British Journal of Pharmacology (2003) 139, 661–671
www.nature.com/bjp

Endothelium-dependent relaxation and endothelial hyperpolarization by P2Y receptor agonists in rat-isolated mesenteric artery

[1]Hammit Mistry, [2]Jonathan M. Gitlin, [2]Jane A. Mitchell & *,[1]C. Robin Hiley

[1]Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1PD and [2]Unit of Critical Care Medicine, Imperial College School of Medicine, National Heart and Lung Institute, Dovehouse Street, London SW3 6LY

1 Vasorelaxation and hyperpolarization of endothelial cells by adenosine 5′-[β-thio]diphosphate (ADPβS) and adenosine 5′-[γ-thio]triphosphate (ATPγS) were studied in rat-isolated mesenteric artery. Effects from stimulation of P2X receptors were avoided by desensitization with α,β-methylene adenosine triphosphate.

2 ADPβS caused concentration- and endothelium-dependent relaxations of methoxamine-precontracted small (third generation) and main mesenteric artery. These were inhibited by N^{ω}-nitro-L-arginine methyl ester (L-NAME) or a combination of apamin plus charybdotoxin (inhibitors of Ca^{2+}-activated K^+ channels); L-NAME, apamin and charybdotoxin applied together abolished the response.

3 ATPγS induced limited relaxation (35% of methoxamine-induced tone at 10 μM) of small mesenteric artery, which was sensitive to L-NAME or endothelium denudation. However, it almost completely relaxed the main mesenteric artery over an extended concentration range (>6 orders of magnitude) in an endothelium-dependent manner. This relaxation was inhibited by either L-NAME or a combination of apamin with charybdotoxin, and abolished by a combination of all the three inhibitors.

4 The $P2Y_1$ receptor antagonist MRS 2179 (2′-deoxy-N^6-methyladenosine 3′,5′-bisphosphate; 0.3–3 μM) caused parallel rightward shifts of the concentration/relaxation curve to ADPβS ($pA_2 = 7.1$). However, MRS 2179 did not inhibit, but potentiated, relaxant responses to ATPγS. MRS 2179 did not affect the contractile responses ATPγS in small mesenteric artery; ATPγS did not contract the main mesenteric artery.

5 ADPβS hyperpolarized the endothelium of the main mesenteric artery in a concentration-dependent manner. This was unaffected by L-NAME but antagonized by MRS 2179. ATPγS also hyperpolarized the mesenteric artery endothelium in a concentration-dependent manner but, when ATPγS was applied at 10 μM, its effect was potentiated by MRS 2179 (3 μM).

6 It is concluded that both relaxation and hyperpolarization to ADPβS are mediated by $P2Y_1$ receptors and that the endothelial hyperpolarization is related to the L-NAME-resistant relaxation. Relaxation to the $P2Y_2$ agonist ATPγS shows regional variation along the mesenteric vasculature. The mechanisms for potentiation of relaxation and hyperpolarization by ATPγS are unknown, but may indicate interactions between P2Y receptor subtypes.
British Journal of Pharmacology (2003) **139,** 661–671. doi:10.1038/sj.bjp.0705271

Keywords: P2Y receptors; endothelium; rat mesenteric artery; EDHF; K^+ channels; MRS 2179

Abbreviations: ADPβS, adenosine 5′-[β-thio]diphosphate; ATP, adenosine 5′-triphosphate; ATPγS, adenosine 5′-[γ-thio]triphosphate; BK_{Ca}, large-conductance Ca^{2+}-activated K^+ channels; EDHF, endothelium-derived hyperpolarizing factor; K_{Ca}, Ca^{2+}-activated K^+ channels; L-NAME, N^{ω}-nitro-L-arginine methyl ester; MRS 2179, 2′-deoxy-N^6-methyladenosine 3′,5′-bisphosphate tetraammonium salt; $pEC_{50\%}$, negative logarithm of the concentration causing 50% relaxation of induced tone; SK_{Ca}, small-conductance Ca^{2+}-activated K^+ channels; V_{max}, calculated maximum change in membrane potential determined by curve fitting

Introduction

P2 receptors on vascular smooth muscle and endothelial cells throughout the cardiovascular system play an important role in the regulation of vascular tone (Burnstock, 1990; Olsson & Pearson, 1990; Ralevic & Burnstock, 1998; Malmsjö *et al.*, 1999; 2000a, b; Ralevic, 2001; Stanford *et al.*, 2001). The endogenous ligands for these receptors are nucleotides, including adenosine 5′-triphosphate (ATP), released from sympathetic nerves, platelets, damaged cells and endothelial cells (Goez *et al.*, 1971; Gordon, 1986; Burnstock, 1989).

In vascular smooth muscle the predominant receptor for ATP is the $P2X_1$ subtype (Vulchanova *et al.*, 1996) that, once activated, typically causes rapid and robust vasoconstriction

*Author for correspondence: Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1PD, U.K.; E-mail: crh1@cam.ac.uk

that is transient in nature (Ralevic & Burnstock, 1991; Evans & Kennedy, 1994). The $P2X_1$ receptor is a rapidly desensitizing, cation-selective ligand-gated ion channel (Kasakov & Burnstock, 1982; North & Surprenant, 2000) at which α,β-methyleneadenosine 5′-triphosphate (α,β-methylene-ATP) is a potent agonist. On the other hand, endothelial cells lining the lumen of arteries possess G-protein-coupled P2Y receptors that can mediate vasorelaxant responses via endothelium-dependent factors (Malmsjö *et al*., 1998; Ralevic & Burnstock, 1998). Among the subtypes of the P2Y receptor, $P2Y_1$ and $P2Y_2$ receptors are known to be present on endothelial cells, while $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors are expressed on vascular smooth muscle cells and mediate contraction (von Kugelgen & Starke, 1990; Ralevic & Burnstock, 1998; Buvinic *et al*., 2002). The development of stable purine and pyrimidine drugs now allows discrimination between P2Y receptor subtypes and elimination of the possible breakdown into alternative P2Y or adenosine receptor ligands (Goody *et al*., 1972; Malmsjö *et al*., 2000a). Thus, the $P2Y_1$ receptor can be selectively activated by adenosine 5′-[β-thio] diphosphate (ADPβS), while adenosine 5′-[γ-thio]triphosphate (ATPγS) is known to activate the $P2Y_2$ receptor; however, neither ADPβS nor ATPγS are specific to these receptors and both can act at other P2Y and P2X receptors (Ralevic & Burnstock, 1998; Bianchi *et al*., 1999). Nevertheless, in the absence of selective antagonists, these compounds have allowed considerable advances in the study of endogenous P2 receptors that were previously not possible with the nonselective purines and pyrimidines.

Evidence suggests that P2Y receptor-mediated arterial relaxation is endothelium dependent and involves nitric oxide, cyclooxygenase products and endothelium-derived hyperpolarizing factor (EDHF) (Malmsjö *et al*., 1998; Ralevic & Burnstock, 1998; Malmsjö *et al*., 1999; Stanford *et a*l., 2001). Upon activation, EDHF hyperpolarizes vascular smooth muscle resulting in vasorelaxation, but the exact nature of the EDHF-mediated phenomenon is not yet resolved (see Busse *et al*., 2002 for review). Hypotheses as to the identity of EDHF include: K^+-ion-induced hyperpolarization and the 'K^+ cloud' theory (Edwards *et al*., 1998; 1999b; Richards *et al*., 2001), gap-junctional communication between endothelial and smooth muscle cells involving the electrotonic spread of current (Chaytor *et al*., 2001; Coleman *et al*., 2001) and epoxyeicosatrienoic acids acting as EDHF (Hecker *et al*., 1994; Campbell *et al*., 1996; Quilley & McGiff, 2000). Nevertheless, a combination of apamin (an inhibitor of small-conductance Ca^{2+}-activated K^+ channels [SK_{Ca}]) and charybdotoxin (an inhibitor of large-conductance [BK_{Ca}] and intermediate-conductance [IK_{Ca}]Ca^{2+}-activated K^+ channels as well as voltage-sensitive K^+ channels [K_v]) can abolish EDHF-mediated responses (Corriu *et al*., 1996; Zygmunt & Högestätt, 1996; Chen & Cheung, 1997), and recent evidence suggests that P2Y-mediated vasorelaxation has an EDHF component (Malmsjö *et al*., 1998; Stanford *et al*., 2001). Malmsjö *et al.* (1998) have shown that inhibition of nitric oxide synthase or EDHF can partially attenuate $P2Y_1$- and $P2Y_2$-mediated relaxation of rat mesenteric artery, but relaxation can be abolished with concurrent inhibition of these components.

A recent publication provided the first molecular evidence that the rat arterial mesenteric bed is endowed with both $P2Y_1$ and $P2Y_2$ receptors on the endothelium (Buvinic *et al*., 2002). It was shown that the activation of these endothelial P2Y receptors caused relaxation through a surge of nitric oxide and a subsequent rise in vascular smooth muscle cyclic GMP levels. The present study provides evidence for endothelial P2Y receptors mediating not only nitric oxide-independent relaxation of rat mesenteric artery, as shown by Malmsjö *et al.* (1998) and Stanford *et al*. (2001), but also hyperpolarization of mesenteric artery endothelial cells. The role of Ca^{2+}-activated K^+ channels (K_{Ca}) in the hyperpolarizing response is also investigated.

Methods

Male Wistar rats (300 – 375 g; Tucks, Rayleigh, Essex, U.K.) were killed with an overdose of sodium pentobarbitone (120 mg kg^{-1}, i.p., Sagatal, Rhone Mérieux, Harlow, Essex, U.K.). The entire gastrointestinal tract was located, removed and placed in ice-cold Krebs – Henseleit solution of the following composition (mM): NaCl, 118; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25; $CaCl_2$, 2.5; D-glucose, 10, and gassed with 95% O_2/5% CO_2. The mesentery was pinned flat on a dissecting dish containing Krebs – Henseleit solution to allow arteries to be cleaned of fat and connective tissue, and then removed to fresh Krebs – Henseleit solution. In this study, third-order branches of mesenteric artery had a mean internal diameter of 0.34 ± 0.04 mm ($n = 61$) and are designated 'small mesenteric artery'. The segment of the mesenteric artery originating from the aorta had a mean internal diameter of 1.24 ± 0.09 mm ($n = 52$) and is referred to as the 'main mesenteric artery'.

Myograph tension recordings

Using tungsten wire, segments approximately 2 mm in length of main or small mesenteric artery were dissected and mounted in a Mulvany–Halpern myograph (Model 500A, Danish Myo Technology, Aarhus, Denmark) as previously described by White & Hiley (1998a). Vessels were allowed to equilibrate at 37°C for 15 min in gassed (95% O_2/5% CO_2) Krebs – Henseleit solution containing indomethacin (10 μM). Following equilibration, vessels were normalized to a tension equivalent to that generated at 90% of the diameter of the vessel at 100 mmHg (Mulvany & Halpern, 1977).

After normalization, endothelial integrity was demonstrated by contracting vessels with methoxamine (10 μM) and adding carbachol (10 μM); a relaxation of $>90\%$ was taken as being indicative of an intact endothelium. Where endothelium was not required, a human hair was carefully rubbed against the intima of vessels to remove endothelial cells. Relaxation of $<10\%$ was taken as being indicative of endothelium denudation.

Cumulative concentration/response curves

The relaxant effects of ADPβS and ATPγS were investigated by adding them cumulatively, in half-log unit increments of concentration, to vessels precontracted with methoxamine (10 μM). Preliminary experiments showed that inclusion of indomethacin (10 μM) in the bathing fluid had no significant effects on the relaxant responses to ADPβS or ATPγS in either the main or small mesenteric artery (data not shown). In experiments using N^{ω}-nitro-L-arginine methyl ester (L-NAME), the concentration of methoxamine used to

precontract arteries was reduced (10 – 5 μM) in order to obtain the same amount of tension as in the absence of nitric oxide synthase inhibition. As contractile P2X receptors on vascular smooth muscle cells would counteract the vasodilator response generated via the P2Y receptor (Ralevic & Burnstock, 1998; Malmsjö *et al*., 2000b), P2X receptors were desensitized by 20 min preincubation with α,β-methylene-ATP (10 μM) for all relaxant studies (Kasakov & Burnstock, 1982; Malmsjö *et al*., 1999; 2000b). Studies were carried out either alone or in the presence of L-NAME (300 μM), apamin (50 nM), charybdotoxin (50 nM), MRS 2179 (0.3, 1 and 3 μM) or combinations of these inhibitors. All inhibitors were added 30 min before, and were present throughout, the determination of a concentration/response curve for relaxant drugs.

In order to assess the effects of the $P2Y_2$ receptor antagonist 2′-deoxy-N^6-methyladenosine 3′,5′-bisphosphate (MRS 2179) on the contractile responses to ATPγS, intact vessels without endothelium were mounted as described above. Then, after testing for the absence of the endothelium and washing the vessels, ATPγS was added in a cumulative fashion over a concentration range of 10 nM–30 μM. The effects of MRS 2179 were assessed by adding the antagonist to the bath 30 min before construction of the concentration/response curve for ATPγS.

Endothelial cell membrane potential recordings

The effect of P2Y receptor agonists on the membrane potential of rat mesenteric artery endothelial cells was investigated by a method previously described by White & Hiley (1998b). Briefly, 5 mm long segments of the main mesenteric artery were cut and transferred to a 0.5 ml organ bath for electrophysiological experiments. The segment of artery was cut open longitudinally and pinned to the silicone rubber base of the organ bath with the intimal surface exposed. The preparation was superfused continually with Krebs – Henseleit solution at room temperature.

Sharp electrodes were pulled from borosilicate glass capillaries (GC120F-10, Harvard Apparatus, Edenbridge, Kent, U.K.) using a Flaming – Brown-type horizontal puller (P-87, Sutter Instruments, San Rafael, CA, U.S.A.) in order to produce electrodes with a long taper (tip resistance 50 – 80 MΩ when filled with 3 M KCl). The electrodes were connected to the headstage of an Axoclamp-2B amplifier (Axon Instruments, Foster City, CA, U.S.A.) and electrical responses at the tip of the electrode were monitored by injecting current pulses (0.1 nA, duration 40 ms) and monitoring changes in measured potential on an oscilloscope (Model 3091; Nicolet, Madison, WI, U.S.A.). The electrode, viewed under a light microscope, was then advanced slowly towards the intimal surface of the arterial segment at an angle of approximately 40° from the horizontal using a Huxley-type manipulator (Sutter Instruments, San Rafael, CA, U.S.A.). Successful impalements were seen as a sudden change in voltage to a stable, negative level, and cells were usually left for 5 min after impalement before the addition of drugs.

Upon removal of the electrode from an impaled cell, whether manually or by loss of the impalement, the electrode resistance was examined in order to ensure that any changes were not caused by blocking of the electrode tip during recording. In all electrophysiological experiments, drugs were added to the organ bath by addition to the reservoir of the superfusing solution. In experiments investigating the effect of inhibitors on responses to hyperpolarizing agents, these inhibitors were added for 5 min before the addition of carbachol, ADPβS or ATPγS, and were present throughout determination of the response. As with relaxation data, all preparations were exposed to α,β-methylene-ATP (10 μM) for 20 min to desensitize P2X receptors before the addition of ADPβS or ATPγS. Tachyphylaxis of the response to ADPβS and ATPγS was observed in both myograph and electrophysiological experiments. Therefore, in all experiments, only one cumulative concentration/response curve was determined in any single preparation.

Data and statistical analysis

Relaxation responses in myograph experiments are expressed as percentage relaxation of the tone induced by methoxamine. Data are given as the mean ± s.e.m. $pEC_{50\%}$ values for individual concentration/response curves were determined as the negative logarithm of the concentration of agent required to give 50% relaxation of the tone induced by methoxamine. Statistical comparison of concentration/response curves was by two-way analysis of variance followed by a Bonferroni/Dunn *post hoc* test using StatView 4.5 (Abacus Concepts, Berkeley, CA, U.S.A.) running on a Macintosh personal computer.

Electrophysiological data are presented as an absolute change in membrane potential. pEC_{50} values were obtained from the mean responses by fitting the data to the logistic equation:

$$V = \frac{V_{max} \cdot (10^A)^{n_H}}{(10^{pEC_{50}})^{n_H} + (10^A)^{n_H}}$$

where V is the change in membrane potential, A the negative logarithm of the concentration of agonist, V_{max} the maximum response, n_H the slope function and pEC_{50} the negative logarithm of the concentration of agonist giving half the maximal response (White & Hiley, 1998a). The curve fitting was carried out using KaleidaGraph (Synergy Software, Reading, PA, U.S.A.) running on a Macintosh computer. Curve-fitting parameters were compared by unpaired t-test, and P-values less than 0.05 were considered to be statistically significant.

Drugs

MRS 2179 (Tocris Cookson Ltd, Avonmouth, U.K.) was dissolved in distilled water. Methoxamine, carbachol, α,β-methylene-ATP, ADPβS, ATPγS, L-NAME, apamin and charybdotoxin (all from Sigma Chemical Co., Gillingham, Dorset, U.K.) were dissolved in distilled water. Indomethacin (Sigma) was dissolved in 5% (w v^{-1}) $NaHCO_3$ solution.

Results

Effect of L-NAME and K^+-channel inhibitors on ADPβS-induced relaxation

Figure 1 shows that ADPβS caused relaxation of the rat precontracted small mesenteric artery in a concentration-dependent manner ($pEC_{50\%} = 6.56 \pm 0.10$, $n = 6$). The response

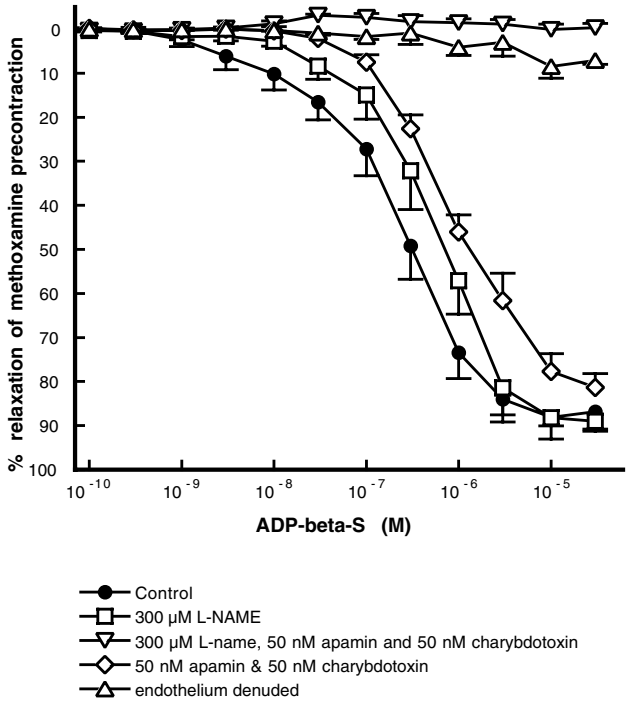

Figure 1 Concentration – response curves showing the effects of endothelium removal, L-NAME (300 μM) and the K^+-channel blockers, apamin and charybdotoxin (both 50 nM), on relaxations of rat intact small mesenteric arteries induced by ADPβS. Values are shown as mean and vertical lines indicate s.e.m. Control + endothelium, $n=6$; with L-NAME, $n=6$; with apamin and charybdotoxin, $n=4$; with L-NAME, apamin and charybdotoxin, $n=4$; after endothelium removal, $n=4$.

was abolished following endothelial cell removal (Figure 1). In contrast, inhibition of nitric oxide synthase by 300 μM L-NAME significantly reduced the potency of ADPβS, but did not affect the maximum response ($pEC_{50\%}=6.16\pm0.04$, $n=6$). A combination of apamin and charybdotoxin (both 50 nM) also significantly reduced the potency of ADPβS at inducing relaxation ($pEC_{50\%}=5.96\pm0.06$, $n=4$). Furthermore, when coadministered with L-NAME (300 μM), the relaxant response was abolished ($n=4$).

The main mesenteric artery, when precontracted with methoxamine, was also relaxed by ADPβS in a concentration-dependent manner (Table 1). Pretreatment with L-NAME (300 μM) again inhibited, but did not abolish the relaxation in intact vessels, but removal of endothelial cells eliminated the response. L-NAME (300 μM), apamin (50 nM) and charybdotoxin (50 nM) in combination abolished ADPβS-induced relaxation of the main mesenteric artery.

Effect of L-NAME and K^+-channel inhibitors on ATPγS-induced relaxation

ATPγS gave only a modest concentration-dependent relaxation of precontracted small mesenteric arteries from the rat. The relaxation at the highest concentration that could be used (30 μM) was small ($35\pm10\%$, $n=6$), compared to the maximum given by ADPβS. L-NAME (300 μM) significantly inhibited the relaxation induced by ATPγS at 30 μM ($10\pm4\%$, $n=4$), and mechanical removal of endothelial cells abolished the relaxant response (Figure 2a).

Table 1 Effects of endothelium removal, MRS 2179, L-NAME and a combination of apamin and charybdotoxin on the relaxation induced by ADPβS in methoxamine-precontracted isolated main mesenteric arteries of the rat

Treatment	$pEC_{50\%}$	*n*
Control	6.29 ± 0.31	6
+ 300 μM L-NAME	6.09 ± 0.23	4
+ 50 nM apamin and 50 nM charybdotoxin	5.13 ± 0.21**	4
+ 300 μM L-NAME, 50 nM apamin and 50 nM charybdotoxin	n.d.	4
endothelium denuded	n.d.	4
+ 0.3 μM MRS 2179	6.08 ± 0.13	4
+ 3 μM MRS 2179	5.68 ± 0.09*	4

Values are shown as mean $\pm$ s.e.m., with *n* indicating the number of animals. Mean internal diameter of arteries was 1.23 ± 0.10, $n=30$. Significant difference from control measurements denoted by *$P<0.05$, **$P<0.01$, n.d. = not determinable.

In contrast, in the main mesenteric artery contracted with methoxamine, ATPγS caused a significantly ($P<0.01$) greater relaxation (response at 30 μM = $82\pm5\%$, $n=8$) than that observed in smaller arteries. Figure 2b shows that the response occurs over a very wide concentration range (over 6 log units of concentration) and it can be seen that the concentration/response curve had a biphasic appearance; unfortunately, it was not possible to analyse the shape of the curve in detail using curve fitting as the response did not reach a clearly defined maximum. Figure 2b also shows that removal of the endothelium abolished the response. Pretreatment of the main artery with L-NAME (300 μM) caused a large rightward shift in the response to ATPγS ($pEC_{50\%}$: control = 6.92 ± 0.21, $n=8$; L-NAME = 4.46 ± 0.27, $n=6$), and a combination of apamin and charybdotoxin (both 50 nM) also significantly attenuated the response ($pEC_{50\%}=5.55\pm0.15$, $n=4$). The relaxation to ATPγS was abolished when L-NAME (300 μM) was coadministered with the toxins.

Effect of MRS 2179 on arterial relaxation to ADPβS and ATPγS

In the methoxamine-precontracted small mesenteric artery, the relaxation to ADPβS (control: $pEC_{50\%}=6.68\pm0.12$, $n=6$) was significantly attenuated in a concentration-dependent manner by pretreatment with MRS 2179 (0.3 μM: $pEC_{50\%}=6.16\pm0.08$, $n=4$; 1 μM: $pEC_{50\%}=5.96\pm0.07$, $n=4$; 3 μM: $pEC_{50\%}=5.69\pm0.04$, $n=4$); there were no significant changes in the maximum observed response. Figure 3 shows that sequential increases in MRS 2179 concentration produced progressively larger rightward shifts of the ADPβS concentration/response curve. Schild analysis gave a pA_2 value of 7.1, and a slope of 0.71 ± 0.09. Inhibition of ADPβS vasorelaxation by MRS 2179 was also observed in the main mesenteric artery (Table 1).

Figure 4 shows that the ATPγS-induced relaxation of methoxamine-precontracted main mesenteric artery in the presence and absence of L-NAME (300 μM) was not inhibited by the additional presence of MRS 2179 (3 μM). In fact, analysis of variance reveals that MRS 2179 significantly ($P<0.01$) increased the relaxant potency of ATPγS in both the presence and absence of L-NAME.

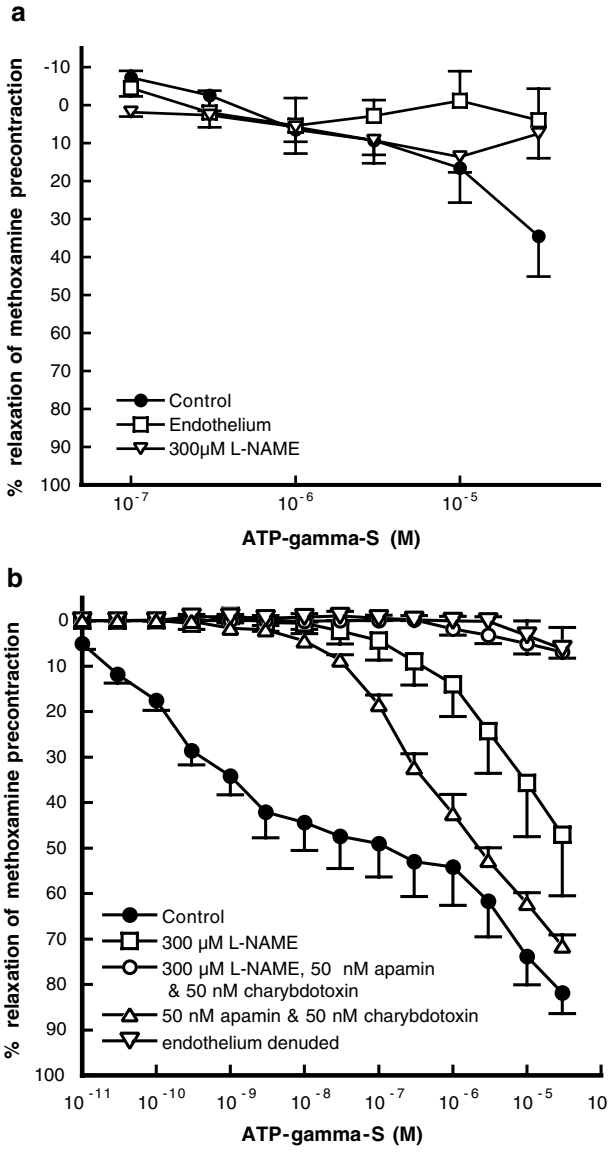

Figure 2 (a) Concentration/response curves showing the effects of endothelium removal and L-NAME (300 μM) on relaxations of rat intact small mesenteric arteries induced by ATPγS. Values are shown as mean and vertical lines indicate s.e.m., $n=6$, for all experiments. (b) Concentration/response curves showing the effects of endothelium removal, L-NAME (300 μM) and the K^+-channel blockers, apamin and charybdotoxin (both 50 nM), on relaxations of rat intact main mesenteric arteries induced by ATPγS. Values are shown as mean and vertical lines indicate s.e.m. Control + endothelium, $n=8$; with L-NAME, $n=6$; with apamin and charybdotoxin, $n=4$; with L-NAME, apamin and charybdotoxin, $n=4$; after endothelium removal, $n=4$.

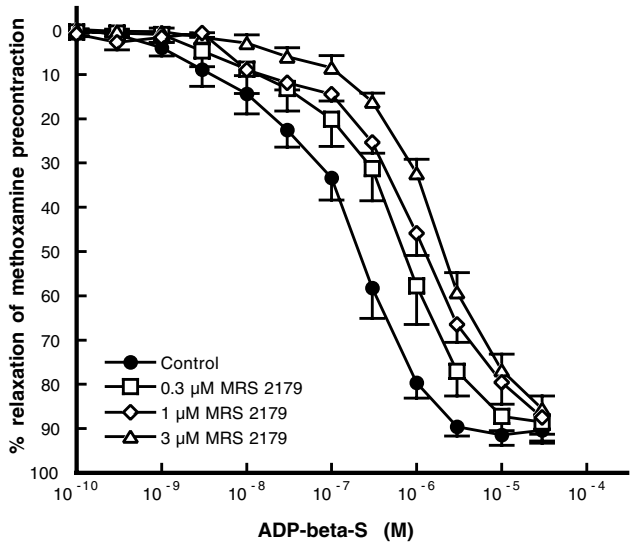

Figure 3 Effects of the $P2Y_1$ -receptor antagonist, MRS 2179 (0.3, 1 and 3 μM), on ADPβS-induced relaxation of rat intact small mesenteric arteries. Values are shown as mean and vertical lines indicate s.e.m., $n=4$, for all experiments except controls where $n=6$.

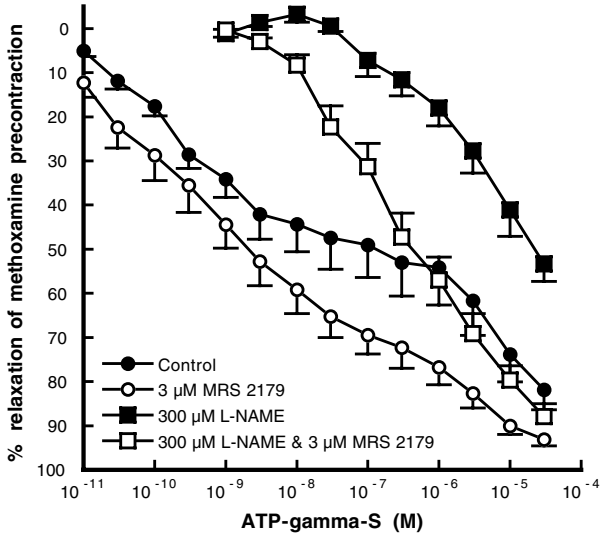

Figure 4 Concentration/response curves showing the effects of 3 μM MRS 2179 on the ATPγS-induced relaxation of main mesenteric artery, in the presence and absence of 300 μM L-NAME. Values are shown as mean and vertical lines indicate s.e.m., $n=6$, for all experiments.

Effect of MRS 2179 on ATPγS-induced contraction of mesenteric arteries

Application of ATPγS contracted quiescent small mesenteric artery following removal of endothelial cells, with a threshold concentration of 1 μM. The mean responses at 1, 3, 10 and 30 μM, the highest concentration of ATPγS that could be achieved, were 1 ± 0, 7 ± 3, 36 ± 2 and 55 ± 3%, respectively, of the contraction to 10 μM methoxamine ($n=4$ for each). MRS 2179 (3 μM) had no significant effect on the ATPγS-induced contraction of small mesenteric arteries (in the presence of MRS 2179, the responses to 1, 3, 10 and 30 μM ATPγS were 2 ± 1, 12 ± 3, 30 ± 2% and 59 ± 7% of the response to 10 μM methoxamine; $n=4$). ATPγS did not contract the quiescent main mesenteric artery.

Effect of L-NAME on ADPβS and ATPγS hyperpolarization of endothelial cells

Using sharp electrode impalement of the main mesenteric artery endothelial cells *in situ*, the mean resting membrane potential was found to be -53.6 ± 0.1 mV ($n=186$). ADPβS (10 μM) produced rapid but transient hyperpolarization of the

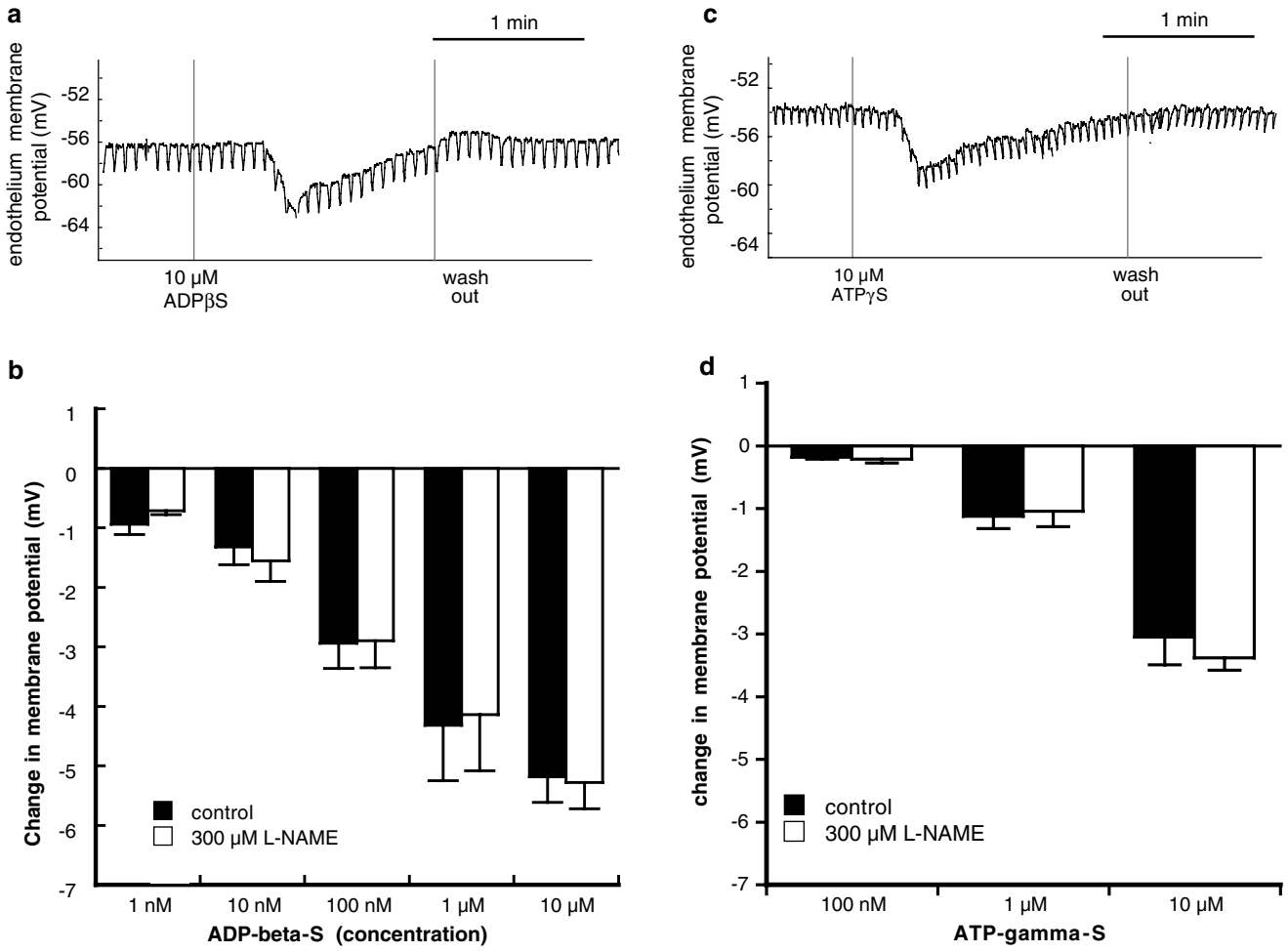

Figure 5 Original records showing hyperpolarization of rat mesenteric endothelial cells by (a) ADPβS (10 μM) and (c) ATPγS (10 μM), elicited in the presence of 300 μM L-NAME. Horizontal bars denote the time course of drug additions at the concentrations indicated. The vertical deflections on the recording are caused by current injections through the electrode (0.1 nA). Concentration/response data for the hyperpolarization effects of ADPβS and ATPγS in the absence and presence of L-NAME (300 μM) are shown in (b) and (d) respectively. Values are shown as the mean hyperpolarization response and vertical lines indicate s.e.m., $n = 4 - 6$.

cells ($V_{max} = 5.1 \pm 0.4$ mV, $n = 6$) and a typical response is shown in Figure 5a. The hyperpolarization response to ADPβS was concentration-dependent and unaffected by pretreatment with L-NAME (300 μM), as shown in Figure 5b. Fitting the mean concentration – response data of ADPβS-induced hyperpolarization in the presence of L-NAME to a logistic function yielded a pEC_{50} value of 6.35 ± 0.26 and $V_{max} = 5.2 \pm 0.4$ mV ($n = 4 - 6$).

ATPγS also hyperpolarized the endothelium of the main mesenteric artery (Figure 5c), but responses were only clearly distinguishable at concentrations of 1 μM and above. Figure 5d shows the response to ATPγS to be both concentration-dependent and unaffected by the presence of L-NAME (300 μM). ATPγS-induced hyperpolarization data in the presence of L-NAME were fitted to a logistic function and generated a pEC_{50} of 5.64 ± 0.21 and $V_{max} = 3.4 \pm 0.2$ mV ($n = 4 - 6$).

Effect of apamin and charybdotoxin on endothelial cell hyperpolarization to ADPβS and ATPγS

The maximum endothelial cell hyperpolarization achieved by ADPβS (10 μM) in the presence of L-NAME (control: $V_{max} = 5.3 \pm 0.5$ mV, $n = 4$) was significantly ($P < 0.001$) reduced when apamin and charybdotoxin were also present ($V_{max} = 0.9 \pm 0.1$ mV, $n = 7$). Similarly, apamin and charybdotoxin significantly ($P < 0.001$) attenuated the maximal endothelial cell hyperpolarization induced by ATPγS in the presence of L-NAME from a control response of 3.4 ± 0.2 mV ($n = 5$) to 0.9 ± 0.1 mV ($n = 6$).

Effect of MRS 2179 on endothelial cell hyperpolarization to ADPβS and ATPγS

MRS 2179 inhibited hyperpolarization of main mesenteric artery endothelial cells by ADPβS, while hyperpolarization to a submaximal concentration of carbachol was unaffected (Figure 6a). It can be seen from Figure 6a that MRS 2179 significantly attenuated the ADPβS-induced endothelial cell hyperpolarization in a concentration-dependent manner.

Figure 6b shows that MRS 2179 does not inhibit the hyperpolarization caused by ATPγS in endothelial cells from the main mesenteric artery. In fact, the presence of MRS 2179 (3 μM) significantly ($P < 0.05$) increased the maximum hyperpolarization achieved by ATPγS (10 μM).

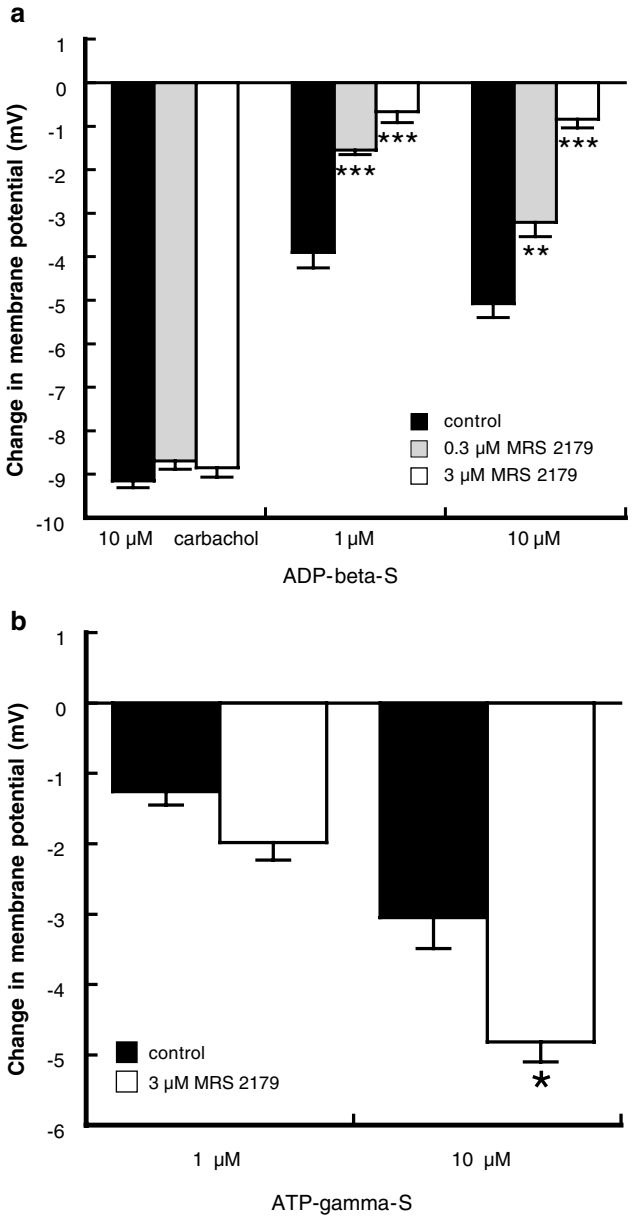

Figure 6 (a) The effects of the $P2Y_1$-receptor antagonist, MRS 2179 (0.3 and 3 μM), on the magnitude of endothelial hyperpolarization evoked by carbachol (10 μM) and ADPβS (1 and 10 μM) in the presence of 300 μM L-NAME. Values are shown as mean and vertical lines indicate s.e.m., $n=4-6$. $^{**}P<0.01$ and $^{***}P<0.001$ indicate statistically significant differences from the control hyperpolarization in the absence of MRS 2179, as determined by Student's unpaired *t*-test. (b) The effects of 3 μM MRS 2179 on the magnitude of endothelial hyperpolarization caused by ATPγS (1 and 10 μM) in the presence of 300 μM L-NAME. Values are shown as mean and vertical lines indicate s.e.m., $n=5-6$. $^{*}P<0.05$ indicates statistically significant difference from the control hyperpolarization in the absence of MRS 2179, as determined by Student's unpaired *t*-test.

Discussion

The results presented in this study show that both ADPβS (a $P2Y_1$-selective receptor agonist; Goody *et al*., 1972; Malmsjö *et al*., 1999) and ATPγS (a $P2Y_2$ receptor agonist; Goody *et al*., 1972; Malmsjö *et al*., 2000a) cause vasorelaxation of precontracted mesenteric artery and concentration-dependent hyperpolarization of vascular endothelial cells *in situ*. Interestingly, ATPγS had a relatively weak vasorelaxant effect in the small mesenteric artery, as compared to the main vessel, which suggests a regional variability in the distribution of P2Y receptors (specifically $P2Y_2$ receptors) within the mesenteric circulation. The antagonist MRS 2179 could only inhibit responses to ADPβS, confirming its selectivity for $P2Y_1$ receptors.

The development of stable ADP and ATP analogues has allowed a more confident identification of P2 receptor subtypes without the problems of agonist breakdown products activating additional receptors. In this study, ADPβS was used as an agonist for the $P2Y_1$ receptor as previous studies have shown it to be highly potent and selective for this receptor subtype in the rat mesenteric vascular system (Malmsjö *et al*., 1999; 2000c). Although ADPβS has some activity at $P2X_1$, $P2X_3$ and $P2X_4$ receptors (Bianchi *et al*., 1999) it was preferred here as being somewhat more selective than 2-methylthioadenosine triphosphate. The latter is a widely used $P2Y_1$ receptor agonist, but it has been reported to have activity at $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$ and several P2X receptors (Ralevic & Burnstock, 1998; Bianchi *et al*., 1999). ATPγS was chosen as the $P2Y_2$ receptor agonist since it has been reported to have good potency and selectivity at this receptor (Malmsjö *et al*., 2000a), although $P2Y_4$, $P2Y_{11}$ and $P2X_{1-4}$ receptors can also be activated by higher concentrations (Ralevic & Burnstock, 1998; Bianchi *et al*., 1999). Currently, there are no more highly selective $P2Y_2$ receptor agonists available, with compounds such as UDP and UTP also affecting the $P2Y_4$, and occasionally the $P2Y_6$, receptors (Ralevic & Burnstock, 1998; Malmsjö *et al*., 2000a) and their use is complicated by their breakdown by ectonucleotidases in the tissues being studied.

Until more highly selective $P2Y_2$ receptor compounds are developed, ATPγS represents one of the best tools available for study of this receptor. In the context of the present study, the activity of ATPγS at $P2X_4$ receptors could be thought to be a problem as Yamamoto *et al*. (2000) have reported functional $P2X_4$ receptors on cultured human endothelial cells. However, there have been no reports of expression of this subtype on rat mesenteric endothelium and, even though Lewis & Evans (2000) found immunohistochemical staining of $P2X_1$, $P2X_4$, $P2X_5$ and $P2X_7$ receptors in the smooth muscle layer of rat small mesenteric artery, none was found in the endothelial cell layer. It therefore seems unlikely that the endothelium-dependent responses to ATPγS reported here are mediated by these receptors.

ADPβS and ATPγS both caused concentration-dependent relaxation of the isolated precontracted mesenteric artery of the rat that was entirely dependent on a functional endothelium. With the $P2Y_1$ receptor agonist, ADPβS, the presence of L-NAME shifted the concentration/response curve to the right revealing a role for nitric oxide; however, the response was not abolished suggesting the existence of another relaxant mechanism. A combination of known K_{Ca} inhibitors, namely apamin (an SK_{Ca} blocker; Adeagbo & Triggle, 1993; Doughty *et al*., 1999) and charybdotoxin (a blocker of IK_{Ca}, BK_{Ca} and K_v; Schweitz *et al*., 1989; Tauc *et al*., 1993; Vogalis *et al*., 1998), can abolish EDHF-mediated relaxation (Corriu *et al*., 1996; Zygmunt & Högestätt, 1996; Chen & Cheung, 1997; White & Hiley, 1997). This toxin combination inhibited the relaxation to ADPβS and when administered together

with L-NAME, the relaxation was abolished, suggesting a role for K_{Ca} as well as nitric oxide in this response. These results confirm previous findings that endothelial $P2Y_1$ receptor activation results in K_{Ca}-dependent relaxation of rat mesenteric artery (Malmsjö *et al*., 1998; Malmsjö *et al*., 1999).

Several vasoactive substances have shown either regional variability (Bigaud & Pelton, 1992; Zygmunt *et al*., 1995) or changes dependent on vessel diameter (Shimokawa *et al*., 1996; Gitterman & Evans, 2000). Interestingly, in the present study the $P2Y_2$ receptor agonist, ATPγS, had a considerably greater relaxant activity in the large diameter main mesenteric artery than in the small, third-generation, mesenteric artery. In addition, the modest relaxation achieved by ATPγS in the small artery was abolished solely by nitric oxide synthase inhibition, suggesting the absence, or relative insignificance, of a K_{Ca}-mediated response (generally ascribed to EDHF) under these circumstances. This is unusual as the importance of EDHF versus nitric oxide generally increases as the vessel diameter is reduced (Shimokawa *et al*., 1996). Although $P2Y_2$ receptors are known to be present on endothelial cells within the mesenteric circulation (Buvinic *et al*., 2002), it is possible that endothelial $P2Y_2$ receptors are not universally expressed within this system and that the small mesenteric artery is devoid of, or possesses fewer, $P2Y_2$ receptors. If this is the case, the relaxation observed to ATPγS in the small artery may be mediated by $P2Y_4$ receptors which are known to be active in the mesenteric circulation (Malmsjö *et al*., 2000a; Kaiser & Buxton, 2002). However, recent evidence suggests that endothelial cells lack $P2Y_4$ receptors (Buvinic *et al*., 2002) therefore arguing against this hypothesis. Alternatively, contractile $P2Y_2$ receptors on vascular smooth muscle (von Kugelgen & Starke, 1990; Erlinge *et al*., 1998; Ralevic & Burnstock, 1998; Malmsjö *et al*., 2000a) may oppose the endothelium-dependent relaxation in small arteries. Indeed, small mesenteric arteries display a contractile response to both of the $P2Y_2$ receptor agonists ATPγS (in the present study) and UTP (Gitterman & Evans, 2000), while the main artery does not give a contractile response (this study). At high concentrations ATPγS can activate a number of receptors in addition to the $P2Y_2$ receptor and it may be another P2 receptor, such as the $P2Y_4$ subtype, that is responsible for the observed contractile activity, although P2X receptors are not likely to be involved as they were desensitized in our study. These suggestions could explain the differences in vasorelaxation to ATPγS found between the small and main mesenteric artery in the present study.

In the main mesenteric artery, ATPγS-induced vasorelaxation was sensitive to both nitric oxide synthase- and K^+-channel inhibitors alone, but the response could only be abolished when both systems were simultaneously inhibited. These results at the $P2Y_2$ receptor confirm the findings of Malmsjö *et al*. (1998; 1999) and are similar to those found with the $P2Y_1$ receptor agonist, ADPβS, in the present study. However, whereas ADPβS relaxed both small and main mesenteric arteries in a similar manner, ATPγS was relatively ineffective in small arteries. In the main artery, the relaxation to ATPγS is biphasic and occurs over an extended concentration range (0.01 nM – 10 μM) and this might be explained by the activation of two distinct receptor subtypes or signalling pathways. The vasorelaxant effect to ATPγS was entirely dependent on a functional endothelium and it is therefore likely to act via endothelial cell receptor activation, presumably involving intracellular calcium elevation. Reports of the presence of both $P2Y_2$ and $P2Y_4$ receptors on endothelial cells (Malmsjö *et al*., 1998; 2000a) may offer an explanation for the biphasic relaxant response. Thus, $P2Y_2$ receptors could be involved at lower concentrations but $P2Y_4$ at higher concentrations. However, recent evidence suggests that $P2Y_4$ receptor mRNA is not expressed in rat mesenteric endothelium (Buvinic *et al*., 2002). Although, mRNA detection studies are helpful in determining the P2 receptors that might be expressed on endothelial cells, species and regional variability (Erlinge *et al*., 1998; von Kugelgen & Wetter, 2000) often means it may be difficult to compare functional and molecular findings between studies. In addition, separation of endothelial cells from vascular smooth muscle cells must be accurately accomplished in order to determine the exact location of receptors, as many P2 receptors are present on both types of cell (Ralevic & Burnstock, 1998).

An important aspect of the present study is that it is the first to show the concentration-dependent hyperpolarization of rat mesenteric artery endothelial cells by $P2Y_1$ and $P2Y_2$ receptor agonists. Hyperpolarization induced by ADPβS or ATPγS was rapid in onset, but transient in nature as the membrane potential returned to baseline levels within approximately 60 s. The responses were similar in profile, but the hyperpolarization achieved by ADPβS was significantly larger than that produced by ATPγS over the concentration range used. The hyperpolarization was abolished by a combination of apamin and charybdotoxin, while the additional presence of L-NAME had no effect. Furthermore, nitric oxide did not contribute to this response since inhibition of nitric oxide synthase alone had no effect on the endothelial hyperpolarization to either ADPβS or ATPγS. The lack of effect of L-NAME could not be attributed to its failure to inhibit nitric oxide synthase completely since application of the nitric oxide donor, sodium nitroprusside, does not cause endothelial hyperpolarization (Mistry and Hiley, unpublished observations). The results therefore suggest that activation of G-protein-coupled $P2Y_1$ or $P2Y_2$ receptors on the endothelial cell membrane brings about the opening of apamin- and charybdotoxin-sensitive channels that cause hyperpolarization independent of nitric oxide.

In contrast to vasorelaxation studies, experiments investigating the concentration-dependent nature of endothelial hyperpolarization by ATPγS did not show biphasic characteristics and occurred at concentrations in the micromolar range. This suggests that vasorelaxation observed at lower concentrations of ATPγS is not associated with opening of K_{Ca} and that another mechanism, possibly nitric oxide, mediates the dilatation. This hypothesis is supported both by the steepening of the concentration – relaxation curve to ATPγS in the presence of L-NAME (presumably revealing the response mediated by K_{Ca} mechanisms) and by the fact that it lies to the right of that in the presence of apamin plus charybdotoxin (which is presumably caused by nitric oxide signalling). Interestingly, Marrelli (2001) showed that activation of endothelial $P2Y_1$ and $P2Y_2$ receptors produced differential intracellular Ca^{2+} mobilization and the threshold level of intracellular Ca^{2+} required to produce nitric oxide-dependent dilatation was significantly lower than that required for K_{Ca}-dependent dilation. Thus, nitric oxide-mediated responses may require fewer endothelial P2 receptors to be activated compared to K_{Ca}-mediated dilatation, which requires a higher intracellular Ca^{2+} concentration. Collectively, these observa-

tions indicate a greater importance of nitric oxide at lower agonist concentrations, with K_{Ca} being important at higher concentrations. This might explain the biphasic nature of ATPγS-induced relaxation of the main mesenteric artery.

Examination of both the ADPβS- and ATPγS-mediated responses in the presence of L-NAME reveals that endothelial cell hyperpolarization and L-NAME-resistant relaxation occur at a similar concentration range. Therefore, it is possible that the receptors or mechanisms involved in endothelial hyperpolarization are the same as those responsible for nitric oxide-independent arterial relaxation. In the presence of L-NAME, the pEC_{50} for ADPβS-induced hyperpolarization was 6.35 ± 0.26, which is in close agreement to that obtained with relaxant data from both small ($pEC_{50\%} = 6.15 \pm 0.04$) and large ($pEC_{50\%} = 6.09 \pm 0.23$) mesenteric artery in the presence of L-NAME. In the case of ATPγS-induced hyperpolarization, the correspondence is less precise as the pEC_{50} was 5.64 ± 0.21 in the presence of L-NAME as compared to a $pEC_{50\%}$ value of 4.46 ± 0.27 for relaxation of the main mesenteric artery in the presence of L-NAME. These results support a relation between endothelial cell hyperpolarization and L-NAME-resistant arterial relaxation, which might be associated with EDHF.

EDHF-induced vasorelaxation is associated with hyperpolarization of vascular smooth muscle cells (Malmsjö *et al*., 1999; Félétou & Vanhoutte, 2000). A functional endothelium is essential for the hyperpolarization response, since in its absence, ADPβS is unable to hyperpolarize vascular smooth muscle cells (Malmsjö *et al*., 1999). Interestingly, the hyperpolarization by ADPβS in both vascular smooth muscle (Malmsjö *et al*., 1999) and endothelial cells reported here is of a similar magnitude and time course. Thus, there may be transfer or electrotonic spread of hyperpolarization between endothelium and smooth muscle cells associated with the vascular response to ADPβS and ATPγS. Gap junctions are known to form between endothelium and smooth muscle (Chaytor *et al*., 1998; Dora, 2001), and communication between these gap junctions has been suggested to contribute to the EDHF response (Edwards *et al*., 1999a; Harris *et al*., 2000; Chaytor *et al*., 2001; Dora, 2001; Edwards & Weston, 2001; Xu *et al*., 2002). It could be argued that the endothelial hyperpolarization recorded here was caused by activation of $P2Y_1$ (by ADPβS) or $P2Y_2$ receptors (by ATPγS) on vascular smooth muscle cells that caused direct hyperpolarization of the smooth muscle, which was in turn conducted to the endothelium through gap junctions. This is unlikely as there is no evidence for $P2Y_1$ receptors on the vascular smooth muscle cells of the mesenteric artery (Buvinic *et al*., 2002), and any $P2Y_2$ receptors are known to cause arterial contraction (Gitterman & Evans, 2000). Most tellingly, hyperpolarization of the vascular smooth muscle would be expected to lead to vasorelaxation; however, P2Y-mediated relaxation was only possible when endothelial cells were present.

Alternative explanations linking hyperpolarization of endothelial cells to vascular relaxation state that endothelial cell hyperpolarization is caused by an efflux of K^+ ions (which in turn hyperpolarize smooth muscle cells by activating the inwardly rectifying K^+ conductance and the Na^+/K^+ pump) or that hyperpolarized endothelial cells release vasodilator epoxyeicosatrienoic acids (Félétou & Vanhoutte, 2000; Busse *et al*., 2002). The identity of EDHF, or the mechanism by which it causes vascular relaxation, is still unknown, and evidence suggests that there may be multiple mechanisms depending on the species and vascular region being investigated (Busse *et al*., 2002; Triggle & Ding, 2002).

Hitherto, the lack of selective P2 receptor agonists and antagonists has hindered the study of these receptors. However, MRS 2179 is a selective $P2Y_1$ receptor antagonist (Boyer *et al*., 1998), which inhibits $P2Y_1$ receptor-mediated vasorelaxant responses in the guinea-pig aorta (Kaiser & Buxton, 2002) and the rat perfused mesenteric bed (Buvinic *et al*., 2002). In the present study, MRS 2179 significantly inhibited ADPβS-mediated vasorelaxation and hyperpolarization of mesenteric artery endothelial cells in a concentration-dependent manner, suggesting $P2Y_1$ receptor involvement. Schild analysis of MRS 2179 antagonism of ADPβS relaxant responses in small arteries revealed a pA_2 value of 7.1, a value similar to that (6.99 ± 0.13) reported by Boyer *et al.* (1998) for MRS 2179 inhibition of 2-methylthioadenosine triphosphate responses at $P2Y_1$ receptors on turkey erythrocyte membranes. However, it should be noted that the compound is only about 11-fold selective for the $P2Y_1$ over the $P2X_1$ receptor (Brown *et a*l., 2000), but as we desensitized the latter receptor before carrying out our experiments with the antagonist, it is unlikely that antagonism of these receptors would contribute to the effects seen. MRS 2179 (3 μM) did not inhibit the concentration-dependent arterial relaxation or endothelial hyperpolarization to either carbachol or ATPγS, suggesting a specific inhibition of $P2Y_1$-mediated responses. Surprisingly, MRS 2179 potentiated the response of ATPγS in both relaxation and hyperpolarization studies, while no such augmentation was found when using carbachol (1 nM – 10 μM). Augmentation of the relaxant effect could be caused by MRS 2179 blocking a contractile response, which is masked by the predominant relaxation effect. However, MRS 2179 did not affect the contractile response of ATPγS in the small mesenteric artery which eliminates this possibility. These findings could therefore indicate that a relationship exists between $P2Y_1$- and $P2Y_2$-mediated responses under certain circumstances; for example, antagonism of $P2Y_1$ receptors by MRS 2179 might cause enhancement of $P2Y_2$ receptor signalling, resulting in potentiation of ATPγS-induced responses. Interestingly, similar observations have been made by other groups when using the $P2Y_2$ receptor agonists, UTP and UDP, in the presence of $P2Y_1$ receptor antagonists such as A3P5P or PPADS (Dol-Gleizes *et al*., 1999; Chootip *et al*., 2002; Payne *et al*., 2002). If this is the case, then it remains to be determined how the stimulation of $P2Y_2$ receptors is being augmented by $P2Y_1$ receptor antagonism.

In conclusion, this study shows that $P2Y_1$ and $P2Y_2$ receptors on endothelial cells mediate relaxation of rat mesenteric arteries and cause a hyperpolarization in the endothelium. The response to the $P2Y_2$ receptor agonist, ATPγS, showed regional variation between the vessels studied; the small mesenteric artery displayed little relaxant activity to ATPγS, while the main mesenteric artery was relaxed in a biphasic manner. Relaxant responses to ADPβS and ATPγS were inhibited by L-NAME and could be abolished by the additional presence of apamin and charybdotoxin, a combination that also eliminates endothelial cell hyperpolarization to these agents and has previously been shown to block EDHF-induced relaxation of rat mesenteric artery (Chen & Cheung, 1997; White & Hiley, 1997). The selective $P2Y_1$ receptor antagonist, MRS 2179, inhibited both the arterial relaxation

and endothelial cell hyperpolarization to ADPβS, supporting a role for actions at $P2Y_1$ receptors. However, responses to ATPγS were potentiated in the presence of MRS 2179, and this may indicate interactions between P2Y receptor subtypes though the mechanism for the potentiation requires elucidation.

References

ADEAGBO, A.S. & TRIGGLE, C.R. (1993). Varying extracellular [K^+]: a functional approach to separating EDHF- and EDNO-related mechanisms in perfused rat mesenteric arterial bed. *J. Cardiovasc. Pharmacol.,* **21,** 423 – 429.

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.

BIGAUD, M. & PELTON, J.T. (1992). Discrimination between ET_A- and ET_B-receptor-mediated effects of endothelin-1 and [$Ala^{1,3,11,15}$]endothelin-1 by BQ-123 in the anaesthetized rat. *Br. J. Pharmacol.,* **107,** 912 – 918.

BOYER, J.L., MOHANRAM, A., CAMAIONI, E., JACOBSON, K.A. & HARDEN, T.K. (1998). Competitive and selective antagonism of $P2Y_1$ receptors by N^6-methyl 2′-deoxyadenosine 3′,5′-bisphosphate. *Br. J. Pharmacol.,* **124,** 1 – 3.

BROWN, S.G., KING, B.F., KIM, Y.-C., JANG, S.Y., BURNSTOCK, G. & JACOBSON, K.A. (2000). Activity of novel adenine nucleotide derivatives as agonists and antagonists at recombinant rat P2X receptors. *Drug Dev. Res.,* **49,** 253 – 259.

BURNSTOCK, G. (1989). Vascular control by purines with emphasis on the coronary system. *Eur Heart J.,* **10,** 15 – 21.

BURNSTOCK, G. (1990). Dual control of local blood flow by purines. *Ann. N. Y. Acad. Sci.,* **603,** 31 – 44.

BUSSE, R., EDWARDS, G., FÉLÉTOU, M., FLEMING, I., VANHOUTTE, P.M. & WESTON, A.H. (2002). EDHF: bringing the concepts together. *Trends Pharmacol. Sci.,* **23,** 374 – 380.

BUVINIC, S., BRIONES, R. & HUIDOBRO-TORO, J.P. (2002). $P2Y_1$ and $P2Y_2$ receptors are coupled to the NO/cGMP pathway to vasodilate the rat arterial mesenteric bed. *Br. J. Pharmacol.,* **136,** 847 – 856.

CAMPBELL, W.B., GEBREMEDHIN, D., PRATT, P.F. & HARDER, D.R. (1996). Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. *Circ. Res.,* **78,** 415 – 423.

CHAYTOR, A.T., EVANS, W.H. & GRIFFITH, T.M. (1998). Central role of heterocellular gap junctional communication in endothelium-dependent relaxations of rabbit arteries. *J. Physiol.,* **508,** 561 – 573.

CHAYTOR, A.T., MARTIN, P.E., EDWARDS, D.H. & GRIFFITH, T.M. (2001). Gap junctional communication underpins EDHF-type relaxations evoked by ACh in the rat hepatic artery. *Am. J. Physiol.,* **280,** H2441 – H2450.

CHEN, G. & CHEUNG, D.W. (1997). Effect of K^+-channel blockers on ACh-induced hyperpolarization and relaxation in mesenteric arteries. *Am. J. Physiol.,* **272**, H2306 – H2312.

CHOOTIP, K., NESS, K.F., WANG, Y., GURNEY, A.M. & KENNEDY, C. (2002). Regional variation in P2 receptor expression in the rat pulmonary arterial circulation. *Br. J. Pharmacol.,* **137,** 637 – 646.

COLEMAN, H.A., TARE, M. & PARKINGTON, H.C. (2001). EDHF is not K^+ but may be due to spread of current from the endothelium in guinea pig arterioles. *Am. J. Physiol.,* **280,** H2478 – H2483.

CORRIU, C., FÉLÉTOU, M., CANET, E. & VANHOUTTE, P.M. (1996). Endothelium-derived factors and hyperpolarization of the carotid artery of the guinea-pig. *Br. J. Pharmacol.,* **119,** 959 – 964.

DOL-GLEIZES, F., MARES, A.M., SAVI, P. & HERBERT, J.M. (1999). Relaxant effect of 2-methyl-thio-adenosine diphosphate on rat thoracic aorta: effect of clopidogrel. *Eur. J. Pharmacol.,* **367,** 247 – 253.

DORA, K.A. (2001). Cell – cell communication in the vessel wall. *Vasc. Med.,* **6,** 43 – 50.

DOUGHTY, J.M., PLANE, F. & LANGTON, P.D. (1999). Charybdotoxin and apamin block EDHF in rat mesenteric artery if selectively applied to the endothelium. *Am. J. Physiol.,* **276,** H1107 – H1112.

EDWARDS, G., DORA, K.A., GARDENER, M.J., GARLAND, C.J. & WESTON, A.H. (1998). K^+ is an endothelium-derived hyperpolarizing factor in rat arteries. *Nature,* **396,** 269 – 272.

EDWARDS, G., FÉLÉTOU, M., GARDENER, M.J., THOLLON, C., VANHOUTTE, P.M. & WESTON, A.H. (1999a). Role of gap junctions in the responses to EDHF in rat and guinea-pig small arteries. *Br. J. Pharmacol.,* **128,** 1788 – 1794.

EDWARDS, G., GARDENER, M.J., FÉLÉTOU, M., BRADY, G., VANHOUTTE, P.M. & WESTON, A.H. (1999b). Further investigation of endothelium-derived hyperpolarizing factor (EDHF) in rat hepatic artery: studies using 1-EBIO and ouabain. *Br. J. Pharmacol.,* **128,** 1064 – 1070.

EDWARDS, G. & WESTON, A.H. (2001). EDHF – are there gaps in the pathway? *J. Physiol.,* **531,** 299.

ERLINGE, D., HOU, M., WEBB, T.E., BARNARD, E.A. & MOLLER, S. (1998). Phenotype changes of the vascular smooth muscle cell regulate P2 receptor expression as measured by quantitative RT – PCR. *Biochem. Biophys. Res. Commun.,* **248,** 864 – 870.

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.

FÉLÉTOU, M. & VANHOUTTE, P.M. (2000). Endothelium-dependent hyperpolarization of vascular smooth muscle cells. *Acta Pharmacol. Sin.,* **21,** 1 – 18.

GITTERMAN, D.P. & EVANS, R.J. (2000). Properties of P2X and P2Y receptors are dependent on artery diameter in the rat mesenteric bed. *Br. J. Pharmacol.,* **131,** 1561 – 1568.

GOEZ, U., PRADA, D. & PLETSCHER, A. (1971). Adenine-, guanine- and uridine-5′-phosphonucleotides in blood platelets and storage organelles of various species. *J. Pharmacol. Exp. Ther.,* **178,** 210 – 215.

GOODY, R.S., ECKSTEIN, F. & SCHIRMER, R.H. (1972). The enzymatic synthesis of thiophosphate analogs of nucleotides. *Biochim. Biophys. Acta,* **276,** 155 – 161.

GORDON, J.L. (1986). Extracellular ATP: effects, sources and fate. *Biochem. J.,* **233,** 309 – 319.

HARRIS, D., MARTIN, P.E., EVANS, W.H., KENDALL, D.A., GRIFFITH, T.M. & RANDALL, M.D. (2000). Role of gap junctions in endothelium-derived hyperpolarizing factor responses and mechanisms of K^+-relaxation. *Eur. J. Pharmacol.,* **402,** 119 – 128.

HECKER, M., BARA, A.T., BAUERSACHS, J. & BUSSE, R. (1994). Characterization of endothelium-derived hyperpolarizing factor as a cytochrome P450-derived arachidonic acid metabolite in mammals. *J. Physiol.,* **481,** 407 – 414.

KAISER, R.A. & BUXTON, I.L. (2002). Nucleotide-mediated relaxation in guinea-pig aorta: selective inhibition by MRS2179. *Br. J. Pharmacol.,* **135,** 537 – 545.

KASAKOV, L. & BURNSTOCK, G. (1982). The use of the slowly degradable analog, α,β-methylene ATP, to produce desensitisation of the P2-purinoceptor: effect on non-adrenergic, non-cholinergic responses of the guinea-pig urinary bladder. *Eur. J. Pharmacol.,* **86,** 291 – 294.

LEWIS, C.J. & EVANS, R.J. (2000). Lack of run-down of smooth muscle P2X receptor currents recorded with the amphotericin permeabilized patch technique, physiological and pharmacological characterization of the properties of mesenteric artery P2X receptor ion channels. *Br. J. Pharmacol.,* **131,** 1659 – 1666.

MALMSJÖ, M., ADNER, M., HARDEN, T.K., PENDERGAST, W., EDVINSSON, L. & ERLINGE, D. (2000a). The stable pyrimidines UDPβS and UTPγS discriminate between the P2 receptors that mediate vascular contraction and relaxation of the rat mesenteric artery. *Br. J. Pharmacol.,* **131,** 51 – 56.

MALMSJÖ, M., EDVINSSON, L. & ERLINGE, D. (1998). P2U-receptor mediated endothelium-dependent but nitric oxide-independent vascular relaxation. *Br. J. Pharmacol.,* **123,** 719 – 729.

MALMSJÖ, M., EDVINSSON, L. & ERLINGE, D. (2000b). P2X receptors counteract the vasodilatory effects of endothelium derived hyperpolarising factor. *Eur. J. Pharmacol.,* **390,** 173 – 180

MALMSJÖ, M., ERLINGE, D., HÖGESTÄTT, E.D. & ZYGMUNT, P.M. (1999). Endothelial P2Y receptors induce hyperpolarisation of vascular smooth muscle by release of endothelium-derived hyperpolarising factor. *Eur. J. Pharmacol.,* **364,** 169 – 173.

MALMSJÖ, M., HOU, M., HARDEN, T.K., PENDERGAST, W., PANTEV, E., EDVINSSON, L. & ERLINGE, D. (2000c). Characterization of contractile P2 receptors in human coronary arteries by use of the stable pyrimidines uridine 5′-O-thiodiphosphate and uridine 5′-O-3-thiotriphosphate. *J. Pharmacol. Exp. Ther.,* **293,** 755 – 760.

MARRELLI, S.P. (2001). Mechanisms of endothelial $P2Y_1$- and $P2Y_2$-mediated vasodilatation involve differential $[Ca^{2+}]_i$ responses. *Am. J. Physiol.,* **281,** H1759 – H1766.

MULVANY, M.J. & HALPERN, W. (1977). Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ. Res.,* **41,** 19 – 26.

NORTH, R.A. & SURPRENANT, A. (2000). Pharmacology of cloned P2X receptors. *Annu. Rev. Pharmacol. Toxicol.,* **40,** 563 – 580.

OLSSON, R.A. & PEARSON, J.D. (1990). Cardiovascular purinoceptors. *Physiol. Rev.,* **70,** 761 – 845.

PAYNE, S.J., BENJAMIN, I.S. & ALEXANDER, B. (2002). Cold storage of rabbit thoracic aorta in University of Wisconsin solution attenuates $P2Y_2$ purine receptors. *Cryobiology,* **44,** 91 – 102.

QUILLEY, J. & MCGIFF, J.C. (2000). Is EDHF an epoxyeicosatrienoic acid? *Trends Pharmacol. Sci.,* **21,** 121 – 124.

RALEVIC, V. (2001). Mechanism of prolonged vasorelaxation to ATP in the rat isolated mesenteric arterial bed. *Br. J. Pharmacol.,* **132,** 685 – 692.

RALEVIC, V. & BURNSTOCK, G. (1991). Effects of purines and pyrimidines on the rat mesenteric arterial bed. *Circ. Res.,* **69,** 1583 – 1590.

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

RICHARDS, G.R., WESTON, A.H., BURNHAM, M.P., FÉLÉTOU, M., VANHOUTTE, P.M. & EDWARDS, G. (2001). Suppression of K^+-induced hyperpolarization by phenylephrine in rat mesenteric artery: relevance to studies of endothelium-derived hyperpolarizing factor. *Br. J. Pharmacol.,* **134,** 1 – 5.

SCHWEITZ, H., STANSFELD, C.E., BIDARD, J.N., FAGNI, L., MAES, P. & LAZDUNSKI, M. (1989). Charybdotoxin blocks dendrotoxin-sensitive voltage-activated K^+ channels. *FEBS Lett.,* **250,** 519 – 522.

SHIMOKAWA, H., YASUTAKE, H., FUJII, K., OWADA, M.K., NAKAIKE, R., FUKUMOTO, Y., TAKAYANAGI, T., NAGAO, T., EGASHIRA, K., FUJISHIMA, M. & TAKESHITA, A. (1996). The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium-dependent relaxations in rat mesenteric circulation. *J. Cardiovasc. Pharmacol.,* **28,** 703 – 711.

STANFORD, S., GITLIN, J. & MITCHELL, J.A. (2001). Identification of a novel second phase dilation induced by ATP in the rat isolated mesenteric bed. *Br. J. Pharmacol.,* **133,** 825 – 832

TAUC, M., CONGAR, P., PONCET, V., MEROT, J., VITA, C. & POUJEOL, P. (1993). Toxin pharmacology of the large-conductance Ca^{2+}-activated K^+ channel in the apical membrane of rabbit proximal convoluted tubule in primary culture. *Pflugers Arch.,* **425,** 126 – 133.

TRIGGLE, C.R. & DING, H. (2002). Endothelium-derived hyperpolarizing factor: is there a novel chemical mediator? *Clin. Exp. Pharmacol. Physiol.,* **29,** 153 – 160.

VOGALIS, F., ZHANG, Y. & GOYAL, R.K. (1998). An intermediate conductance K^+ channel in the cell membrane of mouse intestinal smooth muscle. *Biochim. Biophys. Acta,* **1371,** 309 – 316.

VON KUGELGEN, I. & STARKE, K. (1990). Evidence for two separate vasoconstriction-mediating nucleotide receptors, both distinct from the P2X-receptor, in rabbit basilar artery: a receptor for pyrimidine nucleotides and a receptor for purine nucleotides. *Naunyn Schmiedeberg's Arch. Pharmacol.,* **341,** 538 – 546.

VON KUGELGEN, I. & WETTER, A. (2000). Molecular pharmacology of P2Y-receptors. *Naunyn Schmiedeberg's Arch. Pharmacol.,* **362,** 310 – 323.

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

WHITE, R. & HILEY, C.R. (1997). A comparison of EDHF-mediated and anandamide-induced relaxations in the rat isolated mesenteric artery. *Br. J. Pharmacol.,* **122,** 1573 – 1584.

WHITE, R. & HILEY, C.R. (1998a). The actions of some cannabinoid receptor ligands in the rat isolated mesenteric artery. *Br. J. Pharmacol.,* **125,** 533 – 541.

WHITE, R. & HILEY, C.R. (1998b). Effects of K^+ channel openers on relaxations to nitric oxide and endothelium-derived hyperpolarizing factor in rat mesenteric artery. *Eur. J. Pharmacol.,* **357,** 41 – 51.

YAMAMOTO, K., KORENAGA, R., KAMIYA, A., QI, Z., SOKABE, M. & ANDO, J. (2000). $P2X_4$ receptors mediate ATP-induced calcium influx in human vascular endothelial cells. *Am. J. Physiol.,* **279,** H285 – H292.

XU, H.L., SANTIZO, R.A., BAUGHMAN, V.L. & PELLIGRINO, D.A. (2002). ADP-induced pial arteriolar dilation in ovariectomized rats involves gap junctional communication. *Am. J. Physiol.,* **283,** H1082 – H1091.

ZYGMUNT, P.M. & HÖGESTÄTT, E.D. (1996). Role of potassium channels in endothelium-dependent relaxation to nitroarginine in the rat hepatic artery. *Br. J. Pharmacol.,* **117,** 1600 – 1606.

ZYGMUNT, P.M., RYMAN, T. & HÖGESTÄTT, E.D. (1995). Regional differences in endothelium-dependent relaxation in the rat: contribution of nitric oxide and nitric oxide-independent mechanisms. *Acta Physiol. Scand.,* **155,** 257 – 266.

(Received January 23, 2003
Revised March 4, 2003
Accepted March 6, 2003)