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P2U-receptor mediated endothelium-dependent but nitric oxide-independent vascular relaxation

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1 The dilator effect of extracellular adenosine triphosphate (ATP) has mainly been characterized as a direct effect on smooth muscle or as an endothelium-dependent effect mediated by nitric oxide (NO) or prostaglandins. We tested the hypothesis that endothelium-derived hyperpolarizing factor (EDHF) may also be involved. Dilator effects were studied *in vitro* by continuous recording of isomeric tension in cylindrical segments of rat blood vessels precontracted by noradrenaline (NA), in the presence of indomethacin (10 μM).

2 By screening different blood vessels in the rat we found that both acetylcholine (ACh) and ATP dilate mesenteric arteries with a resting tone of 1 mN by an endothelium-dependent non-NO mechanism. With an increased resting tone (4 mN) the dilatation was mediated by NO. Thus by varying the resting tension the different dilator mechanisms could be examined. However, in the carotid artery the dilatation was solely mediated by an endothelium-dependent NO mechanism, even at different resting tones (1 and 4 mN).

3 The N-nitro-L-arginine methyl ester (L-NAME)-resistant dilatation to ACh and ATP was further inhibited by the NO-scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO), indicating L-NAME insensitive NO-synthesis.

4 In carotid arteries and mesenteric arteries at high resting tones (4 mN) the ATP-dilatation was totally inhibited by endothelium removal or L-NAME (10^{-3} M). In mesenteric arteries at low resting tone (1 mN) the ATP, UTP (uridine-triphosphate) and 2-MeSATP (2methylthioATP)-dilatation was totally inhibited by endothelium removal. However, L-NAME in combination with indomethacin attenuated only 5% of the UTP dilatation, 70% of the ATP dilatation but all of the 2-MeSATP-dilatation. The inhibitors of Ca^{2+}-activated K^+ channels charybdotoxin (0.5×10^{-7} M) together with apamin (10^{-6} M), and the cytochrome P_{450} inhibitor, SKF 525A (10^{-4} M), each in combination with indomethacin, L-NAME and PTIO (0.5×10^{-3} M) totally abolished the remaining ATP and UTP-dilatation. This indicates a dilatation mediated by an endothelium-dependent non-NO factor, probably EDHF.

5 Agonist potency (UTP > ATP $\gg$ 2-MeSATP), indicates that the EDHF-mediated dilatation was stimulated by a P2U-receptor, possibly by a selective pyrimidine-receptor. In contrast, a P2Y-receptor stimulated NO-mediated dilatation (2-MeSATP = ATP > UTP).

6 In conclusion, the dilator effects of ATP and especially UTP can be mediated by an endothelium-dependent non-NO-mediated mechanism, probably EDHF, mediated by a P2U-receptor, possibly a selective pyrimidine-receptor, while NO-mediated dilatation is stimulated mainly by a $P2Y_1$-receptor. Furthermore, the EDHF-dilatation is dependent on the resting tone of the blood vessel.

Keywords: Vascular reactivity; EDHF; nitric oxide; P2Y-, P2U-purinoceptor; pyrimidine-receptor; dilatation; endothelium

Introduction

Endothelial cells that line the lumen of all blood vessels are essential in vascular physiology. The many roles of endothelial cells include the modulation of vascular smooth muscle tone through release of vasoactive agents such as nitric oxide (NO), prostacyclin and thromboxane (Davidge *et al*., 1995). The obligatory role of an intact endothelium for the relaxation induced by acetylcholine (ACh), in the rat and rabbit isolated aorta was originally demonstrated by Furchgott and Zawadzki (1980). The mediator initially referred to as EDRF was later shown to be identical to NO or a labile NO-containing molecule (Palmer *et al*., 1987). NO promotes smooth muscle relaxation by binding to the haem moiety of soluble guanylate cyclase, leading to activation of the enzyme and in turn an increase of the intracellular guanosine 3′:5′-cyclic monophosphate (cyclic GMP) level (Moncada *et al*., 1991).

In 1988, Taylor and Weston suggested that endothelium-dependent relaxation of vascular smooth muscle reflected the release of an additional factor to NO, which causes relaxation by increasing the membrane potential of the muscle cells (Taylor & Weston, 1988). This additional factor was termed endothelium-derived hyperpolarizing factor (EDHF) (Chen *et al*., 1988). So far the identity of EDHF remains unknown although it has been proposed that epoxyeicosatrienoic acids (EETs) may represent EDHF (Campbell *et al*., 1996). EDHF may be distinguished from NO by the use of arginine analogues, such as L-NAME, that inhibit NO-synthesis, but do not alter EDHF-mediated relaxations (Huang *et al*., 1988; Chen *et al*., 1991; Fujii *et al*., 1992). Furthermore, a selective NO-scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) has been discovered with the potential to inhibit L-NAME-resistant NO-synthesis (Akaike *et al*. 1993). In large conducting arteries, EDHF may provide a secondary system to NO, while in small resistance arteries (100–300 μm in diameter), EDHF appears to be a major determinant of

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vascular calibre, and may therefore be of primary importance in the regulation of vascular resistance (Garland *et al*., 1995). So far vascular smooth muscle endothelium-dependent relaxation via EDHF has been shown for cholinomimetics, bradykinin and substance P.

Adenosine 5′-triphosphate (ATP) is stored in and can be released from sympathetic nerves, platelets, endothelial cells and all cell types when damaged. It can induce both vasodilatation as well as vasoconstriction in blood vessels from most regions of the body. Generally, the contractile effect is mediated by receptors on the vascular smooth muscle cell while the dilator effect is known to be mediated through activation of endothelial cells and the subsequent release of nitric oxide or prostacyclin (Burnstock, 1990). The receptors through which these effects are manifested have been classified into A (P1)- and P2-receptors. At the A-receptor, adenosine is more potent than ATP, whereas at the P2-receptor the reverse is true. There are many different subtypes of P2-receptors, of which P2Y- and P2U-receptors stimulate endothelium-dependent relaxation. The P2Y-receptor is characterized by the more potent effects of 2-methylthioATP (2-MeSATP) in comparison to ATP and is considered to be rather insensitive to the pyrimidine uridine triphosphate (UTP), while potent effects of UTP as compared to ATP and 2-MeSATP characterizes the P2U-receptor (O'Connor *et al*., 1991). In the present study we have examined the hypothesis that EDHF is involved in the ATP-mediated vascular relaxation and characterized the receptors involved.

Methods

Tissue preparation

Female Sprague-Dawley rats (200–300 g) were anaesthetised by inhalation of CO_2 (carbon dioxide), after which they were killed by a cardiac cut. The carotid and mesenteric arteries were removed gently and immersed in cold oxygenated buffer solution and dissected free of adhering tissue under a microscope. The vessels were cut into cylindrical segments (2–3 mm long), with intact endothelium which were immediately used in the experiments. Each cylindrical segment was mounted on two L-shaped metal prongs, one of which was connected to a force displacement transducer (FT03C) for continuous recording of the isomeric tension, and the other to a displacement device (Högestätt *et al*., 1983). The position of the holder could be changed by means of a movable unit allowing fine adjustments of the vascular resting tension by varying the distance between the metal prongs. The mounted specimens were immersed in temperature controlled (37°C) tissue baths containing a buffer solution of the following composition (mM): NaCl 119, $NaHCO_3$ 15, KCl 4.6, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $CaCl_2$ 1.5 and glucose 5.5. The solution was continuously gassed with 5% CO_2 in O_2 giving a pH of 7.4.

Special care was taken to ensure that the endothelial layer was not damaged. This was checked by monitoring dilator responses to acetylcholine (ACh) in arteries precontracted with noradrenaline. ACh induced dilator responses in both carotid and mesenteric arteries with a maximal effect of 50–100% of the precontraction.

A tension of 1 or 4 mN was applied to the arterial segments and they were allowed to stabilize at this level of tension for 1 h. The contractile capacity of each vessel segment was examined by exposure to a potassium-rich (60 mM) buffer solution in which NaCl was exchanged for an equimolar concentration of KCl. When two reproducible contractions had been achieved the vessels were used for further studies.

Measurement of mechanical responses

Relaxation was studied in preparations precontracted by NA. The NA concentration was titred to give a contraction amounting to 90% of the maximum NA response (10^{-6}–10^{-5} M). Thirty minutes before each series of experiments antagonists were added. All experiments were performed with indomethacin present to abolish prostacyclin-induced dilator effects. Endothelium-removal was achieved by perfusion of the vessel for 5 s with 0.1% Triton X followed by another 5 s of perfusion with a buffer solution with a fine needle. Endothelium removal was checked by examining ACh-induced dilatation (see Results).

Agonists were added cumulatively to determine concentration-response relationships. Eight ring segments were studied at the same time in separate tissue baths. Between each series the preparations were allowed to stabilize for 40–60 min. No desensitization was observed.

SIN-1, which is active through guanylyl cyclase, and papaverin, which is not dependent on guanylyl cyclase, were used as endothelium-independent vasodilator controls.

The combinations of antagonists used

Indomethacin + L-NAME; indomethacin + L-NAME + PTIO; indomethacin + L-NAME + PTIO + SKF 525A; indomethacin + L-NAME + PTIO + charybdotoxin + apamin; indomethacin + L-NAME + PTIO + TEA; indomethacin + L-NAME + PTIO + glibenclamide. Indomethacin was always used to eliminate any prostaglandin-mediated dilatation.

Length-tension measurements

The mesenteric arteries from two rats were removed and divided into eight segments each. Three segments from each rat (randomly chosen) were immediately suspended in the standard buffer solution (for composition, see above) and mounted for continuous recording of isomeric tension (see above). The vessels were allowed to stabilize in a relaxed state for 1 h after which they were activated by a potassium-rich (60 mM) buffer solution. Following a short accommodation period (10 min), the distance between the metal prongs was increased, each time by 0.25 mm, and the tension recorded at different internal diameters. The tension was always allowed to reach a steady level in between the extensions. The magnitude of the steady state tension was used in the calculations.

The other six segments were transferred into a Ca^{2+} deficient buffer solution from which the CaCl solution had been left out. The vessels were stored in this for 24 h at 8°C in order to deplete the smooth muscle cells of activator Ca^{2+}, and therefore reduce the activity of the contractile system to a minimum. The relaxed arterial segments were subsequently suspended in this Ca^{2+} free solution. After being mounted, the vessels were allowed to stabilize in a relaxed state for 1 h and then subjected to stretches as described above.

Drugs

ATP (10^{-9}–5×10^{-5} M), 2-MeSATP (10^{-9}–10^{-5} M), UTP (10^{-9}–5×10^{-3} M), ACh (10^{-10}–10^{-5} M), indomethacin (10^{-5} M), NA (10^{-6}–10^{-5} M), papaverine (10^{-7}–10^{-3} M) and

3-morpholino-synonimine (SIN-1 $10^{-7}-10^{-3}$ M) (Sigma Co., USA). L-NAME (10^{-3} M), PTIO (5×10^{-3} M), SKF 525A (β-diethylaminoethyl-diphenyl-propyl-acetate HCl; 10^{-4} M), tetraethylammonium chloride (TEA, 10^{-4} M), charybdotoxin (5×10^{-7} M), apamin (10^{-6} M) and glibenclamide (10^{-5} M) (Research Biochemicals International, U.S.A.). All drugs were dissolved in 0.9% saline.

Calculations and statistics

When we studied the relaxation, the amplitude of contraction before application of agonists was set to 100%. The negative logarithm of the drug concentration eliciting 50% relaxation (pD_2) was determined by linear regression analysis by use of the values immediately above and below half-maximum response. R_{max} refers to maximum relaxation. Values are presented as mean $\pm$ s.e.mean. All experiments were performed on at least six segments (animals). Statistical significance was accepted when $P<0.05$, ANOVA. All differences referred to in the text have been statistically verified.

Results

The dilator responses were examined in carotid and mesenteric arteries at high (4 mN) and low (1 mN) resting tones. The results were similar in the carotid artery (at both tensions) as for the mesenteric artery at high resting tension. For clarity and to save space only the results from the mesenteric artery are presented.

The contractile response of the mesenteric artery to 60 mM K^+ at a low resting tone (1 mN) was 5.2 ± 0.3 mN and at a high resting tone was 4.8 ± 0.3 mN.

Indomethacin

Indomethacin (10^{-5} M) did not have any effect in the absence of L-NAME. At low resting tension, in the presence of L-NAME, indomethacin attenuated the ATP-induced dilatation by 10%, indicating a minor contribution of cyclo-oxygenase products.

Dilator responses to adenosine

The dilator effects of adenosine were only seen at high concentrations (10^{-4} M).

Dilator responses to ACh

ACh induced a concentration-dependent and almost complete relaxation in both mesenteric arteries at high (4 mN) and low (1 mN) resting tones (Figure 1 and Table 1). The relaxation was totally abolished in endothelium-denuded vessels (Table 1). In mesenteric artery at a low resting tone L-NAME had only minor effects, while in mesenteric artery at a high resting tone L-NAME virtually abolished the dilatation (Figure 1 and Table 1). When L-NAME was used together with PTIO in mesenteric arteries at a low resting tone the dilatation was further antagonized with a rightward shift of the dose-response curve indicating an L-NAME-resistant dilatation remaining, but with a major part of the maximum dilatation still present (Figure 1 and Table 1).

SKF 525A or charybdotoxin combined with apamin used in the presence of L-NAME abolished the dilatation in mesenteric artery at a low resting tone (Table 1). When PTIO was added the minimal remaining relaxation disappeared totally. In the presence of L-NAME, TEA and glibenclamide had no effect, but when PTIO was added, TEA or glibenclamide partly inhibited the relaxation (Figure 1a and Table 1).

SKF 525A or charybdotoxin and apamin used in the absence of L-NAME in mesenteric artery at a high resting

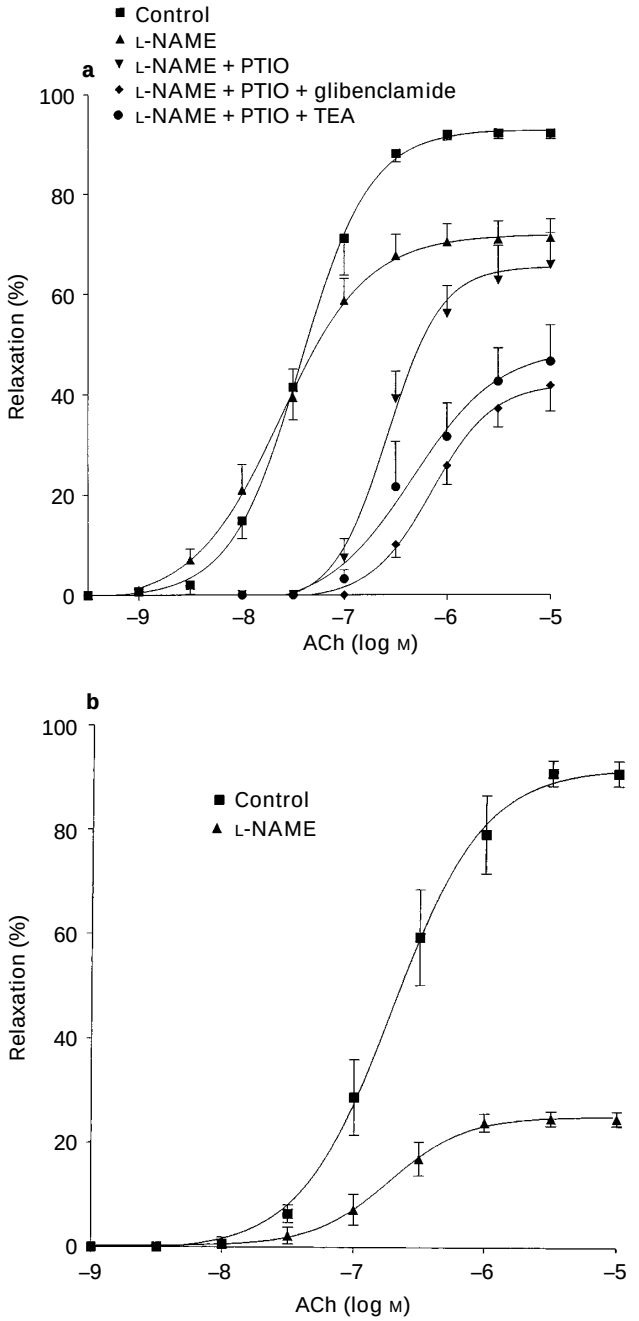

Figure 1 Concentration-dependent relaxations to ACh in segments of mesenteric artery at a resting tone of 1 mN (a) and 4 mN (b). Concentration-response curves were constructed in the absence (control) and in the presence of L-NAME (10^{-3} M). In mesenteric artery at a low resting tone (a) preparations were also exposed to the combinations of L-NAME and PTIO (5×10^{-3} M), L-NAME, PTIO and glibenclamide (10^{-5} M) and L-NAME, PTIO and TEA (5×10^{-2} M). L-NAME and PTIO in combination with charybdotoxin (5×10^{-7} M) and apamin (10^{-6} M) or SKF 525A (10^{-4} M) totally abolished the dilator response and are not shown in this diagram. Dilator responses are expressed as percentage of an initial contraction induced by NA. Data are shown as means of six to ten experiments; vertical lines show s.e.mean.

Table 1 Dilator effects of ACh on mesenteric artery at high (4 mN) and low (1 mN) resting tone

	Low tone		*High tone*	
	R_{max}	pD_2	R_{max}	pD_2
In the absence of L-NAME (10^{-3} M)				
Control	92.1±1.2	7.4±0.1	91.3±2.5	6.7±0.1
Denuded	0±0	–	0±0	–
SKF 525A (10^{-4} M)			84.5±3.9	5.6±0.1
Charybdotoxin (10^{-7} M) and apamin (10^{-6} M)			85.9±1.9	6.6±0.1
K^+ (30 mM)			24.3±3.2	6.9±0.3
In the presence of L-NAME				
Control	71.8±3.3	7.5±0.2	25.1±1.4	6.8±0.2
SKF 525A	1.2±0.6	2.2±1.1		
Charybdotoxin and apamin	0±0	–		
In the presence of L-NAME and PTIO (5×10^{-3} M)				
Control	66.3±6.6	6.5±0.1		
SKF 525A	0±0	–		
Charybdotoxin and apamin	0±0	–		
TEA (10^{-4} M)	48.8±7.5	6.3±0.2		
Glibenclamide (10^{-5} M)	41.9±5.1	5.2±0.9		

The relaxations are expressed as percentage of an initial contraction induced by NA. Data are shown as R_{max}±s.e.mean and pD_2±s.e.mean.

tone did not affect the maximum dilatation but caused a rightward shift of the dose-response curves (Table 1, Figure 2). K^+ (30 mM) non-specifically reduced the maximum relaxation markedly from 91.3±2.5% to 24.3±3.2% (Figure 2 and Table 1).

Dilator responses to ATP

As for ACh, ATP induced a concentration-dependent and almost complete relaxation in both mesenteric arteries at high (R_{max} 75.0±9.9%) and low (R_{max} 94.2±1.5%) resting tension (Figure 2 and Table 2). The relaxation was abolished in endothelium-denuded vessels (Table 2). At a high resting tone L-NAME and PTIO virtually abolished the relaxation (Figure 3 and Table 2). At a low resting tone L-NAME and PTIO was not able to inhibit all of the ATP-induced dilatation, leaving 15.5±3.0%. SKF 525A, charybdotoxin and apamin, TEA or glibenclamide used in the presence of L-NAME and PTIO abolished the remaining dilatation totally (Figure 3a and Table 2).

Dilator responses to UTP

UTP induced a concentration-dependent relaxation in both mesenteric arteries at high (R_{max} 61.0±4.0%) and low (R_{max} 69.2±4.6%) resting tension (Figure 4 and Table 3). The relaxation was abolished in endothelium-denuded vessels (Table 3). At high resting tone, L-NAME and PTIO abolished the relaxation (Figure 4b and Table 3). At low resting tone L-NAME had minor effects on the UTP-dilatation, with a minor reduction from 69.2±4.6% to 64.7±3.7%. Addition of PTIO did not reduce the dilatation further (67.4±6.9%), but caused a minor rightward shift of the curve. SKF 525A or charybdotoxin and apamin used in the presence of L-NAME and PTIO abolished the remaining dilatation (Figure 3a and Table 2). In the presence of L-NAME and PTIO, TEA or glibenclamide partly attenuated the dilatation.

Dilator responses to 2-MeSATP

2-MeSATP induced a concentration-dependent relaxation in both mesenteric arteries at high (R_{max} 40.2±3.0%) and low (R_{max} 46.0±1.3%) resting tension (Figure 5 and Table 4). The relaxation was abolished in endothelium-denuded vessels (Table 4). At a high resting tone L-NAME abolished the relaxation (Figure 5 and Table 4). At a low resting tone L-NAME reduced the 2-MeSATP-dilatation from 46.0±1.3% to 24.1±3.7%. Addition of PTIO abolished the dilatation totally. SKF 525A or charybdotoxin and apamin, TEA or glibenclamide used in the presence of L-NAME and PTIO could not affect the response further since the dilatation already was abolished.

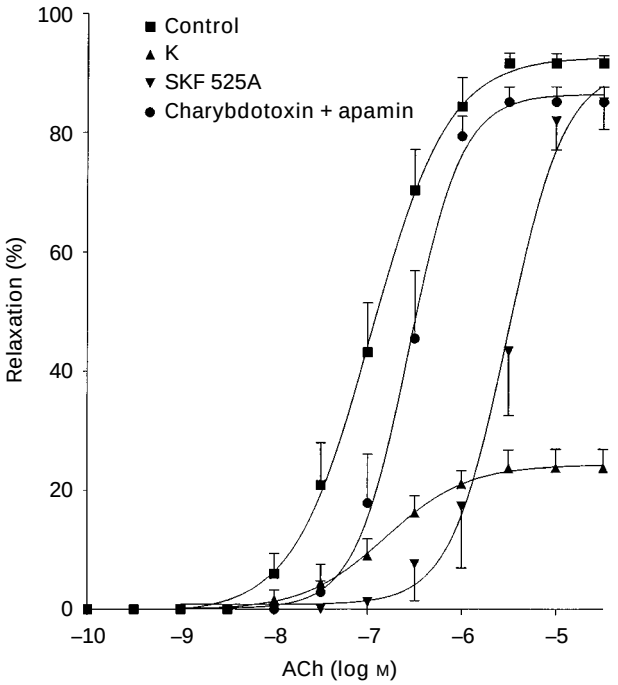

Figure 2 Relaxation induced by ACh in mesenteric artery at a high resting tone in the absence and in the presence of 30 mM K^+, SKF 525A (10^{-4} M) and charybdotoxin (5×10^{-7} M) together with apamin (10^{-6} M). Dilator responses are expressed as percentage of an initial contraction induced by NA. Data are shown as means of six to ten experiments; vertical lines indicate s.e.mean.

Length-tension characteristics

The length-tension diagram (Figure 6) shows a maximum response to K^+ (60 mM) at a passive wall tension of 1.7 ± 0.4 mN. At passive tensions of 1 and 4 mN the reactivity of the vessel was similar to each other, 1.2 ± 0.1 mN and 1.2 ± 0.9 mN respectively. Laplace law was used to calculate the corresponding blood pressure which was 43 mmHg for a passive wall tension of 1 mN, 57 mmHg for 1.7 mN and 105 mmHg for 4 mN.

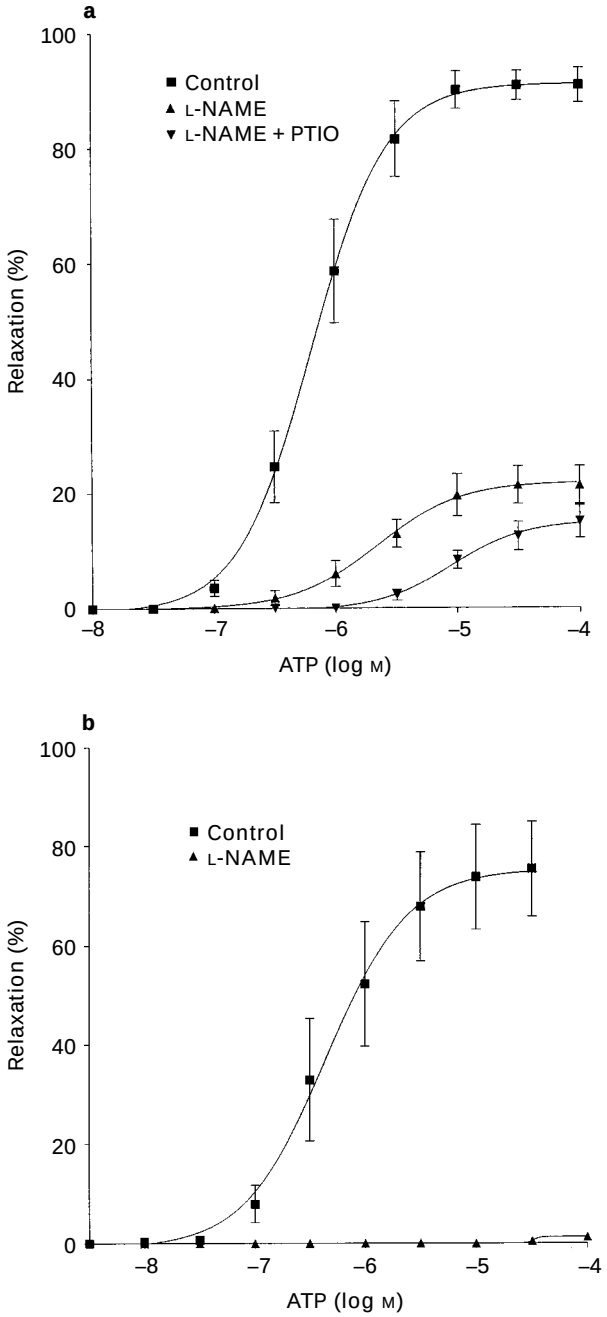

Figure 3 Concentration-dependent relaxations to ATP in segments of mesenteric artery at a resting tone of 1 mN (a) and 4 mN (b). Concentration-response curves were constructed in the absence (control) and in the presence of L-NAME (10^{-3} M). In mesenteric artery at a low resting tone (a) preparations were also exposed to L-NAME and PTIO (5×10^{-3} M). L-NAME and PTIO in combination with charybdotoxin (5×10^{-7} M) and apamin (10^{-6} M), SKF 525A (10^{-4} M), TEA (5×10^{-2} M) or glibenclamide (10^{-5} M) totally abolished the dilator response and are not shown in this diagram. Dilator responses are expressed as percentage of an initial contraction induced by NA. Data are shown as means of six to ten experiments; vertical lines show s.e.mean.

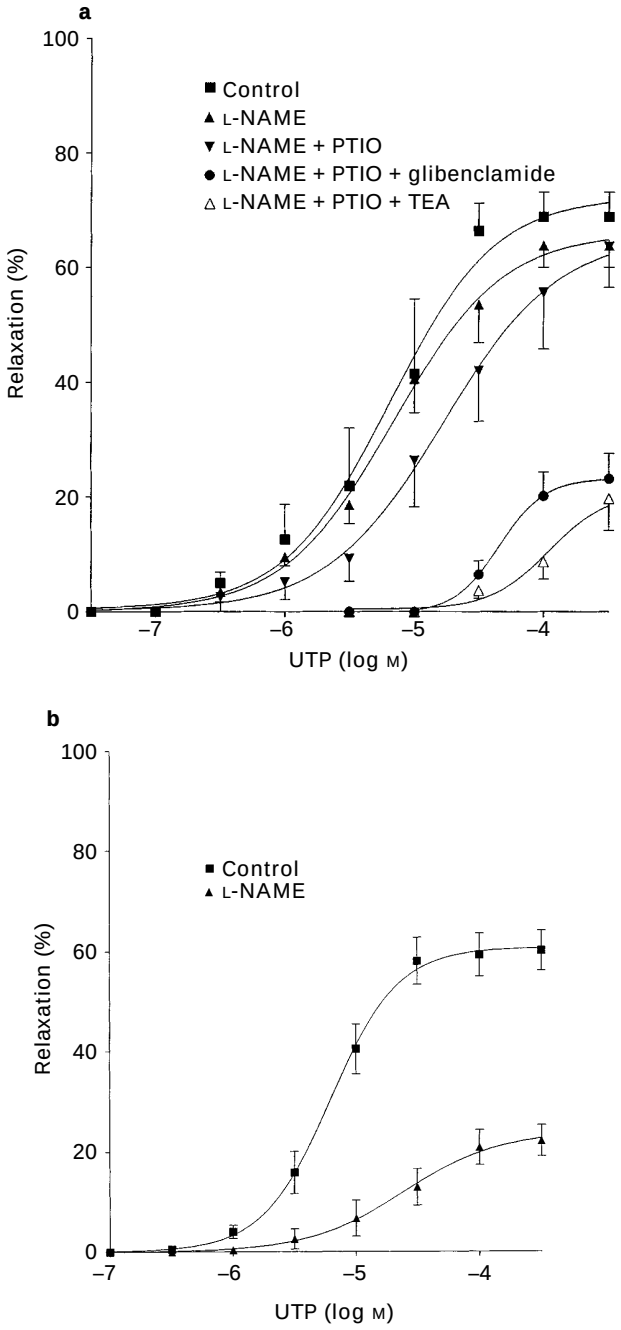

Figure 4 Concentration-dependent relaxations to UTP in segments of mesenteric artery at a resting tone of 1 mN (a) and 4 mN (b). Concentration-response curves were constructed in the absence (control) and in the presence of L-NAME (10^{-3} M). In mesenteric artery at a low resting tone (a) preparations were also exposed to the combinations of L-NAME and PTIO (5×10^{-3} M), L-NAME, PTIO and glibenclamide (10^{-5} M) and L-NAME, PTIO and TEA (5×10^{-2} M). L-NAME and PTIO in combination with charybdotoxin (5×10^{-7} M) and apamin (10^{-6} M) or SKF 525A (10^{-4} M) totally abolished the dilator response and are not shown in this diagram. Dilator responses are expressed as percentage of an initial contraction induced by NA. Data are shown as means of six to ten experiments; vertical lines show s.e.mean.

Table 2 Dilator effects of ATP on mesenteric artery at high (4 mN) and low (1 mN) resting tone

	Low tone		*High tone*	
	R_{max}	pD_2	R_{max}	pD_2
In the absence of L-NAME (10^{-5} M)				
Control	94.2±1.5	6.1±0.1	75.0±9.9	6.3±0.2
Denuded	0±0	–	0±0	–
In the presence of L-NAME				
Control	22.1±3.5	5.5±0.3	1.4±1.0	1.5±0.9
In the presence of L-NAME and PTIO (5×10^{-3} M)				
Control	15.5±3.0	5.0±0.1		
SKF 525A (10^{-4} M)	0±0	–		
Charybdotoxin (5×10^{-7} M) and apamin (10^{-6} M)	0±0	–		
TEA (10^{-4} M)	0±0	–		
Glibenclamide (10^{-5} M)	0±0	–		

The relaxations are expressed as percentage of an initial contraction induced by NA. Data are shown as $R_{max}\pm$s.e.mean and $pD_2\pm$s.e.mean.

Table 3 Dilator effects of UTP on mesenteric artery at high (4 mN) and low (1 mN) resting tone

	Low tone		*High tone*	
	R_{max}	pD_2	R_{max}	pD_2
In the absence of L-NAME (10^{-5} M)				
Control	69.2±4.6	5.2±0.2	61.0±4.0	5.2±0.1
Denuded	0±0	–	0±0	–
In the presence of L-NAME				
Control	64.7±3.7	5.1±0.1	22.7±3.1	4.7±0.2
In the presence of L-NAME and PTIO (5×10^{-3} M)				
Control	67.4±6.9	4.7±0.2	0±0	–
SKF 525A (10^{-4} M)	0±0	–		
Charybdotoxin (5×10^{-7} M) and apamin (10^{-6} M)	0±0	–		
TEA (10^{-4} M)	20.3±5.7	4.1±0.1		
Glibenclamide (10^{-5} M)	23.2±4.3	4.4±0.1		

The relaxations are expressed as percentage of an initial contraction induced by NA. Data are shown as $R_{max}\pm$s.e.mean and $pD_2\pm$s.e.mean.

Table 4 Dilator effects of 2-MeSATP on mesenteric artery at high (4 mN) and low (1 mN) resting tone

	Low tone		*High tone*	
	R_{max}	pD_2	R_{max}	pD_2
In the absence of L-NAME (10^{-5} M)				
Control	46.0±1.3	6.6±0.3	40.2±3.0	6.8±0.1
Denuded	0±0	–	0±0	–
In the presence of L-NAME				
Control	24.1±3.7	6.4±0.2	5.8±3.0	2.6±1.2
In the presence of L-NAME and PTIO (5×10^{-3} M)				
Control	0±0	–		
SKF 525A (10^{-4} M)	0±0	–		
Charybdotoxin (5×10^{-7} M) and apamin (10^{-6} M)	0±0	–		
TEA (10^{-4} M)	0±0	–		
Glibenclamide (10^{-5} M)	0±0	–		

The relaxations are expressed as percentage of an initial contraction induced by NA. Data are shown as $R_{max}\pm$s.e.mean and $pD_2\pm$s.e.mean.

Table 5 Dilator effects of SIN-1 and papaverine at a low resting tension (1 mN)

	SIN-1		*Papaverine*	
	R_{max}	pD_2	R_{max}	pD_2
Control	98.0±1.1	5.6±0.1	101.3±1.3	4.7±0.0
SKF 525A (10^{-4} M)	100.5±1.7	5.5±0.1	100.2±0.2	4.7±0.0
Charybdotoxin (5×10^{-7} M) and apamin (10^{-6} M)	97.5±1.1	5.7±0.1	100.0±0.0	4.8±0.0

The relaxations are expressed as a percentage of an initial contraction induced by NA. Data are shown as $R_{max}\pm$s.e.mean and $pD_2\pm$s.e.mean.

Endothelium-independent vasodilators

Neither SKF 525A or charybdotoxin and apamin inhibited the dilator response to SIN-1 or papaverin in the mesenteric arteries at a low resting tension (Table 5).

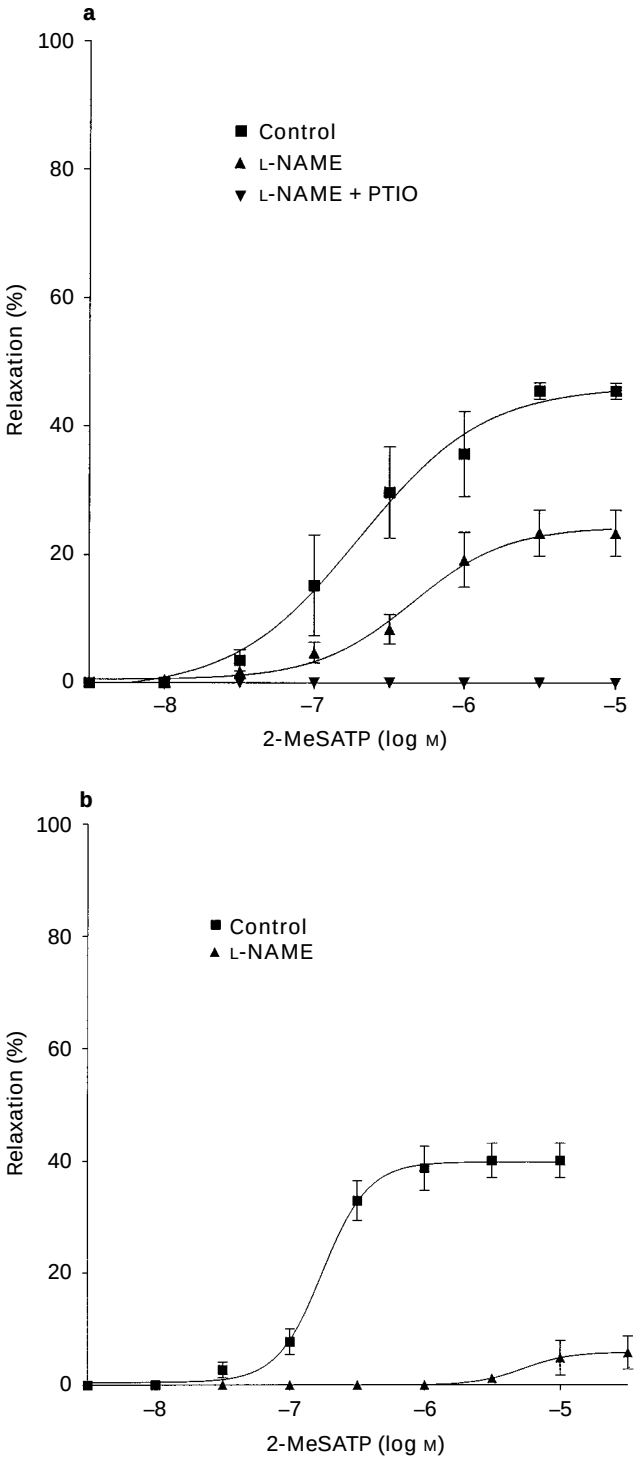

Figure 5 Concentration-dependent relaxations to 2-MeSATP in segments of mesenteric artery at a resting tone of 1 mN (a) and 4 mN (b). Concentration-response curves were constructed in the absence (control) and in the presence of L-NAME (10^{-3} M). In mesenteric artery at a low resting tone (a) preparations were also made with L-NAME and PTIO (10^{-3} M). L-NAME and PTIO in combination with charybdotoxin (10^{-7} M) and apamin (10^{-6} M), SKF 525A (10^{-4} M), TEA (10^{-2} M) or glibenclamide (10^{-5} M) totally abolished the dilator response and are not shown in this diagram. Dilator responses are expressed as percentage of an initial contraction induced by NA. Data are shown as means of six to ten experiments; vertical lines show s.e.mean.

Discussion

The endothelium has emerged as an important regulator of vascular tone (Furchgott & Vanhoutte, 1989; Moncada *et al*., 1991). Several soluble mediators released by the endothelium are involved in these vascular effects. These mediators include prostacyclin, EDRF or NO, and EDHF. The dilator effects of ATP and its analogues have so far mainly been characterized as a direct effect on smooth muscle or as an endothelium-dependent effect mediated by NO or prostaglandins. However, the ATP-induced vasodilator response in human forearm is not mediated by NO-release (Rongen *et al*., 1994). To examine the possibility of a purinergic mediated EDHF-dilation rat blood vessels were examined.

EDHF is dependent on resting tension

As shown previously (Garland & McPherson, 1992), the dilator response to ACh in the mesenteric artery was endothelium-dependent but L-NAME-resistant. Surprisingly, this non-NO-induced dilatation was dependent on the resting tone of the blood vessel. The relaxation was only slightly attenuated by the NO-synthase inhibitor L-NAME or even by addition of the NO-scavenger PTIO at a low resting tone (1 mN) of the mesenteric artery, indicating an endothelium-dependent non-NO mechanism. In mesenteric arteries at a high resting tone (4 mN) and in the carotid arteries at different resting tones (1 and 4 mN) the dilatation was preferentially mediated by NO, as most of it could be blocked by L-NAME and PTIO. The results are similar to the findings of Zygmunt *et al*. (1994), in rat hepatic artery and aorta where the L-NAME resistant dilatation was more pronounced at a weak NA-precontraction. The reason for this is not known but evidence suggests that endothelial release of vasodilator substances is dependent on stretch of

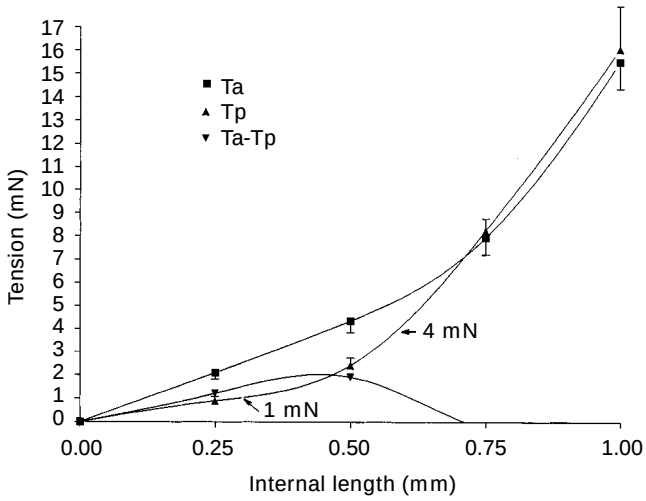

Figure 6 Relation between effective vessel lumen length and wall tension in relaxed (T_p) and K^+ activated (T_a) rat mesenteric artery. The T_p at the various lengths was derived from arteries immersed in Ca^{2+}-free buffer solution. The vessels were stepwise extended from slack length to a length of 1 mm and the wall tension measured at each length. A passive wall tension of 1 mN corresponds to 43 mmHg and 4 mm to 105 mmHg according to Laplace law. Separate arterial segments were initally contracted by K^+ before being subjected to identical streches and the active tension (T_a) was recorded. A subtraction was made between the mean T_a and T_p ($T_a - T_p$) to visualize the greatest reactivity of the vessel. Data are shown as means of eight measurements; vertical lines indicate s.e.mean.

the vessel, e.g., being increased by pulsatile flow (Hutcheson & Griffith 1994).

According to the length-tension characteristics of the mesenteric artery, the passive tension used in this system (1 and 4 mN) is within the range of the greatest reactivity of the vessel. The blood pressure calculated from Laplace law for a passive wall tension of 1 mN is 43 mmHg and for 4 mN, 105 mmHg (Figure 6). Thus, the simulated blood pressure in our system is similar to the normal blood pressure in the rat mesenteric artery and therefore of physiological relevance. The NO-independent dilatation was considerably more pronounced at a low vessel wall tension (43 mmHg). This indicates that EDHF is of more importance in low pressure vascular beds, e.g. in small resistance arteries, as previously proposed by Garland *et al*. (1995).

Thus, by varying the resting tone and the type of blood vessel used, the different dilator mechanisms could be examined. Several lines of evidence indicate that dilatation to ACh, ATP and UTP in mesenteric arteries with a low resting tension is to a large extent mediated by EDHF: firstly, the ACh, ATP and especially UTP-induced dilatations were not blocked by L-NAME combined with PTIO. The high concentration of L-NAME used in the present experiments (10^{-3} M) has in previous studies been shown to abolish the

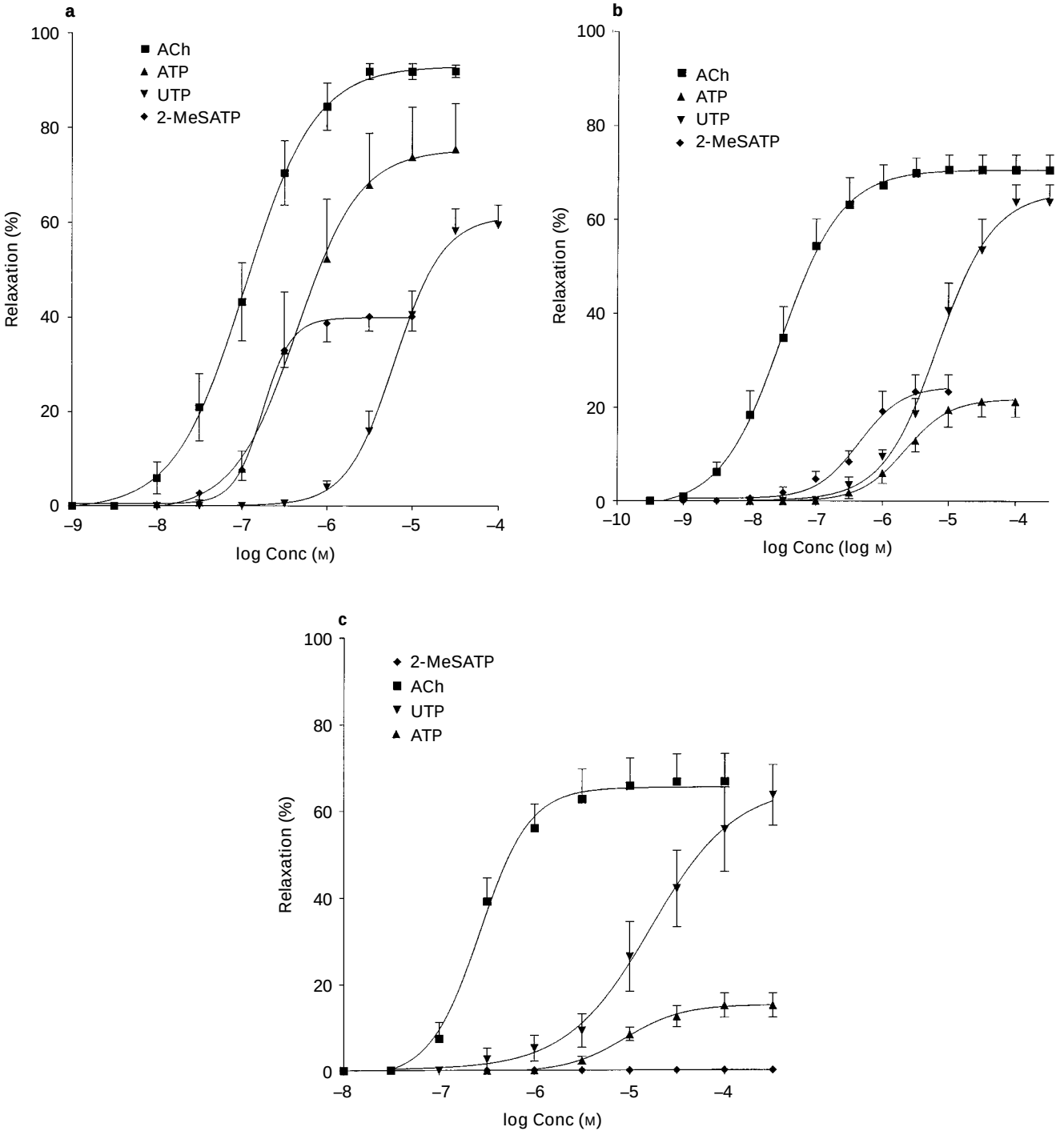

Figure 7 Concentration-dependent relaxation to ACh, ATP, UTP and 2-MeATP in mesenteric artery at a high resting tone (4 mN) (a), and in mesenteric artery at a low resting tone (1 mN) in the presence of L-NAME (10^{-3} M) (b) and in the presence of L-NAME and PTIO (5×10^{-3} M) (c). Dilator responses are expressed as percentage of an initial contraction induced by NA. Data are shown as means of six to ten experiments; vertical lines show s.e.mean.

release of NO from rat aorta (Zygmunt *et al*., 1994). However, in our experiments, addition of the NO-scavenger PTIO further antagonized the dilatation. Hence, both procedures were used to be certain that the NO-dilatation was abolished. Secondly, indomethacin was present excluding the possibility of a cyclo-oxygenase product being responsible for the L-NAME resistant dilatation. Thirdly, the activity of EDHF may be distinguished from NO in that it is blocked by inhibitors of Ca^{2+}-activated K^+ channels although ATP-sensitive K^+ channels may also be involved in some tissues (Garland *et al*., 1995). In rat arteries, both the EDHF relaxation and hyperpolarization were blocked by inhibitors of different Ca^{2+}-activated K^+ channels, TEA or a combination of charybdotoxin and apamin (Zygmunt & Högestätt, 1996). Our results demonstrates that the L-NAME and PTIO resistant dilatation to ACh, ATP and UTP was totally abolished by a combination of charybdotoxin and apamin, indicating a role for Ca^{2+}-activated K^+ channels. High concentrations of TEA (10^{-2} M) had the same inhibitory effect (data not shown). However, at these concentrations TEA is a non-selective potassium-channel blocker. With a dose where it appears to be selective for Ca^{2+}-activated K^+ channels (10^{-4} M), it only partly inhibited the remaining ACh-dilatation, with somewhat better effect against UTP and total inhibition of ATP. The inhibitor of ATP-sensitive K^+ channels, glibenclamide, had very similar effects. The data suggests that both Ca^{2+}-activated K^+ channels and ATP sensitive K^+ channels are involved in the L-NAME and PTIO resistant dilatation to ACh, ATP and UTP. It is interesting that in the absence of PTIO, TEA and glibenclamide had no effect, indicating a L-NAME resistant synthesis of NO. Fourthly, evidence suggests that the generation of EDHF is dependent on cytochrome P_{450}-dependent enzymes and that EDHF-relaxations are inhibited by cytochrome P_{450} inhibitors such as SKF 525A (Bauersachs *et al*., 1994). Chen and Cheung (1996) recently demonstrated that the hyperpolarization response to ACh in rat mesenteric arteries depends on the level of cytochrome P_{450} activity. In our experiments SKF 525A totally abolished the NO-independent ACh, ATP and UTP dilatation.

We examined the specificity for SKF 525A and the combination of charybdotoxin and apamin as EDHF-inhibitors on other vasodilators acting independently of the endothelium. They did not modify relaxation to the NO-donor SIN-1 and therefore do not affect the direct NO-mediated activation of guanylate cyclase in the smooth muscle cell. Neither did they have any effect on the papaverin-mediated dilatation and can therefore not affect ability of papaverin to inhibit cyclic nucleotide phosphodiesterase via an endothelium-independent mechanism.

Taken together, these results indicate a dilatation mediated by an endothelium-derived non-NO factor, activated by cytochrome P_{450}, later acting upon Ca^{2+}-activated K^+ channels, probably EDHF. However, in mesenteric arteries at a high resting tone SKF 525A, and to a lesser extent charybdotoxin and apamin caused a rightward shift of the NO-mediated dilatation without affecting the maximum effect. Thus, the inhibitory effects of these antagonists are not totally specific to EDHF. High potassium (30 mM) caused vascular contraction and acted as a non-specific inhibitor of vascular relaxation in this system and could not be used to discriminate between NO and EDHF-mediated dilatation. Zygmunt *et al*. (1994) found that a stronger precontraction by NA made the EDHF-mediated dilatation vanish. The strong precontraction caused by K^+ could abolish the NO-independent dilatation by a similar mechanism. These non-specific effects of proposed EDHF-'blockers' have not been emphasized in the literature. They may in part be explained by findings that NO also hyperpolarizes vascular smooth muscle and stimulates K^+ channel activity (Tare *et al*., 1990; Bolotina *et al*., 1994; Archer *et al*., 1994; Garland *et al*., 1995). Furthermore, cytochrome P_{450} inhibitors, including SKF 525A (proadifen), exert profound effects on a variety of K^+-channel subtypes, indicating that enzymes depending on this cofactor may regulate K^+-channels (Edwards *et al*., 1996).

L-NAME resistant NO-synthesis

High concentrations of L-NAME (10^{-3} M), are often considered to block NO-synthesis totally. Our experiments with the addition of the NO-scavenger PTIO indicates a remaining L-NAME resistant NO-synthesis and dilatation. Thus, in future studies the addition of PTIO is recommended before concluding that the dilatation is mediated by EDHF.

Differences between species

The NO-resistant dilator mechanisms were studied here in Sprague-Dawley rats. In Wistar rats this dilator response was not observed (data not shown).

ATP and UTP stimulate EDHF-mediated dilatation

To our knowledge this is the first study to indicate an ATP-dilatation mediated by EDHF. Keef *et al*. (1992) demonstrated that ATP hyperpolarized smooth muscle cells in guinea-pig coronary artery. This hyperpolarization was abolished after endothelium removal. However, the hyperpolarization did not appear to be critical for relaxation. Chen and Suzuki (1991) observed that ATP and ADP hyperpolarized smooth muscle cells in an endothelium-dependent way. However, they did not examine any dilator effects of the nucleotides. In our system a significant part of the ATP-induced dilatation seems to be mediated by EDHF. To confirm this further we plan to set up electrophysiological methods to demonstrate hyperpolarization of the smooth muscle cell. This has been demonstrated in rat mesenteric artery for ACh (Garland & McPherson, 1992).

Different endothelium-dependent relaxation for P2Y- and P2U-receptors

In carotid and mesenteric arteries at a high resting tension the dilator effects of the nucleotides (2-MeSATP = ATP > UTP) indicate that the NO-mediated dilatation is stimulated by a P2Y-receptor (Figure 7a). The 2-MeSATP dilatation was abolished by L-NAME and PTIO, while the UTP dilatation was only slightly reduced in the mesenteric arteries at a low resting tension. The effects of the nucleotides indicate that the EDHF-mediated dilatation was stimulated by a P2U-receptor, since the order of potency was UTP > ATP$\gg$2-MeSATP (Figure 7c). This demonstrates that two endothelium-dependent dilator pathways are selectively activated by different P2-receptors. In analogy, EDHF release evoked by ACh has been shown to be mediated by M_1 muscarinic receptors, while the release of NO may be mediated by M_2 muscarinic receptors (Komori & Suzuki, 1987).

Previously, both P2Y- and P2U-receptors have been shown to stimulate endothelium-dependent dilatation via prostaglandins and nitric oxide (O'Connor *et al*., 1991). The receptors coexist on bovine aortic endothelial cells where their second messenger systems have been examined (Purkiss *et al*., 1994; Communi *et al*., 1995a, b). Both P2Y- and P2U-receptors

increase intracellular Ca^{2+} by stimulating protein kinase C and inositol phosphate release. However, the P2U-receptor-mediated response was sensitive to pertussis toxin and the P2Y-receptor-mediated response was more strongly and rapidly inhibited by phorbol 12-myristate 13-acetate. This indicates that the receptor subtypes may use different G-proteins and second messenger pathways, which supports the possibility of selective NO and EDHF-release for $P2Y_1$- and P2U-receptors.

Cloned P2Y- and P2U-receptors

Since the cloning of the first P2-receptor (Webb *et al*., 1993), the numbers of subtypes identified has increased rapidly so that this receptor family has become one of the largest families of G-protein coupled receptors, possibly indicating important physiological functions. It has been suggested that P2Y- and P2U-receptors are included in the same receptor family where at least six G-protein coupled receptor subtypes have been cloned and called $P2Y_1$–$P2Y_6$ (Fredholm *et al*., 1994). Of these at least three are responsive to UTP ($P2Y_2$, $P2Y_4$ and $P2Y_6$) and may be involved in responses previously classified as P2U-mediated.

The agonist potency of the L-NAME and PTIO-resistant dilatation is dominated by the UTP effect with a weak ATP effect and no effect of 2-MeSATP. In fact, the weak ATP-effect indicates a selective 'pyrimidine-receptor', possibly $P2Y_4$ or $P2Y_6$ (Communi *et al*., 1995a, b; Chang *et al*., 1995). To our knowledge, this is the first indication of a selective 'pyrimidine-receptor' in the endothelium.

Conclusions

The dilator effect of ATP and UTP can be mediated by an endothelium-dependent non-NO-mediated mechanism, probably EDHF. The dilator effects are mediated preferably by a P2U receptor, possibly a selective 'pyrimidine-receptor', while NO-mediated dilatation is mainly stimulated by a $P2Y_1$ receptor. Furthermore, the EDHF-dilatation is dependent on the resting tone of the blood vessel.

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