British Journal of Pharmacology (2001) 133, 825–832

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Identification of two distinct vasodilator pathways activated by ATP in the mesenteric bed of the rat

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1 Adenosine 5′-triphosphate (ATP) has important roles in the cardiovascular system, modulating vascular tone by acting as both a vasoconstrictor and a vasodilator.

2 The dilator function of ATP is traditionally thought to be monophasic and mediated primarily by nitric oxide (NO).

3 Here we have identified the endothelium-dependent biphasic nature of ATP-induced vasodilatation of the rat isolated mesenteric bed and investigated the two distinct pathways involved.

4 ATP, at doses of 1×10^{-11} to 1×10^{-8} moles, induced transient relaxations that were inhibited by the NO synthase (NOS) inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME: 1×10^{-4} M), the soluble guanylyl cyclase inhibitor, 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ: 3×10^{-6} M) and KCl ($6 \times 10^{-2} - 1.2 \times 10^{-1}$ M).

5 At doses upwards of 1×10^{-8} moles ($1 \times 10^{-8} - 3 \times 10^{-7}$ moles), ATP also induced prolonged vasodilatations which were unaltered by L-NAME, L-NAME (1×10^{-3} M) and indomethacin (1×10^{-5} M), or by ODQ, but were abolished in the presence of KCl.

6 In addition, the cannabinoid CB_1 receptor antagonist SR141716A (1×10^{-5} M) was found to inhibit the second prolonged phase of vasodilatation. However, at the concentration used SR141716A is reported to be non-selective. A second CB_1 receptor antagonist, AM251 (1×10^{-6} M), had a small but significant inhibitory effect on the second phase of ATP-induced vasodilatation. SR141716A, AM251 and KCl ($6 \times 10^{-2} - 1.2 \times 10^{-1}$ M) all inhibited anandamide-induced relaxation of the isolated mesenteric bed.

7 These observations demonstrate that ATP stimulates vasodilatation of the mesenteric bed by two distinct mechanisms involving the release of NO and an EDHF. In the absence of better pharmacological tools we can only speculate as to the involvement of an endogenous CB_1 receptor ligand in these responses.

British Journal of Pharmacology (2001) **133,** 825–832

Keywords: ATP; nitric oxide; EDHF; rat isolated mesenteric bed; cannabinoid; CB_1 receptor; anandamide

Abbreviations: ACh, acetylcholine; AEA, anandamide; ATP, adenosine 5′-triphosphate; cyclic AMP, adenosine 3′5′-cyclic monophosphate; CAPS, capsaicin; cyclic GMP, guanosine 3′5′-cyclic monophosphate; CGRP, calcitonin gene-related peptide; DMSO, dimethyl sulphoxide; D-NAME, N^G-nitro-D-arginine methyl ester; EDHF, endothelium-derived hyperpolarizing factor; EFS, electric field stimulation; KCl, potassium chloride; L-NAME, N^G-nitro-L-arginine methyl ester; NANC, non-adrenergic non-cholinergic; NO, nitric oxide; NOS, NO synthase; SNP, sodium nitroprusside; ODQ, 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one; 8-SPT, 8-sulphophenyltheophylline.

Introduction

ATP is the ubiquitous energy-carrying molecule of the cell. Free energy, liberated in the hydrolysis of ATP, is required by all cells to perform functions such as ion transport, contraction and the synthesis and release of molecules. Most cells contain ATP in their cytoplasm and mitochondria. In some settings, namely platelets, neurones and cells of the adrenal medulla, ATP is stored in granules and released, under various physiological conditions, by exocytosis. Cultured vascular endothelial and smooth muscle cells (Pearson & Gordon, 1979) have also been shown to release ATP. It has been reported that the release of ATP from endothelial cells is a common receptor-mediated response to several agonists (including bradykinin and ADP: Yang *et al*., 1994). Endothelial cells also release ATP in response to shear stress (Burnstock, 1999). ATP is a particularly important mediator in the cardiovascular system where its actions have been well studied using a range of preparations including the isolated Krebs' perfused mesenteric vascular bed (Ralevic & Burnstock, 1988). Using this preparation, ATP has been identified as both a vasoconstrictor and vasodilator. The ATP-induced vasoconstriction is mediated by P2X-purinoceptors, located on smooth muscle, and vasodilatation by P2Y-purinoceptors usually located on the endothelium (Ralevic & Burnstock, 1988: Corr & Burnstock, 1994). The vasodilator functions of ATP have been attributed to the secondary release of nitric oxide (NO) (Rubino *et al*., 1995). However, at physiologically relevant doses, we have observed a novel vasodilator property of ATP identifying, for the first time, the presence of two components that comprise the response in the rat isolated mesenteric bed (Stanford & Mitchell, 1998). This biphasic relaxant response consists of a

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rapid but transient first phase and a more prolonged second phase. The purpose of this study was to identify the possible mechanisms of action by which ATP induces this biphasic relaxation. Our initial observations have been, published in abstract form (Stanford & Mitchell, 1998).

Methods

Experimental procedure

Male Wistar rats (250–300 g) were anaesthetized with sodium pentobarbitone (100 mg kg^{-1}; i.p.) and killed by cervical dislocation. The superior mesenteric artery was then located, cannulated and the mesentery excised. The bed was perfused at a constant rate (10 ml min^{-1}) with Krebs' buffer (NaCl 11.8×10^{-2} M; KCl 5.9×10^{-3} M; $MgSO_4$ $7H_2O$ 1.2×10^{-3} M; NaH_2PO_4 $2H_2O$ 1.2×10^{-3} M; $CaCl_2$ $2H_2O$ 2.5×10^{-3} M; glucose 5.6×10^{-3} M; $NaHCO_3$ 25.6×10^{-3} M) warmed to 37°C and gassed (95% O_2 : 5% CO_2). In some experiments electric field stimulation (EFS) electrodes were placed on the mesenteric artery. This was done carefully to ensure good contact with the sensory nerves. In other experiments the endothelium was removed by a 30-s infusion of deoxycholic acid (5.0×10^{-3} M). Perfusion pressure, recorded *via* an arterial cannula, was raised to approximately 120 mmHg by titration of the non-selective α-adrenoceptor agonist, methoxamine (1×10^{-6}–1.2×10^{-5} M) added to the Krebs' perfusate. In experiments designed to examine the effects of high K^+, perfusion pressure was raised again to approximately 120 mmHg, by titration of KCl (6×10^{-2}–1.2×10^{-1} M) instead of methoxamine.

Protocols for drug administration

In contrast to studies by Ralevic and co-workers (Ralevic & Burnstock, 1988; Rubino *et al*., 1995), where injection volumes of 50 μl were used, we injected ATP in volumes of 1–3 μl, which did not effect perfusion pressure directly. The effect of bolus injections of ATP (1×10^{-11}–3×10^{-7} moles), administered *via* a side arm in the apparatus, were recorded. In some experiments the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME: 1×10^{-4} M), its inactive isomer N^G-nitro-D-arginine methyl ester (D-NAME: 1×10^{-4} M), L-NAME (1×10^{-3} M) in combination with the cyclo-oxygenase inhibitor indomethacin (1×10^{-5} M), the soluble guanylyl cyclase inhibitor 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ: 3×10^{-6} M), the adenylyl cyclase inhibitor SQ22536 (1×10^{-4} M) or the ODQ/SQ22536 vehicle, dimethyl sulphoxide (DMSO), were added to the Krebs' perfusate prior to constriction of the preparation with methoxamine.

Similarly, the effects of 1–3 μl volume injections of ATP (1×10^{-11}–3×10^{-7} moles) or adenosine (1×10^{-10}–1×10^{-7} moles) were recorded in the presence or absence of the non-selective P_1-purinoceptor antagonist 8-sulphophenyltheophylline (8-SPT: 1×10^{-4} M).

A further set of experiments was designed to address any role of neuropeptides (e.g. calcitonin gene-related peptide (CGRP)) in the dilator effects of ATP. For these experiments capsaicin (1 mg l^{-1}), which activates sensory nerves and releases CGRP in the rat mesenteric bed (Fujimori *et al*., 1990), was added to the Krebs' perfusate following preconstriction of the rat isolated mesenteric bed with methoxamine. Sensory nerve depletion induced by capsaicin was evidenced by a vasodilator response that returned to baseline during the course of the infusion (Figure 3). Simple desensitization of the vanilloid (capsaicin) receptor (VR1) was ruled out by demonstrating that EFS evoked vasodilatation was abolished following capsaicin infusion. Specifically, the effect of EFS (4 Hz, 50 V, 1×10^{-3} s duration, 30 s stimulation) was recorded prior to, and subsequent to, capsaicin addition to the Krebs'. The effects 1–3 μl volume injections of ATP (1×10^{-11}–3×10^{-7} moles) were recorded in both control and capsaicin 'desensitized' preparations.

The effect of 1 μl volume injections of ATP (1×10^{-9}–1×10^{-7} moles) or histamine (1×10^{-7} and 1×10^{-6} moles) were recorded in the presence or absence of the H_1 receptor antagonist mepyramine (1×10^{-5} M).

The effect of 1 μl and 3 μl volume injections of 1×10^{-7} and 3×10^{-7} moles of ATP respectively or 1–3 μl injections of anandamide (1×10^{-8}–1×10^{-7} moles) were recorded. These experiments were performed both in endothelium-intact and endothelium-denuded preparations. In endothelium-intact preparations, further experiments were performed in the presence of the CB_1 receptor antagonists SR141716A (1×10^{-5} M) or AM251 (1×10^{-6} M), the SR141716A/AM251 vehicle (DMSO) or KCl (6×10^{-2}–1.2×10^{-1} M). KCl was used in order to assess the contribution of K^+ conductances in the vasodilator response to both ATP and anandamide. In endothelium denuded preparations, prior to the addition of ATP or anandamide, the effects of acetylcholine (ACh: 1×10^{-8} moles) and the NO donor, sodium nitroprusside (SNP: 1×10^{-9} moles), were determined

Drugs

ATP, L-NAME, D-NAME, indomethacin, anandamide, capsaicin and mepyramine were all purchased from Sigma. KCl was from BDH, ODQ, SR141716A and AM251 were from Tocris Cookson and 8-SPT from RBI. SQ22536 was a kind gift from Dr P. Timmins at Bristol-Myers Squibb.

Statistical analysis

All results are represented in both the text and figures as mean ± s.e.mean. Statistical tests used are fully explained in figure legends and in each case a P value of less than 0.05 was considered to be statistically significant.

Results

ATP induced reductions in perfusion pressure, which were rapid in onset but transient at doses of 1×10^{-11} to 1×10^{-8} moles. At higher doses (1×10^{-8}–3×10^{-7} moles), in addition to the initial transient reduction in perfusion pressure seen at the lower doses, a second and more prolonged phase in the relaxant response to ATP was observed (Figure 1). It should be noted that in addition, and prior to the dose-dependent reductions in perfusion pressure, ATP also induced a transient increase in perfusion pressure (Figure 1a) which has been described previously (Ralevic & Burnstock, 1988) but was not the subject of this investigation.

L-NAME, D-NAME and ODQ

Both the NOS inhibitor, L-NAME (1×10^{-4} M), and the guanylyl cyclase inhibitor, ODQ (3×10^{-6} M) inhibited the first phase of the relaxant response to ATP (Figure 2a, b). Experiments performed in the presence of the ODQ/indomethacin vehicle, DMSO, did not significantly differ from controls. D-NAME had no effect on either phase of the ATP-induced response (Table 1). In contrast to the first phase, the second and more prolonged phase was not affected by ODQ (Table 1). Moreover, L-NAME induced a small but significant potentiation of the second phase ($P<0.05$ by two-way ANOVA, Table 1). In the presence of L-NAME together with indomethacin, the second phase of the ATP-induced relaxation was further potentiated (ATP 1×10^{-7} moles: $35.1\pm4.7\%$, $n=10$ *vs* $62.6\pm3.1\%$, $n=4$).

Adenosine and 8-SPT

The non-selective P_1-purinoceptor antagonist, 8-SPT (1×10^{-4} M) (Evoniuk *et al*., 1987) completely abolished the relaxant response to adenosine but had no such effect on either the first or second phase of ATP-induced vasodilatation (Table 1).

Capsaicin

The addition of capsaicin (1 mg l^{-1}) to the Krebs' perfusate induced a relaxant response in the preconstricted bed. Following a time period of 65.0 ± 3.5 min ($n=4$) the bed became desensitized to the effects of capsaicin and tone was restored, indicative of the depletion of CGRP from sensory nerves (Figure 3). Desensitization of the mesenteric bed to capsaicin also abolished the relaxation of the bed to EFS (4 Hz, 50 V, 1×10^{-3} s duration, 30 s stimulation), however the responses of ATP were not modified (Table 1).

Histamine and mepyramine

The dilator responses of histamine in our study were significantly reduced (at 1×10^{-6} moles: 53.1 ± 6.2 *vs*

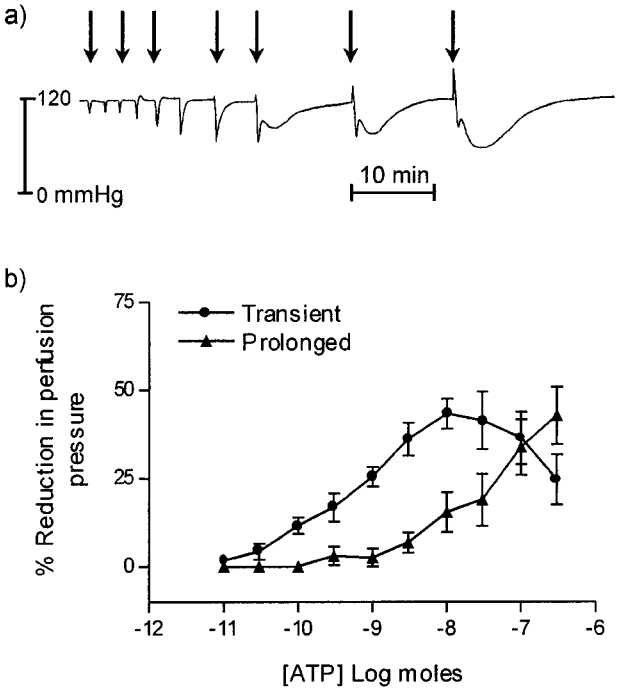

Figure 1 The ATP-induced biphasic reduction in perfusion pressure in the isolated mesenteric bed of the rat as illustrated by (a) a representative recorder trace (↓ 1×10^{-11}, 1×10^{-10}, 1×10^{-9}, 1×10^{-8}, 3×10^{-8}, 1×10^{-7} and 3×10^{-7} moles) and (b) a dose response curve ($n=7$).

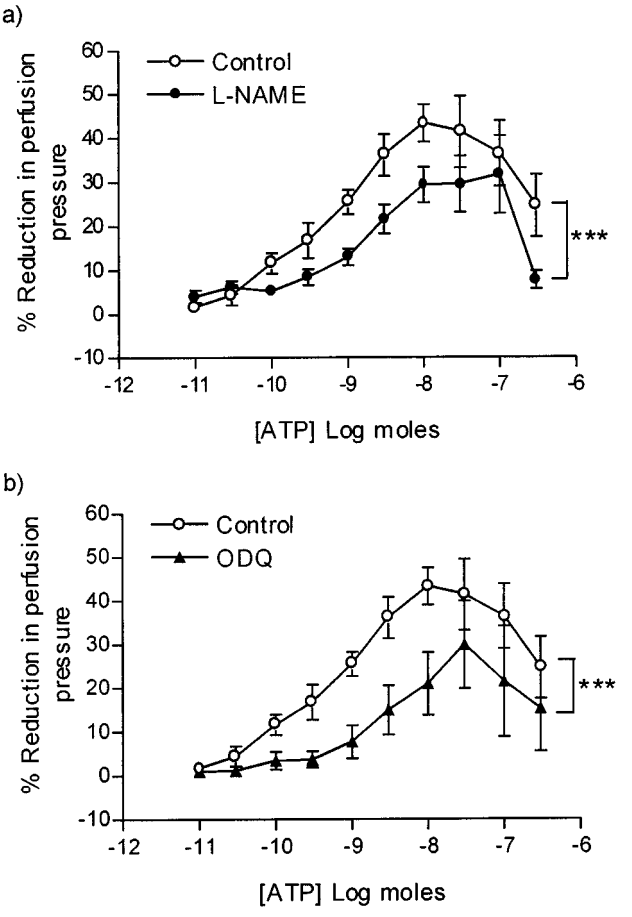

Figure 2 The effect of (a) L-NAME (1×10^{-4} M, $n=7$) or (b) ODQ (3×10^{-6} M, $n=4$) on the first phase of the ATP-induced reduction in perfusion pressure in the isolated mesenteric bed of the rat. ***$P<0.001$ by two-way ANOVA.

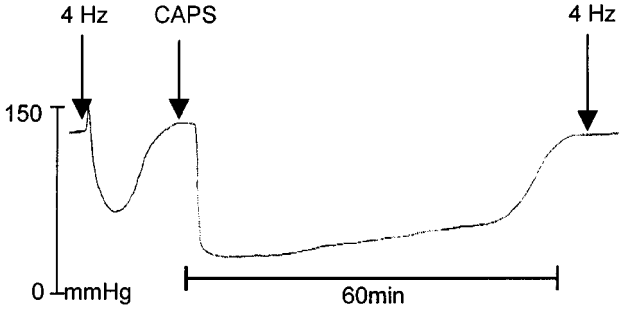

Figure 3 Effect of capsaicin (CAPS: 1 mg l^{1}) on perfusion pressure in the isolated mesenteric bed of the rat illustrated by a representative pen recorder trace. Trace also demonstrates the inhibition of the relaxation in response to EFS following capsaicin desensitization.

Table 1 Effect of various agents on the two phases of ATP-induced relaxation

Agent in perfusate	*1st Phase: ATP 1×10^{-8} moles* (Per cent reduction in perfusion pressure) *Control*	*+ Agent*	*2nd Phase: ATP 1×10^{-7} moles* (Per cent reduction in perfusion pressure) *Control*	*+ Agent*
L-NAME	43.3±4.3	29.3±4.0*	33.8±7.9	52.8±9.2
D-NAME	43.3±4.3	41.4±3.5	33.8±7.9	41.9±7.4
ODQ	43.3±4.3	20.9±7.2*	33.8±7.9	25.5±5.5
8-SPT	43.3±4.3	40.2±9.2	33.8±7.9	34.4±9.2
Capsaicin	43.3±4.3	36.6±6.7	33.8±7.9	28.6±16.6
Mepyramine	46.8±3.3	40.5±3.6	53.5±3.9	58.0±4.0
SQ22536	43.3±4.3	42.8±6.4	33.8±7.9	27.1±6.4

Data represents the maximum per cent reduction in perfusion pressure observed following a bolus injection 1×10^{-8} moles or 1×10^{-7} moles of ATP for the first and second phase respectively. Data are given as mean±s.e.mean. *$P<0.05$ using a 2-tailed, unpaired, non-parametric Mann–Whitney test (control experiments $n=7$: L/D–NAME $n=7$: ODQ, 8-SPT and capsaicin $n=4$: mepyramine and SQ22536 ($n=5$).

17.8±2.7 % $n=6$) by the H_1 receptor antagonist mepyramine (1×10^{-5} M). However, mepyramine did not influence either the first or the second phase of dilation induced by ATP (Table 1).

SQ22536

The adenylyl cyclase inhibitor SQ22536 (Haslam *et al*., 1978) (1×10^{-4} M) did not influence the ATP-induced response (Table 1).

KCl, SR141716A, AM251 and anandamide

Anandamide induced dose-dependent relaxations in the rat mesentery. The relaxant response to anandamide (1×10^{-7} moles) was inhibited by KCl (6×10^{-2}–1.2×10^{-1} M) and by the presence of the CB_1 receptor antagonists SR141716A (Felder *et al*., 1995) (1×10^{-5} M), and AM251 (Lan *et al*., 1999) (1×10^{-4} M) (Figure 4). The vehicle used for these drugs (DMSO) had no effect on relaxations induced by anandamide (Control $n=10$ *vs* DMSO $n=5$: 51.5±2.7 *vs* 50.3±3.4%). The presence of KCl inhibited the first phase and abolished the second phase of the ATP-induced relaxant response (Figure 5). SR141716A had no effect on the first phase of the ATP-induced relaxant response (Figure 6a) but completely blocked the second phase of dilation induced by ATP (Figures 6b, c). AM251, whilst having no effect on the first phase (Figure 7a), had a small but significant effect on the second phase of the relaxant response induced by a 3×10^{-7} mole dose of ATP (Figure 7b).

Endothelium denudation

Following removal of the endothelium, the relaxant responses to ATP (1×10^{-7} moles) and anandamide (1×10^{-8} moles) were abolished, as was that induced by ACh (1×10^{-8} moles). SNP-invoked relaxation was unaffected (Figure 8).

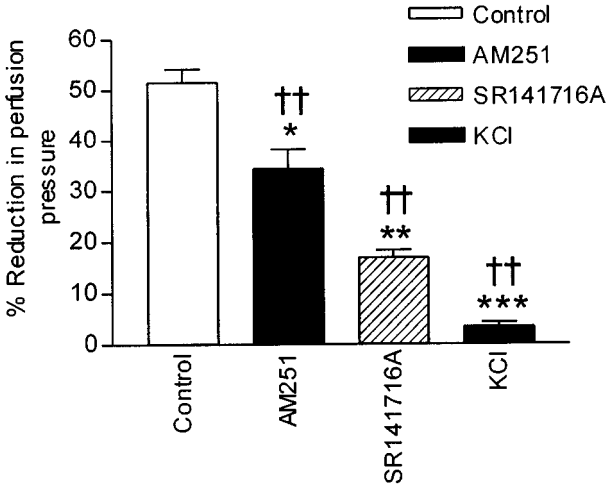

Figure 4 Effect of AM251 (1×10^{-6} M, $n=5$), SR141716A (1×10^{-5} M, $n=4$) and KCl (6×10^{-2}–1.2×10^{-1} M, $n=8$) on the relaxation induced by anandamide (1×10^{-7} moles) in the isolated mesenteric bed of the rat. *$P<0.05$, **$P<0.01$, ***$P<0.001$ using a 2-tailed, unpaired, non-parametric Mann–Whitney test *vs* control. ††$P<0.01$ using a one-way ANOVA *vs* control.

Discussion

Here we have identified two distinct pathways by which ATP produces vasodilatation of the rat isolated mesenteric bed. The NOS inhibitor, L-NAME inhibited the first phase of the relaxant response to ATP suggesting that ATP is producing the initial component of relaxation by stimulating the production of NO. NO relaxes smooth muscle by the activation of soluble guanylyl cyclase and the subsequent rise in the levels of intracellular guanosine 3′5′-cyclic monophosphate (cyclic GMP). Indeed the guanylyl cyclase inhibitor, ODQ like L-NAME, reduced the first phase of the relaxant response. These observations are in keeping with others in the literature showing that ATP stimulates NO release from vascular preparations, including the rat mesenteric bed (Mathie *et al*., 1991; Rubino *et al*., 1995).

At doses of 1×10^{-8} moles and above, ATP stimulated a prolonged vasodilatation. We suggest that these concentrations can be achieved, at least transiently, *in vivo* where cytoplasmic ATP is in the millimolar range and the granules of platelets contain ATP levels in the molar range (Olsson & Pearson, 1990). Previous studies using the isolated perfused mesenteric bed of the rat have similarly identified a NO-dependent vasodilator property of ATP, but failed to reveal the second prolonged phase. This is most probably due to the doses of ATP and the injection protocol used.

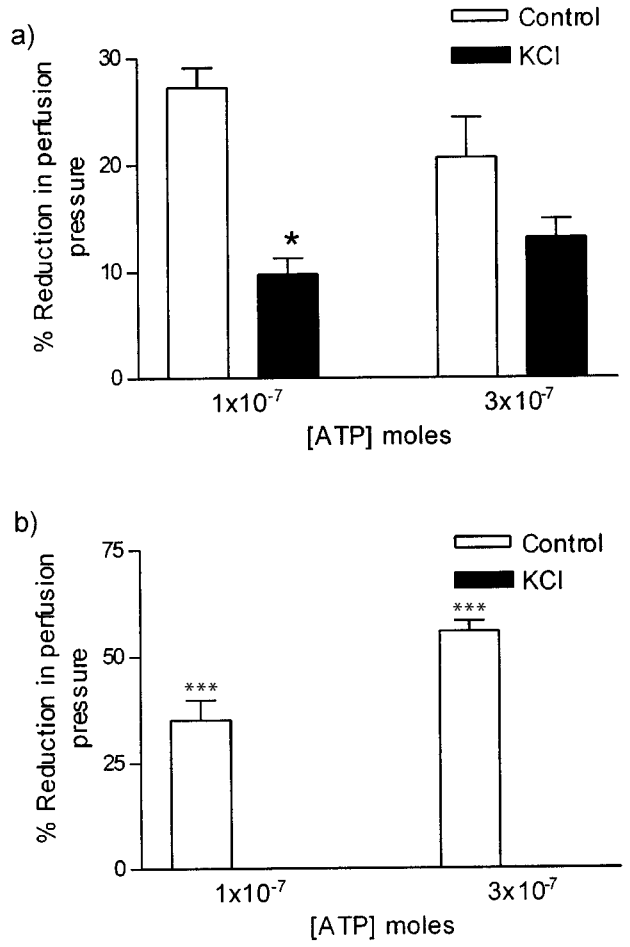

Figure 5 Effect of KCl (6×10^{-2}–1.2×10^{-1} M, $n=8$) on (a) the first transient phase and (b) the second prolonged phase of the ATP-induced reduction in perfusion pressure in the isolated mesenteric bed of the rat. $^{*}P<0.05$, $^{***}P<0.001$ using a 2 tailed, unpaired, non-parametric Mann–Whitney test.

In contrast to the first phase of the ATP-induced relaxant response, the second and more prolonged phase was ODQ resistant. Moreover, L-NAME alone or in combination with indomethacin induced a potentiation of the second phase. This perhaps suggests an inhibitory role for NO/prostacyclin in the mechanism underlying this second phase. How then, is this prolonged dilatation mediated? During the course of this study we have addressed this question by systematically assessing the potential role of each of a number of known dilator mechanisms utilized in the mesenteric bed.

ATP can exert biological effects directly or *via* metabolism to other purines including adenosine. Unlike ATP (which acts on P_2-purinoceptors), adenosine modulates vascular tone by stimulating P_1-purinoceptors (Rubino *et al*., 1995), of which there are a number of subtypes. In order to explore the possibility that the second phase of ATP-induced relaxation was mediated by adenosine, formed after ATP metabolism, we used the non-selective P_1-purinoceptor antagonist, 8-SPT (Evoniuk *et al*., 1987). Whilst 8-SPT completely abolished the relaxant response to adenosine it had no such effect on either the first or second phase of ATP-induced vasodilatation. Thus, ATP metabolism to adenosine and the subsequent activation of P_1-purinoceptors does not contribute to either phase of the ATP-induced response.

One of the most potent dilator mediators in the rat mesenteric bed is the sensory nerve peptide, CGRP. In the rat mesenteric bed this neurotransmitter is reported to mediate the EFS-induced non-adrenergic non-cholinergic (NANC) relaxant response (Kawasaki *et al*., 1988). In addition, the relaxant response to CGRP (Stanford *et al*., 1996) is relatively protracted and similar therefore to that generated by ATP. Thus, in our study ATP may be causing vasodilatation by stimulation of sensory nerves and release of CGRP. The addition of capsaicin to the Krebs' perfusate induced a relaxant response in the preconstricted bed. Following desensitization to the effects of capsaicin, tone was restored, indicative of the depletion of CGRP from sensory nerves. Desensitization of the mesenteric bed to capsaicin also abolished the relaxation of the bed to EFS, however the responses to ATP were not affected. This rules out CGRP as a possible mediator of the second phase of ATP-induced relaxation and is in agreement with recent data from Ralevic (2001).

Histamine is another possible mediator for the second phase of the ATP-induced relaxation. It is released from resident mast cells in the mesentery by stimuli including ATP (Ennis & Pearce, 1980). Histamine evokes potent dilations in mesenteric vessels *via* the activation of H_1 receptors (Guth & Smith, 1978). Indeed, we found the dilator responses of histamine in our study were significantly reduced by the H_1 receptor antagonist mepyramine. However, mepyramine did not influence either the first or the second phase of dilation induced by ATP.

The guanylyl or adenylyl cyclase pathways, leading to the generation of cyclic GMP and cyclic AMP respectively often mediate vasodilatation in blood vessels. As discussed above, the first phase of ATP induced response was identified as being mediated by NO and guanylyl cyclase activation. However, no role for guanylyl cyclase was found in the second phase. Similarly, in separate experiments we found that the adenylyl cyclase inhibitor, SQ22536 (Haslam *et al*., 1978), did not influence the ATP-induced response.

The rat isolated mesenteric bed is reported to be very sensitive to the effects endothelium-derived hyperpolarizing factor (EDHF: McCulloch *et al*., 1997). Furthermore, it has been reported that activation of P2Y-purinoceptors in the rat mesenteric artery induces hyperpolarization of the smooth muscle cells (Malmsjo *et al*., 1999). EDHF is putatively believed to cause hyperpolarization of the cell membrane by the opening of a K^+ conductance ('selective' blockers have implicated the involvement of various different types of K^+ channels although results are sometimes conflicting). Hyperpolarization reduces the open probability of voltage-dependent Ca^{2+} channels attenuating Ca^{2+} influx, lowering intracellular free Ca^{2+} levels, resulting in relaxation. Recent evidence suggests the involvement of heterocellular gap junctions in the transfer of EDHF from the endothelium to the underlying smooth muscle (Hutcheson *et al*., 1999). It has been reported that NO attenuates the release of EDHF (Bauersachs *et al*., 1996). If the second phase were due to EDHF then this might explain the ability of L-NAME to potentiate this response. Indeed the removal of the endothelium abolished both phases of ATP-induced relaxation at all concentrations tested. Thus, we show conclusively

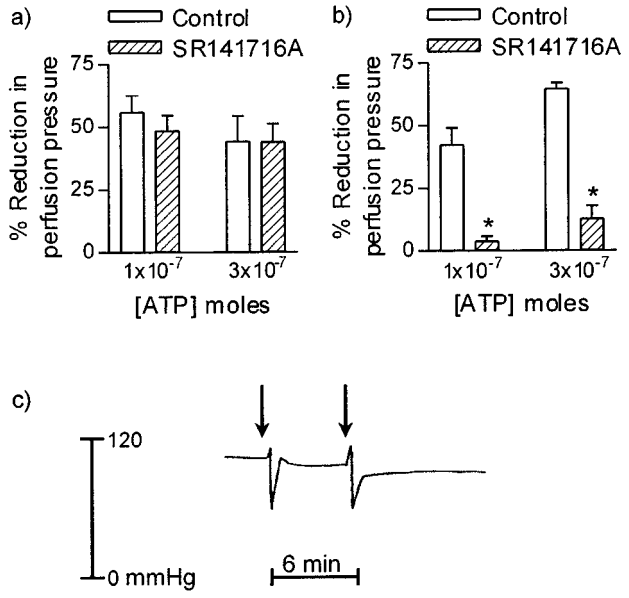

Figure 6 Effect of SR141716A (1×10^{-5} M, $n=4$) on (a) the first phase and (b) the second phase of the ATP-induced reduction in perfusion pressure of the isolated mesenteric bed of the rat. $^*P<0.05$ using a 2-tailed, unpaired, non-parametric Mann–Whitney test. Part (c) shows a representative trace (↓ 1×10^{-7}, 3×10^{-7} moles).

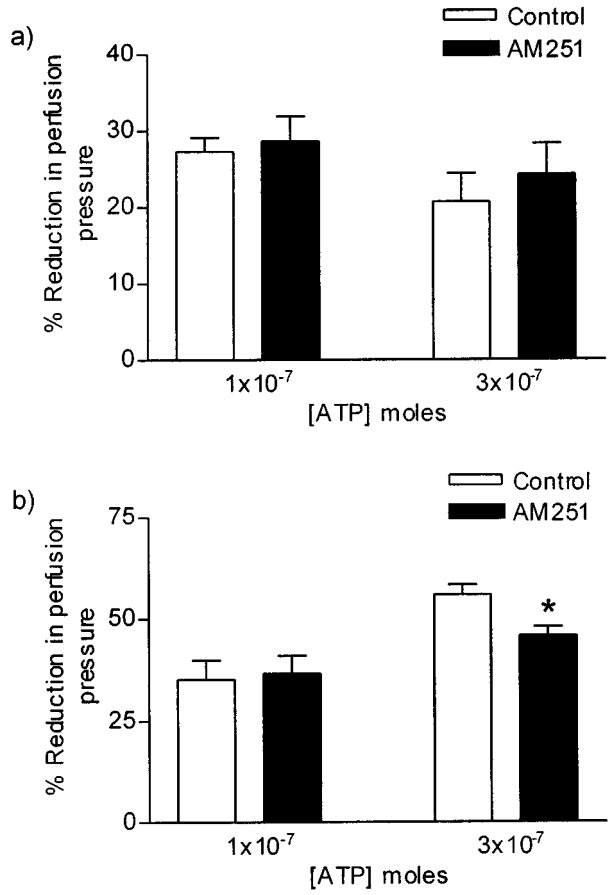

Figure 7 Effect of AM251 (1×10^{-6} M, $n=5$) on (a) the first phase and (b) the second phase of the reduction in perfusion pressure induced by ATP in the isolated mesenteric bed of the rat. $^*P<0.05$ using a 2-tailed, unpaired, non-parametric Mann–Whitney test.

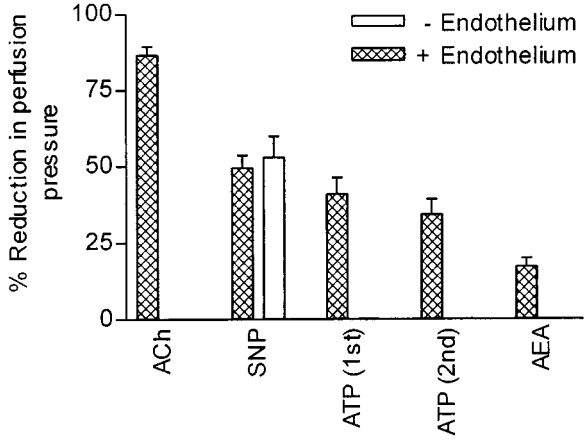

Figure 8 Effect of endothelium denudation on the relaxation induced by ACh (1×10^{-8} moles, $n=5$), SNP (1×10^{-9} moles, $n=5$), ATP (1×10^{-7} moles, $n=5$) and anandamide (AEA, $n=5$) in the isolated mesenteric bed of the rat.

that the second phase of ATP-induced vasodilatation is endothelium-dependent. By contrast the data presented by Ralevic (2001) addressing the endothelium-dependency of this second phase was less conclusive. Ralevic suggested that the second phase of the ATP-induced relaxation was endothelium-dependent at a dose of 5×10^{-8} moles but independent of the endothelium at doses of 5×10^{-7} or 5×10^{-6} moles. One explanation for Ralevic's conflicting observations could be the incomplete removal of the endothelium by the protocol used. In our study endothelial cell removal was achieved with detergent and confirmed by the absence of dilator response to a high dose of ACh but with a preserved response to the endothelium-independent dilator SNP. In Ralevic's study water was used to remove the endothelium and removal was confirmed using a relatively low dose of ATP and without addressing vascular smooth muscle function.

Characterisic of an EDHF, consistent with vasodilatation due to increased K^+ conductance, the presence of high extracellular K^+ abolished the second phase of the ATP-induced relaxant response. Interestingly, high K^+ also had an inhibitory effect on the NO-sensitive, first phase of ATP-induced relaxation.

The identity of EDHF remains elusive but it is thought that in mesenteric arteries arachidonoylethanolamide (anandamide) may be an EDHF (Randall *et al.*, 1996) although others dispute this (Plane *et al.*, 1997: White & Hiley, 1997). Other candidates for EDHF include the cytochrome P450 metabolites of arachidonic acid, epoxyeicosatrienoic acids (Campbell *et al.*, 1996) and potassium ions (Edwards *et al.*, 1998). Anandamide is a phosphatidyl derivative and the putative endogenous ligand for the cannabinoid CB_1 receptor. Like EDHF-induced relaxations, anandamide-induced relaxations are independent of NO or prostanoids but sensitive to potassium channel blockers and high extracellular K^+ levels (Randall *et al.*, 1996; 1997). We investigated the possibility that ATP is acting *via* a CB_1/EDHF pathway. We found anandamide induced dose-dependent relaxations in the rat mesentery that were inhibited by the presence of CB_1

receptor antagonist SR141716A (Felder *et al*., 1995). These observations are in agreement with those of Randall *et al*. (1996). Furthermore, SR141716A had no effect on the first phase of the ATP-induced relaxant response but completely blocked the second phase of dilation induced by ATP. However, there are reports in the literature that at the concentration we used SR141716A is non-selective, interacting with potassium channels (Chataigneau *et al*., 1998) and blocking gap junctions (Chaytor *et al*., 1999). Thus, we have also assessed the effects of a second CB_1 receptor antagonist, AM251 used at a lower concentration. AM251 had a statistically significant, but small inhibitory effect on anandamide-induced vasodilatation. Similarly AM251 inhibited the second phase of ATP-induced dilatation to a significant albeit small extent. It should also be noted that Randall and co-workers have recently suggested that EDHF-mediated relaxations are mediated by a CB receptor that does not fit into the present classification possibly representing a CB_1 receptor subtype (Harris *et al*., 1999). Thus, until we have a better understanding of the pharmacology of CB receptors in the vasculature and until better tools are developed, we cannot conclusively rule in or out a role for CB_1 in the vasodilatation induced by ATP.

Like the ATP-induced relaxation, in our hands the relaxation of the mesenteric bed due to anandamide was inhibited by removal of the endothelium. The literature reports that anandamide is able to induce both endothelium-dependent and independent relaxations of the mesenteric artery. It has been postulated that the endothelium-dependent component, like the EDHF response, is dependent on gap junctional communication (Chaytor *et al*., 1999). Therefore, the action of SR141716A may be attributable to its inhibitory effects on gap junctions rather than antagonism of the CB_1 receptor. The response to anandamide, like the second phase of the ATP-induced response, was inhibited by high levels of K^+.

In conclusion, this study demonstrates that ATP is able to stimulate vasodilatation of the rat isolated mesenteric bed by two distinct mechanisms. The first involving the release of NO and the second phase release of an EDHF. In the absence of better pharmacological tools we can only speculate as to the involvement of an endogenous CB_1 receptor ligand. Many vasoactive mediators including ACh and bradykinin are known to induce relaxation by the release of an EDHF, NO and/or prostaglandins although in these cases just one biological phase or response is observed. The identification of two distinct pathways activated by ATP may provide a means of looking specifically at, and thus characterizing, an EDHF response. This study furthers our understanding of how ATP functions and adds weight to the hypothesis that hyperpolarization is an important pathway for the regulation of mesenteric blood flow.

This work was funded by grants from the British Heart Foundation and the Wellcome Trust. The authors wish to thank Dr P. Anning, Dr M. Belvisi and Prof T. Evans for helpful discussion.

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(Received May 2, 2000
Revised April 9, 2001
Accepted April 30, 2001)