $P2X_1$ receptors; vasoconstriction

Abbreviations: α,β-meATP, α,β-methylene ATP; Ap_nAs, diadenosine polyphosphates; Ap_nGs, adenosine polyphosphoguanosines; FITC, fluorescein isothiocyanate; PBS, phosphate buffered saline

Introduction

Platelets store and release a range of vasoactive nucleosides including diadenosine polyphosphates (Ap_nAs, $n=3-7$) and adenosine polyphospho guanosines (Ap_nGs, $n=2-6$) which can act through P2X receptors for ATP to mediate vasoconstriction (Busse *et al*., 1988; Schlüter *et al*., 1994; 1998; Jankowski *et al*., 1999). P2X receptors are ligand gated cation channels and their activation leads to membrane depolarization, calcium influx and smooth muscle contraction (Evans & Surprenant, 1996). Seven genes encoding P2X receptors have been isolated ($P2X_{1-7}$) (Collo *et al*., 1996; Surprenant *et al*., 1996; Ralevic & Burnstock, 1998). Artery smooth muscle P2X receptors are sensitive to the metabolically stable ATP analogue α,β-methylene ATP (α,β-meATP) and responses rapidly desensitize during the continued presence of agonist. The $P2X_1$ receptor is the predominant isoform expressed by smooth muscle and its properties are similar to those of the native arterial smooth muscle P2X receptor (Valera *et al*., 1994; Collo *et al*., 1996; Evans & Surprenant, 1996; Lewis *et al*., 1998; McLaren *et al*., 1998).

The vasoconstrictor effects of a range of naturally occurring Ap_nAs and Ap_nGs have been determined in a limited number of studies on perfused vascular beds. This work has shown that the activity of these compounds is dependent on the length of the polyphosphate chain (Ralevic *et al*., 1995; van der Giet *et al*., 1997; Schlüter *et al*., 1998). Similar results have been reported for the actions of Ap_nAs at recombinant $P2X_1$ receptors (Evans *et al*., 1995; Wildman *et al*., 1999; Bianchi *et al*., 1999). However a direct quantitative comparison of the effects of Ap_nAs at arterial smooth muscle and recombinant $P2X_1$ receptors is difficult to make as drugs were applied in a bolus in the perfused vascular bed experiments and thus the effective agonist concentration at the receptor was not determined. In addition the metabolism of nucleotides by ecto-enzymes can lead to underestimating agonist potency in organ bath studies (Benham & Tsien, 1987; Inoue & Brading, 1990; Evans & Kennedy, 1994; Trezise *et al*., 1994). For example recent studies have suggested that Ap_4A may be subject to ecto-enzyme breakdown (Westfall *et al*., 1997). Complications associated with agonist breakdown can be overcome in patch clamp studies on acutely dissociated smooth muscle cells where drugs can be applied rapidly under concentration clamp conditions (Evans & Kennedy, 1994). This system has the added advantage that currents recorded in response to drugs give a direct measure of activation of the P2X receptor channels. The primary aim of this study was to characterize the effects of a range of Ap_nAs, Ap_nGs and related compounds on rat mesenteric artery smooth muscle P2X receptors to allow comparisons to be made between native smooth muscle and recombinant $P2X_1$ receptors and to investigate further the structure activity relationships of these compounds.

*Author for correspondence; E-mail: RJE6@le.ac.uk

Methods

Male wistar rats (250–300 g) were killed by cervical dislocation and femoral exsanguination. The mesentery was removed and second and third order mesenteric arteries were dissected. For contraction experiments second order mesenteric artery rings were mounted in a Mulvany myograph (vessel diameter 226.5 ± 11.4 μm, $n=8$) (Lagaud *et al*., 1996). The arteries were superfused at 2 ml min^{-1} with a warmed physiological saline with the following composition (mM): NaCl 150, KCl 5, HEPES 10, $CaCl_2$ 2.5, $MgCl_2$ 1, (pH to 7.3 with NaOH). The organ bath temperature was 37°C. Drugs were added to the superfusate. The vasoconstrictor agonists evoked transient contractions of the artery that returned to baseline diameter in the continued presence of purinergic drugs (Figure 2a). Reproducible contractions were obtained when agonists were applied at 30 min intervals.

Acutely dissociated smooth muscle cells were prepared by a two step papain and collagenase/hyaluronidase enzymatic digestion (see Quayle *et al*., 1996). Cells were plated onto glass coverslips, stored at 4°C and used within 2–36 h. For electrophysiological recording smooth muscle cells were superfused at 2 ml min^{-1} with physiological solution and drugs were applied rapidly using a U-tube perfusion system (Evans & Kennedy, 1994). Amphotericin permeabilized patch recordings were made with an Axopatch 200B amplifier and data was collected using pClamp6 software (Axon Instruments U.S.A.). Holding potential was −60 mV. Patch electrodes (2–5 MΩ) were filled with a solution of the following composition (mM): potassium gluconate 140, NaCl 5, HEPES 10, EGTA 9, (pH adjusted to 7.3 with KOH). Reproducible responses to agonists (200–500 ms pulse duration) were obtained when a 5 min interval was given between agonist applications.

Data are reported throughout as mean ± s.e.mean, n = number of observations. Peak currents in response to drugs are expressed as percentage of the response to 10 μM α,β-meATP, this is a maximal concentration at rat mesenteric artery P2X receptors (Lewis *et al*., 1998). It was not always possible to construct a concentration response curve in a single smooth muscle cell therefore pooled concentration response data for agonists were fitted by the least squares method using Origin software (Microsoft U.S.A.) with the equation; $response = \alpha[A]^H/([A]^H + [A_{50}]^H)$ where α and H are the asymptote and Hill coefficient, [A] is the agonist concentration, A_{50} is the agonist concentration producing 50% of the maximum agonist response (EC_{50}). Differences between means were determined by Student's unpaired t-test (two tailed) and were considered significant when $P<0.05$.

For immunohistochemical studies second and third order mesenteric arteries were dissected as for electrophysiology experiments and embedded in Tissue-Tek (Miles Inc, Elkhart, In.) and frozen over dry ice and hexane. Frozen transverse arterial 12 μm sections were cut and mounted on pre-subbed slides. The sections were fixed in a 2% paraformaldehyde solution in Sorensons buffer for 10 min. After washing in a phosphate buffered saline (PBS) solution the slides were incubated in a blocking solution (10% donkey serum (Jackson Immunoresearch), 0.5% Triton-X (Sigma) in PBS for 30 min at room temperature to permeabilize the tissue and reduce non-specific binding. Sections were then washed and incubated overnight in primary antisera (Anti-$P2X_1$ ± control antigen blocking peptide, Alomone Lab. Israel) at 4°C. Anti-$P2X_1$ is a polyclonal antibody raised in rabbit against the C-terminal residues 382–399 of the $P2X_1$ receptor. The anti-$P2X_1$ receptor antibody was used at a dilution of 1 : 200 in 10% donkey serum in PBS. When blocking peptides were used, the primary antibody and its corresponding antigen peptide were incubated together (1 mg of each) for 1 h at room temperature. After washing in PBS, sections were incubated in secondary antisera for visualization, which in each case was fluorescein isothiocyanate (FITC) labelled anti-rabbit IgG raised in donkey (Jackson Immunoresearch) (1 : 100 in 10% donkey serum in PBS) for 2 h at room temperature. As a control to determine the amount of non-specific binding, slides were incubated in 10% donkey serum in PBS only or secondary antisera only. Slides were washed in PBS and mounted in Citifluor (UKC Chem Lab, U.K.). Arterial sections were examined under epifluorescence with neutral density filters. Images were captured using Scionimage software.

Drugs: papain, dithioerythritol, collagenase, hyaluronidase, α,β methylene ATP (α,β-meATP, Ap_2A, Ap_3A, Ap_4A, Ap_5A, Ap_6A, GTP, adenosine tetraphosphate, tetrapolyphosphate (Hexa-ammonium salt), and trisodium trimetaphosphate (Sigma). HPLC analysis of diadenosine polyphosphates from Sigma has shown that they are of high purity and are not contaminated with ATP (Pintor *et al*., 1996). Ap_7A, Ap_2G, Ap_3G, Ap_4G, Ap_5G, and Ap_6G (synthesized and HPLC purified by H. Schlüter).

Results

Effects of Ap_nAs on acutely dissociated rat mesenteric smooth muscle cells

The diadenosine polyphosphates Ap_4A, Ap_5A and Ap_6A evoked concentration dependent inward currents which declined during the continued presence of high concentrations of agonist (Figure 1a). Ap_5A evoked responses had an EC_{50} of 6.7 μM (pA_{50} 5.23 ± 0.12) and a slope of 1.1 ± 0.23, the maximal response to Ap_5A was 80.8 ± 7.2% of the response to 10 μM α,β-meATP ($n=5$). The mean concentration response relationship for Ap_6A gave an EC_{50} of ~13 μM and a maximum of 43 ± 7.5% of the response to 10 μM α,β-meATP ($n=5$–7) (Figure 1a,b). No clear maximum response to Ap_4A was recorded at concentrations up to 300 μM ($n=4$) (~50 μM Ap_4A evoked a response equivalent to 50% of the maximal α,β-meATP response). Ap_3A evoked a small current at 100 μM which was 21.3 ± 5.7% of the maximal α,β-meATP evoked response. In cross desensitization studies following α,β-meATP (10 μM) treatment no response to Ap_nAs (all 30 μM, $n=3$–6) was recorded indicating that all these agonists acted at the same receptor. No response was recorded to applications of Ap_2A or Ap_7A (30 and 100 μM) ($n=4$–6).

Comparison of Ap_nA evoked contractions in intact arteries and currents recorded from isolated smooth muscle cells

The majority of studies on arterial smooth muscle P2X receptors have recorded contractile responses. These whole tissue pharmacological studies may be complicated by problems associated with agonist metabolism by ecto-enzymes and the contractile response only gives an indirect measure of P2X receptor activation. It was therefore of interest to compare Ap_nA mediated P2X receptor inward currents and contractions in the rat mesenteric artery. Ap_5A evoked rapid, concentration dependent, transient constrictions of rat mesenteric artery wings which returned to baseline during the continued presence of the agonist (Figure 2a). The EC_{50} values for evoking contraction and inward currents were essentially the same (6.5 and 8.5 μM respectively, Figure 2b). The

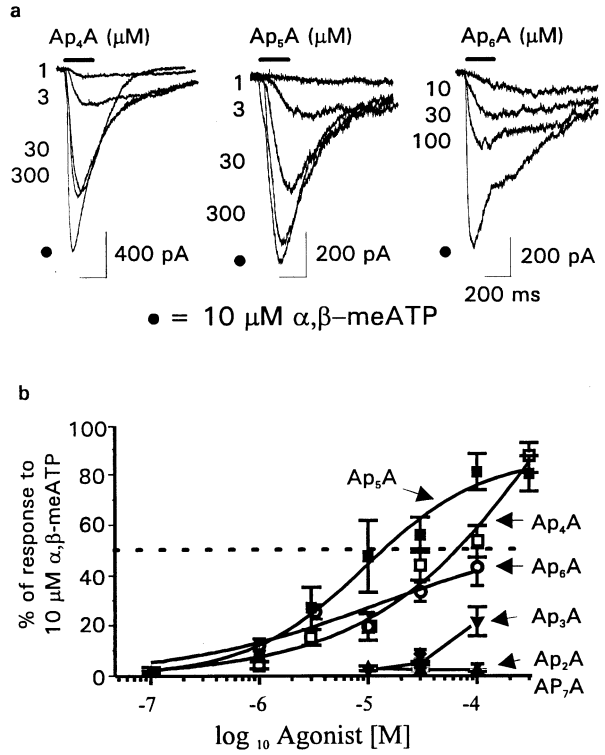

Figure 1 P2X receptor mediated inward currents evoked by Ap_nAs in acutely dissociated rat mesenteric smooth muscle cells. (a) Representative traces of inward currents evoked by Ap_4A, Ap_5A and Ap_6A compared to the current evoked by 10 μM α,β-meATP. Drugs were applied for 200 ms indicated by the bar. (b) Concentration response relationships for inward currents evoked by Ap_nA ($n=2-8$). Each point is the mean percentage of the response to 10 μM α,β-meATP ($n=4-7$ for each point).

maximal constrictor response was $113\pm11.6\%$ of the response to 10 μM α,β-meATP compared to $80.8\pm7.2\%$ for inward currents. Following desensitization of smooth muscle P2X receptors in the continued presence of 10 μM α,β-meATP the response to 100 μM Ap_5A was abolished ($n=4$) (Figure 2c). These cross desensitization experiments suggest that Ap_5A and α,β-meATP are acting through the same P2X receptor to mediate contraction.

The effects of 10 μM Ap_nAs ($n=2-6$) in contractile and patch clamp studies are shown in Figure 2d. Ap_2A (upto 100 μM) and Ap_3A (upto 30 μM) did not evoke contractions or inward currents in the mesenteric artery. Ap_2A (10 μM) had no effect on contractions evoked by an EC_{50} concentration of α,β-meATP (1 μM) (responses $103\pm2.6\%$ of control, $n=4$). In contrast Ap_3A (10 μM) potentiated by $59\pm15\%$ ($n=6$) contractions evoked by α,β-meATP (1 μM). Ap_4A appeared less effective in mediating vasoconstriction than inward currents ($8.8\pm1.3\%$ and $19.1\pm5.2\%$ respectively of the response to 10 μM α,β-meATP, $n=6$). Although this effect is not significant, given that Ap_5A and Ap_6A were more effective in evoking contractions than inward currents, it may indicate that Ap_4A is subject to ecto-enzyme breakdown in whole tissue studies as has been reported previously (Westfall *et al*., 1997). Ap_6A was significantly more effective in mediating vasoconstriction than inward currents ($50.3\pm4.3\%$ and $21.2\pm4\%$ of the response to 10 μM α,β-meATP $n=6$ and 5 respectively $P<0.005$). Ap_7A was an effective agonist in mediating transient contractions (response to 10 μM Ap_7A was $50.1\pm11.2\%$ of response to 10 μM α,β-meATP, $n=4$) (Figure 2d). In cross desensitization studies following pre-treatment with α,β-meATP (10 μM) contractile responses to Ap_7A were abolished ($n=3$). These results indicate there is a major difference in the activity of Ap_7A in contractile and patch clamp studies and suggest that Ap_nAs may be subject to metabolism in whole tissue studies.

Effects of Ap_nGs on acutely dissociated rat mesenteric smooth muscle cells

The Ap_nGs mediate vasoconstriction in the rat isolated perfused kidney preparation and have been suggested to mediate this response through the activation of P2X receptors (Schlüter *et al*., 1998). In the present study Ap_nGs ($n=2-6$, 30 μM) evoked P2X receptor mediated inward currents in isolated smooth muscle cells (Figure 3). Ap_3G was significantly more effective than Ap_3A in evoking inward currents ($P<0.001$). In contrast Ap_4G, Ap_5G and Ap_6G were significantly less effective at evoking inward currents than the corresponding diadenosine polyphosphate compound ($P<0.05$). In cross desensitization studies following α,β-meATP treatment (10 μM) Ap_nAs ($n=4-6$, 30 μM) had no effect on holding current indicating that they were acting through the same P2X receptor ($n=3-6$). In whole tissue experiments Ap_nGs ($n=4-6$) were effective in evoking contractions, the response to 30 μM Ap_nG ($n=4-6$) were 7.3 ± 1.6, 44.1 ± 10.3 and $55.4\pm7.9\%$ respectively of the contractile response to 10 μM α,β-meATP ($n=4$). Thirty μM Ap_3G failed to evoke a contractile response ($n=4$).

Effects of other 'polyphosphate' compounds on P2X receptors

The action of Ap_nAs and Ap_nGs at P2X receptors raises questions about ligand recognition by P2X receptors and possible metabolism of these compounds in whole tissue studies. In order to investigate this further we have determined the effects of a number of related 'polyphosphate' compounds. GTP, trimetaphosphate and tetrapolyphosphate (100 μM) were ineffective as agonists at mesenteric artery P2X receptors ($n=4-6$) and ineffective as antagonists (Cook & Evans, 1999 unpublished observations) (Figure 4a). These results show that an adenosine moiety and a phosphate chain are required for ligand recognition by mesenteric artery P2X receptors. Adenosine tetraphosphate evoked similar currents to those seen with α,β-meATP (Figure 4b,c) with an EC_{50} of 0.51 μM. Responses to adenosine tetraphosphate (10 μM) were abolished following desensitization with 10 μM α,β-meATP ($n=3$). At adenosine tetraphosphate concentrations $\geqslant1$ μM recovery from receptor inactivation was prolonged, for example following an application of 1 μM adenosine tetraphosphate a subsequent response to 10 μM α,β-meATP required 15 min to return to pre adenosine tetraphosphate control levels, and at concentrations of 3 and 10 μM adenosine tetraphosphate responses to α,β-meATP (10 μM) were only $\sim70\%$ of control values after 30 min ($n=4$). The maximal adenosine tetraphosphate response was 159.5% of the response to 10 μM α,β-meATP.

Immunohistochemical localization of $P2X_1$ receptors in rat mesenteric artery smooth muscle cells

A polyclonal antibody raised against the carboxy terminus of the $P2X_1$ receptor produced selective staining of the smooth muscle cell layer of mesenteric artery rings that was abolished by pre-incubation of the antibody with a sequence specific blocking peptide, similar detection of $P2X_1$ receptor immuno-

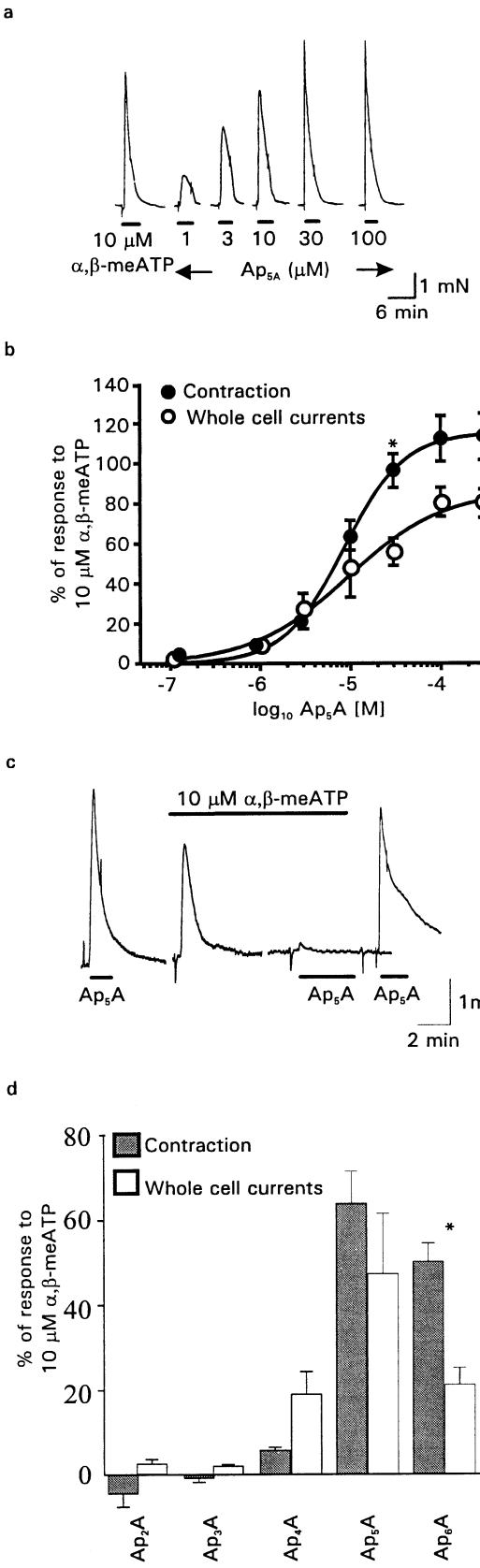

Figure 2 Comparison between responses to Ap_nAs in whole mesenteric artery contraction studies and patch clamp recordings from acutely dissociated mesenteric artery smooth muscle cells. (a) Ap_5A evoked concentration dependent contractions which fade during the continued presence of the agonist. A response to 10 μM α,β-meATP in the same vessel is shown. Drugs were applied for the time indicated by bars. (b) Comparison of concentration response relationships for contractions and whole cell currents evoked by Ap_5A. Each point is the mean percentage of the response to 10 μM α,β-meATP ($n=4-6$ for each point). (c) Ap_5A (100 μM) evoked transient contraction of the rat mesenteric artery, similar responses were evoked by α,β-meATP (10 μM). In the continued presence of α,β-meATP the response to Ap_5A was reduced by >95%. Drug application periods indicated by bars. (d) Comparison between contractions and whole cell currents evoked by 10 μM Ap_nAs ($n=2-7$). Each column is the mean response as a percentage of the maximal response to 10 μM α,β-meATP ($n=4-7$). (*$P<0.05$, **$P<0.01$).

reactivity in smooth muscle has been detected previously (Vulchanova *et al*., 1996) (Figure 5). A high level of background autofluorescence was associated with the endothelial cell layer (Figure 5b).

Discussion

This study has shown that a range of Ap_nAs evoked inward currents in rat mesenteric artery smooth muscle cells through the activation of P2X receptor cation channels and that activity of these compounds is dependent on the length of the phosphate chain. The study extends on previous work and documents the effective concentration range of agonists and presents new data on the structure activity relationships of P2X receptors. These data show that at concentrations likely to be present physiologically Ap_nAs and Ap_nGs may contribute to the control of blood pressure through the direct activation of $P2X_1$-like receptor cation channels.

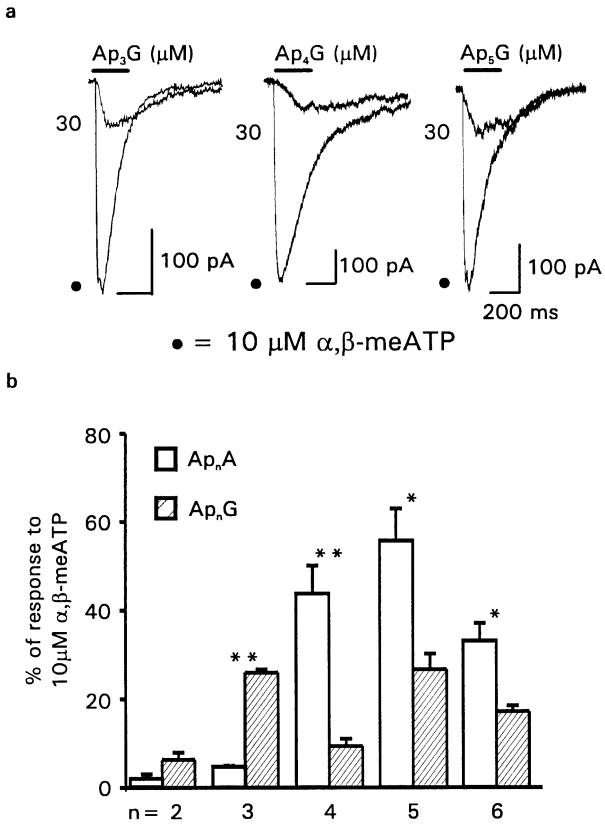

Figure 3 P2X receptor mediated currents evoked by Ap_nGs in isolated rat mesenteric arterial smooth muscle cells. (a) Representative traces of inward currents evoked by Ap_3G, Ap_4G and Ap_5G (all 30 μM) compared to currents evoked by 10 μM α,β-meATP (•) in individual cells. Drugs were applied for 200 ms as indicated by the bar. (b) Comparison between current amplitude of responses evoked by the diadenosine phosphates (30 μM) and the adenosine polyphospho guanosines (30 μM). Each column is the mean response as a percentage of the maximal response to 10 μM α,β-meATP ($n=4-7$). (*$P<0.05$, **$P<0.01$).

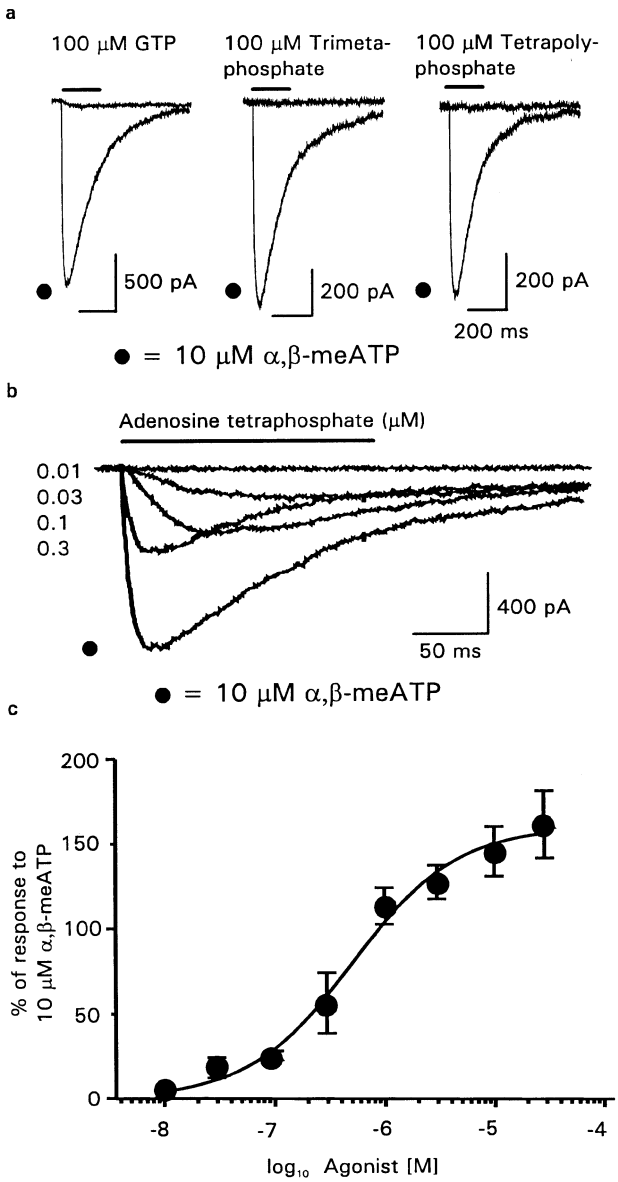

Figure 4 Effects of nucleotides and polyphosphate compounds on dissociated rat mesenteric artery smooth muscle cells. (a) GTP, trimetaphosphate and tetrapolyphosphate (all 100 μM) had no effect on the holding current in cells where α,β-meATP (10 μM, indicated by •) evoked an inward current. (b) Adenosine tetraphosphate evoked concentration dependent inward currents, the response to 10 μM α,β-meATP (indicated by •) is shown for comparison. (c) Concentration dependence of inward currents evoked by adenosine tetraphosphate. Each point is the mean percentage of the response to 10 μM α,β-meATP ($n=4-7$ for each point). Drugs were applied for 200 ms as indicated by the bar.

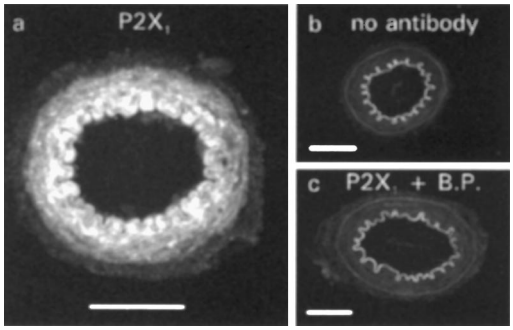

Figure 5 Immunohistochemical localization of $P2X_1$ receptors on rat mesenteric artery rings. (a) $P2X_1$ receptor specific immunoreactivity is localized to the smooth muscle layer of mesenteric artery rings. (b) Autofluorescence of the endothelial cell layer in fixed sections with no antibody. (c) $P2X_1$ receptor specific immunoreactivity is blocked by pre-incubation of the antibody with antigen blocking peptide (B.P.). Scale bar = 50 μm.

The use of patch clamp methods in this study has allowed a direct comparison of the effects of Ap_nAs between P2X receptor mediated currents in rat mesenteric smooth artery cells and at recombinant $P2X_1$ receptors (Evans *et al*., 1995; Wildman *et al*., 1999; Bianchi *et al*., 1999) to be made. Previous comparisons of native smooth muscle and recombinant $P2X_1$ receptors have been based on the timecourse of response and sensitivity to the agonists α,β-meATP, 1-β,γ-methylene ATP and the antagonist trinitrophenyl ATP. We extended this to compare further native smooth muscle P2X receptors and recombinant $P2X_1$ receptors. Our results are similar to those reported for human $P2X_1$ receptors expressed in mammalian HEK293 or 1321N1 astrocytoma cells (Evans *et al*., 1995; Bianchi *et al*., 1999). There are however marked differences in the actions of Ap_4A and Ap_6A in rat mesenteric artery smooth muscle cells and at recombinant rat $P2X_1$ receptors expressed in *Xenopus* oocytes (Wildman *et al*., 1999). A degree of caution however must be used in drawing conclusions based on these findings as discrepancies between the properties of recombinant P2X receptors expressed in mammalian and amphibian systems have been reported (e.g. comparison of the studies of Brake *et al*., 1994; Evans *et al*., 1995; Pintor *et al*., 1996). Thus it remains to be confirmed whether the native rat mesenteric artery smooth muscle P2X receptor is different from recombinant rat $P2X_1$ receptors or if the differences can be accounted for by the difference in the expression system. However the general properties of rat mesenteric artery P2X receptors and the presence of $P2X_1$ receptor immunoreactivity on these vessels suggest strongly that the $P2X_1$ receptor characteristics dominate this native phenotype (Evans & Surprenant, 1996; Lewis *et al*., 1998).

Ap_7A has been shown recently to be released by platelets and has a vasoconstrictor action in the isolated perfused rat kidney (Jankowski *et al*., 1999). In the present study we show that Ap_7A evokes α,β-meATP sensitive contractions of the rat mesenteric artery. However Ap_7A failed to evoke inward currents in patch clamp studies. Similarly Ap_6A was more effective in evoking contractions than P2X receptor currents. These results suggest that in whole tissue studies Ap_6A and Ap_7A may be subject to metabolism, as has been shown for Ap_4A (Westfall *et al*., 1997), and that when added under concentration clamp conditions in patch clamp studies the true potency of the agonist is revealed. Cleavage of the phosphate chain could give rise to ATP and adenosine tetraphosphate (Mateo *et al*., 1997) which then act locally to activate P2X receptor channels (Evans *et al*., 1995 and this study). As Ap_6A is an agonist of P2X receptor currents it is likely that in contraction studies the response results from activation of the P2X receptor by both Ap_6A and metabolic products. Thus the activity of Ap_6A and particularly Ap_7A directly at P2X receptors may be overestimated in whole tissue studies due to metabolism.

The Ap_nGs evoked inward currents and contractions through mesenteric artery P2X receptors. Previously Ap_nGs have been shown to mediate vasoconstriction in the perfused kidney preparation (Schlüter *et al*., 1998) however this is the first time a direct role for P2X receptors in this process has been demonstrated. Ap_2G and Ap_3G were more effective than the corresponding Ap_nA at evoking inward currents. The

increased efficacy of Ap_3G relative to Ap_3A may reflect that binding of the γ-phosphate to the P2X receptor is influenced by the chemical group substituted at the ester oxygen. When this group is a phosphate i.e. adenosine tetraphosphate the potency is equivalent to α,β-meATP. The potency order of the chemical groups at this position is phosphate > > guanosine > adenosine. In contrast Ap_4G, Ap_5G and Ap_6G were less effective than the corresponding Ap_nA. The reduced potency of Ap_nG ($n=4-6$) compared to the corresponding Ap_nA may simply reflect the reduced probability of the adenosine moiety binding with the P2X receptor.

A number of 'polyphosphate' compounds including Ap_2A, Ap_3A, GTP, trimetaphosphate and tetrapolyphosphate were ineffective as agonists at the P2X receptor. When these compounds were co-applied with α,β-meATP (1 μM, an EC_{50} concentration) they either had no effect on the amplitude of the P2X receptor mediated responses or slightly potentiated them. These results demonstrate that binding of an adenine group in the case of Ap_2A or a triphosphate group in the case of GTP is ineffective in activating the receptor. The fact that these compounds are ineffective as antagonists suggests that for high affinity binding at the P2X receptor both an adenine group and a triphosphate moiety are required.

The activation of platelets leads to the release of a variety of vasoactive nucleotides. For example Ap_nAs and Ap_nGs have been estimated to be present at 0.5–3 μM in the supernatant following platelet aggregation (Schlüter *et al*., 1998). Recent studies on platelets have shown that it is likely that the nucleotide concentration at the local microenvironment of the cell surface may be at least an order of magnitude higher than that measured in the supernatant (Beigi *et al*., 1999). Ap_nAs and Ap_nGs may therefore be present locally in the 5–30 μM range, concentrations that were effective in activating P2X receptors in this study. When the endothelium is damaged there may be a local rise in Ap_nAs and Ap_nGs following platelet activation resulting in the activation of P2X receptors and subsequent vasoconstriction.

In summary these results have shown that diadenosine polyphosphates and adenosine polyphospho guanosines at physiologically relevant concentrations can act directly at $P2X_1$-like receptor ion channels to mediate vasoconstriction in rat mesenteric artery. The activity of these compounds is dependent on the length of the polyphosphate chain. In addition it is possible that Ap_nAs and Ap_nGs ($n=6-7$) may be metabolised in whole tissue studies to yield the P2X receptor agonists ATP and adenosine tetraphosphate that may mediate some of their vasoconstrictor actions.

We would like to thank the Wellcome Trust, the MRC and DFG Schl 406/1–2 for support.

References

BEIGI, R., KOBATAKE, E., AIZAWA, M. & DUBYAK, G.R. (1999). Detection of local ATP release from activated platelets using cell surface-attached firefly luciferase. *Am. J. Physiol.,* **276,** C267–C278.

BENHAM, C.D. & TSIEN, R.W. (1987). A novel receptor-operated Ca^{2+}-permeable channel activated by ATP in smooth muscle. *Nature,* **328,** 275–278.

BIANCHI, B.R., LYNCH, K.J., TOUMA, E., NIFORATOS, W., BURGARD, E.C., ALEXANDER, K.M., PARK, H.S., YU, H., METZGER, R., KOWALUK, E., JARVIS, M.F. & VAN BIESEN T. (1999). Pharmacological characterization of recombinant human and rat P2X receptor subtypes. *Eur. J. Pharmacol.,* **376,** 127–138.

BRAKE, A.J., WAGENBACH, M.J. & JULIUS, D. (1994). New structural motif for ligand-gated ion channels defined by an ionotropic ATP receptor. *Nature,* **371,** 519–523.

BUSSE, R., OGILVIE, A. & POHL, U. (1988). Vasomotor activity of diadenosine triphosphate and diadenosine tetraphosphate in isolated arteries. *Am. J. Physiol.,* **254,** H828–H832.

COLLO, G., NORTH, R.A., KAWASHIMA, E., MERLO-PICH, E., NEIDHART, S., SURPRENANT, A. & BUELL, G. (1996). Cloning of $P2X_5$ and $P2X_6$ receptors and the distribution and properties of an extended family of ATP-gated ion channels. *J. Neurosci.,* **16,** 2495–2507.

EVANS, R.J. & KENNEDY, C. (1994). Characterization of P2-purinoceptors in the smooth muscle of the rat tail artery: a comparison between contractile and electrophysiological responses. *Br. J. Pharmacol.,* **113,** 853–860.

EVANS, R.J., LEWIS, C., BUELL, G., VALERA, S., NORTH, R.A. & SURPRENANT, A. (1995). Pharmacological characterization of heterologously expressed ATP-gated cation channels (P2X purinoceptors). *Mol. Pharmacol.,* **48,** 178–183.

EVANS, R.J. & SURPRENANT, A. (1996). P2X receptors in autonomic and sensory neurons. *Semin. Neurosci.,* **8,** 217–223.

INOUE, R. & BRADING, A.F. (1990). The properties of the ATP-induced depolarization and current in single cells isolated from the guinea-pig urinary bladder. *Br. J. Pharmacol.,* **100,** 619–625.

JANKOWSKI, J., TEPEL, M., VAN DER GEIT, M., TENTE, I., HENNING, L., JUNKER, R., ZIDEK, W. & SCHLÜTER, H. (1999). Identification and characterization of P1,P7-Di(adenosine-5′)-heptaphosphate from human platelets. *J. Biol. Chem.* in press.

LAGAUD, G.J.L., STOCLET, J.C. & ANDRIANTSITOHAINA, R. (1996). Calcium handling and purinoceptor subtypes involved in ATP-induced contraction in rat small mesenteric arteries. *J. Physiol.,* **492,** 689–703.

LEWIS, C., SURPRENANT, A. & EVANS, R.J. (1998). 2′,3′-O-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate (TNP-ATP) a nanomolar affinity antagonist at rat mesenteric artery P2X receptor ion channels. *Br. J. Pharmacol.,* **124,** 1463–1466.

MATEO, J., MIRAS-PORTUGAL, M. & ROTLLAN, P. (1997). Ecto-enzymatic hydrolysis of diadenosine polyphosphates by cultured adrenomedullary vascular endothelial cells. *Am. J. Physiol.,* **273,** C918–C927.

MCLAREN, G.J., SNEDDON, P. & KENNEDY, C. (1998). Comparison of the actions of ATP and UTP at $P2X_1$ receptors in smooth muscle of the rat tail artery. *Eur. J. Pharmacol.,* **351,** 139–144.

PINTOR, J., KING, B.F., MIRAS-PORTUGAL, M.T. & BURNSTOCK, G. (1996). Selectivity and activity of adenine dinucleotides at recombinant $P2X_2$ and $P2Y_1$ purinoceptors. *Br. J. Pharmacol.,* **119,** 1006–1012.

QUAYLE, J.M., DART, C. & STANDEN, N.B. (1996). The properties and distribution of inward rectifier potassium currents in pig coronary arterial smooth muscle. *J. Physiol.,* **494,** 715–726.

RALEVIC, V. & BURNSTOCK, G. (1998). Receptors for purines and pyrimidines. *Pharmacol. Rev.,* **50,** 413–492.

RALEVIC, V., HOYLE, C.H.V. & BURNSTOCK, G. (1995). Pivotal role of phosphate chain length in vasoconstrictor versus vasodilator actions of adenine dinucleotides in rat mesenteric arteries. *J. Physiol.,* **483,** 703–713.

SCHLÜTER, H., GROSS, I., BACHMANN, J., KAUFMANN, R., VAN DER GEIT, M., TEPEL, M., NOFER, J., ASSMANN, G., KARAS, M., JANOWSKI, J. & ZIDEK, W. (1998). Adenosine(5′) oligophospho-(5′) guanosines and guanosine(5′) oligophospho-(5′) guanosines in human platelets. *J. Clin. Invest.,* **101,** 682–688.

SCHLÜTER, H., OFFERS, E., BRUGGEMANN, G., VAN DER GEIT, M., TEPEL, M., NORDHOFF, E., KARAS, M., SPIEKER, C., WITZEL, H. & ZIDEK, W. (1994). Diadenosine phosphates and the physiological control of blood pressure. *Nature,* **367,** 186–188.

SURPRENANT, A., RASSENDREN, F., KAWASHIMA, E., NORTH, R.A. & BUELL, G. (1996). The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor $P2X_7$. *Science,* **272,** 735–738.

TREZISE, D.J., BELL, N.J., KENNEDY, I. & HUMPHREY, P.P.A. (1994). Effects of divalent cations on the potency of ATP and related agonists in rat isolated vagus nerve: implications for P2 purinoceptor classification. *Br. J. Pharmacol.,* **113,** 463–470.

VALERA, S., HUSSY, N., EVANS, R.J., ADAMI, N., NORTH, R.A., SURPRENANT, A. & BUELL, G. (1994). A new class of ligand-gated ion channel defined by P2X receptor for extraxcellular ATP. *Nature,* **371,** 516 – 519.

VAN DER GIET, M., KHATTAB, M., BORGEL, J., SCHLÜTER, H. & ZIDEK, W. (1997). Differential effects of diadenosine phosphates on purinoceptors in the rat isolated perfused kidney. *Br. J. Pharmacol.,* **120,** 1453 – 1460.

VULCHANOVA, L., ARVIDSSON, U., RIEDL, M., BUELL, G., SURPRENANT, A., NORTH, R.A. & ELDE, R.P. (1996). Differential distribution of two ATP-gated ion channels (P2X receptors) determined by immunohistochemistry. *Proc. Natl. Acad. Sci. U.S.A.,* **93,** 8063 – 8067.

WESTFALL, T.D., MCINTYRE, C.A., OBEID, S., BOWES, J., KENNEDY, C. & SNEDDON, P. (1997). The interaction of diadenosine polyphosphates with P2X-receptors in the guinea-pig isolated vas deferens. *Br. J. Pharmacol.,* **121,** 57 – 62.

WILDMAN, S.S., BROWN, S.G., KING, B.F. & BURNSTOCK, G. (1999). Selectivity of diadenosine polyphosphates for rat P2X receptor subunits. *Eur. J. Pharmacol.,* **367,** 119 – 123.

(Received August 20, 1999
Accepted October 6, 1999)