Br. J. Pharmacol. (1990), **100**, 427–434

Endothelium-dependent and BRL 34915-induced vasodilatation in rat isolated perfused mesenteric arteries: role of G-proteins, K^+ and calcium channels

[1]Ayotunde S.O. Adeagbo & [2]Kafait U. Malik

Department of Pharmacology, University of Tennessee, Memphis, TN 38163, U.S.A.

1 In the isolated perfused, noradrenaline (NA)–constricted mesenteric arteries of the rat, acetylcholine (0.003–1 nmol), histamine (0.01–10 nmol) and the calcium ionophore A23187 (0.01–1 nmol), caused endothelium-dependent vasodilatation while the vasodilatation by the K^+ channel activator BRL 34915 (0.1–1 nmol) was independent of endothelium.

2 The guanylate cyclase inhibitor, methylene blue at 10 μM did not inhibit the action of any of the vasodilators but at 50 μM reduced the vasodilator effect of acetylcholine (ACh), histamine and A23187.

3 Infusion of ouabain or perfusion with K^+-free or excess K^+ (50 mM) Krebs solution reduced the vasodilator effect of ACh, histamine and A23187, suggesting the action of these agents involves, at least in part, activation of Na^+/K^+-ATPase. The vasodilator effect of BRL 34915 was not affected by ouabain, but abolished during perfusion with Krebs solution containing excess K^+ or depleted of K^+.

4 Five structurally distinct K^+ channel blockers (apamin, crude scorpion venom, procaine, quinidine and tetraethylammonium) attenuated the vasodilator effect of ACh, histamine and A23187. The K^+ channel blockers, except apamin and crude scorpion venom, also inhibited the vasodilatation produced by BRL 34915.

5 The vasodilator effect of ACh, histamine or A23187 was not altered in mesenteric vessels of pertussis toxin-treated rats, suggesting that the K^+ channels associated with the endothelium-dependent vasodilator effect of these agents are either not coupled to G-proteins or are coupled to G-proteins that are insensitive to pertussis toxin.

6 The calcium channel blockers, diltiazem (0.1 or 1 μM), nifedipine (0.01 or 0.1 μM) or nitrendipine (1 nM) attenuated the vasodilatation produced by ACh, histamine, A23187 and also that by BRL 34915.

7 We conclude that endothelium-dependent vasodilatation induced by ACh, histamine and A23187 is mediated via activation of membrane K^+ channels and Na^+/K^+-ATPase. The K^+ channels involved in the vasodilator action of these agents are not coupled to pertussis toxin-sensitive G-proteins and appear to be regulated by Ca^{2+}.

Introduction

Vascular relaxation in response to acetylcholine (ACh) and a variety of other vasodilators has been shown to be dependent on an intact, functional endothelium (Furchgott & Zawadzki, 1980). In the rat, the endothelium-dependent nature of the vasorelaxation induced by ACh, calcium ionophore A23187 and histamine has been demonstrated in the aorta and pulmonary artery (Van De Voorde & Leusen, 1983; Chen *et al.*, 1988), perfused mesenteric (Burdet *et al.*, 1986; Furchgott *et al.*, 1987; Randall & Hiley, 1988; Bhardwaj & Moore, 1988) and tail (Spokas & Folco, 1984) arteries. These agents are believed to act through the release of endothelium-derived relaxing factor (EDRF). EDRF (and agents that cause its release) relaxes vascular smooth muscle via activation of guanylate cyclase. The evidence for this is derived from the observations that the action of agents that release EDRF is often accompanied by elevation of guanosine 3′:5′-cyclic monophospate (cyclic GMP) levels (Moncada *et al.*, 1988) and can be blocked by inhibitors of guanylate cyclase, methylene blue and oxyhaemoglobin (Murad *et al.*, 1978; Martin *et al.*, 1985). The relative insensitivity of endothelium-dependent effects to guanylate cyclase inhibitors in the rat mesenteric artery (Furchgott *et al.*, 1987) raises the possibility that this effect may be mediated via another mechanism, notably increased potassium permeability as suggested by Bolton *et al.* (1983) through activation of either the Na^+/K^+ pump (Haddy, 1978; Webb & Bohr, 1978; De Mey & Vanhoutte, 1980; Rubanyi & Vanhoutte, 1985) or directly via K^+ channels (Gordon & Martin, 1983; Petersen & Maruyama, 1984; Gebremedhin *et al.*, 1987).

ACh which acts on muscarinic receptors and certain other receptor ligands are known to elicit their effects by activation or enhancement of the activity of resting K^+ channels and recent evidence suggests that a guanine nucleotide binding-protein (G-protein) couples the specific receptors of these ligands to K^+ channels (Pfaffinger *et al.*, 1985; Andrade *et al.*, 1986; Codina *et al.*, 1987; Yamashita *et al.*, 1987; Nakajima *et al.*, 1988). Since muscarinic receptors involved in endothelium-dependent vasodilatation to ACh have also been linked to K^+ channels in bovine aortic endothelial cells (Olesen *et al.*, 1988), it is reasonable to expect modulation by K^+ channel blockers of endothelium-dependent responses. Furthermore, since G-proteins mediate the signal transduction of some K^+ channel-linked responses (Stryer & Bourne, 1986; Gilman, 1987; Sasaki & Sato, 1987), procedures that interfere with GTP-binding proteins, such as treatment with pertussis toxin, could also be expected to affect biological functions coupled to these channels. Accordingly, the objectives of the present study were two fold: first, to ascertain by use of K^+ channel blocking agents, the role of K^+ channels in endothelium-dependent vasodilatation elicited by ACh, A23187 and histamine. Second, to determine the influence of pertussis toxin treatment on the vasodilator effect of these agents in order to characterize further the contribution of G-protein coupled K^+ channels to endothelium-dependent responses. In addition, since the role of calcium in the release of EDRF is unequivocal, we

[1] Present address: Division of Basic Medical Sciences, Faculty of Medicine, Memorial University of Newfoundland, St. John's, Newfoundland, Canada A1B 3V6.
[2] Author for correspondence.

have also examined the action of calcium channel antagonists on endothelium-dependent vasodilatation in rat mesenteric arteries.

Methods

Experiments were performed on male rats (Charles River, Indianapolis, IN) which weighed 250–300 g. Under ether anesthesia, midline laparotomy was performed on the rats and the aorta ligated both proximally and distally to the superior mesenteric artery. A small cannula was inserted into the superior mesenteric artery, and the vessels were flushed with heparinized Krebs solution (100 u ml^{-1}) and subsequently isolated, as described by McGregor (1965). Following isolation, the arterial preparation was transferred to a chamber and perfused with Krebs solution (maintained at 37°C and gassed with 95% O_2, 5% CO_2 mixture) at a constant flow rate of 5 ml min^{-1} by a peristaltic pump (Harvard Apparatus, Millis, MA). Changes in perfusion pressure were measured with a Statham pressure transducer and recorded on a Rikadenki polygraph recorder (KA-12 series; Soltec, Elcino CA). Since the flow was maintained at a constant rate, alterations in perfusion pressure were taken to reflect vascular resistance. The Krebs solution used had the following composition (mM): NaCl 118; KCl 4.7, $CaCl_2$ 2.5, NaH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 11.5. High K^+ Krebs was prepared by substituting an equimolar amount of K^+ for Na^+, while K^+-free Krebs solution was made without any added K^+ and osmolarity was maintained with sucrose. Arterial perfusion pressure was elevated by continuous infusion of noradrenaline (NA, 2–5 μM) in the presence of ascorbic acid (20 μg ml^{-1}) as an antioxidant, or in some cases with KCl (50 mM).

Experimental protocol

Series 1 In the first series of experiments, the dependence or otherwise of the vasodilator actions of ACh, A23187 and histamine or a K^+ channel activator (BRL 34915) on an intact functional endothelium was examined. Dose-response curves were constructed to each of these substances in arteries preconstricted with NA with or without endothelium. The arteries were denuded of their endothelium by perfusion for 10 min with either distilled water (Criscione *et al.*, 1984) or 10 μM *p*-bromophenacyl bromide (*p*-BPB, Furchgott, 1983). After either treatment, the arteries were re-equilibrated in Krebs solution and subsequently tone was elevated with NA infusion. Dose-response curves to each of the vasodilators were established in this set of arteries, and the responses obtained were compared with those in control arteries. In some arteries with intact endothelium, we also examined the effect of methylene blue (MB, a guanylate cyclase inhibitor) which is widely stated to block endothelium-dependent responses of vascular tissues. In such experiments, the effects of vasodilators were tested before and after 1 h infusion of MB.

Series 2 This series of experiments was conducted in order to assess the contributions of Na^+/K^+-ATPase enzyme and K^+ channels to the vasodilator actions of the various agents used in this study. To achieve this objective, the vasodilators were tested during perfusion with either normal (with or without ouabain, 0.1 mM), 0-K^+, or excess K^+ (50 mM) Krebs solution. In experiments where normal or 0-K^+ Krebs solution was used, arterial tone was elevated with NA (2 μM). The selectivity of ouabain on Na^+/K^+-ATPase was assessed against K^+-induced vasodilatation according to Webb & Bohr (1978). Studies on the role of K^+ channels in the vasodilator action of the agents under investigation employed apamin (0.1 or 0.5 μM), crude scorpion venom (*Leiurus quinquestraitus hebraeus*, 2.5 μg ml^{-1}), procaine (0.1 or 0.5 mM), tetraethylammonium (TEA, 5 or 10 mM) or quinidine (5 or 10 μM) as inhibitors of K^+ channels. In these studies, each inhibitor was tested against the vasodilator response to a submaximal dose of each of ACh, A23187 and histamine. Each inhibitor was infused for 30 min, except TEA which (because of its quaternary ammonium chemical nature) was infused for 1 h.

Series 3 In these experiments, we examined the contribution of calcium channels to the vasodilator action of ACh, histamine, A23187 and BRL 34915 by studying the effect of calcium channel blockers (nifedipine, nitrendipine and diltiazem) on their effects; the experimental protocol was similar to that described for the K^+ channel blockers above.

Treatment of rats with pertussis toxin

Rats were injected with pertussis toxin (PTX), 25 μg 100 g^{-1} body weight intraperitoneally (i.p.) in volumes not exceeding 200 μl per rat. This dose has previously been shown (Lynch *et al.*, 1986) to be effective in causing adenosine diphosphate (ADP) ribosylation in rat hepatic plasma membranes. Control rats were administered saline. Twenty-four or seventy-two hours later, mesenteric arteries were isolated and set up as described earlier. The effect of PTX treatment was examined on the vasoconstrictor actions of NA, arginine vasopressin (AVP) and KCl for the purpose of determining the agent most suitable for elevation of arterial tone. Thereafter, vasodilator responses to ACh, histamine and A23187 during infusion of the constrictor agent were obtained in saline- (control) and PTX-treated rats, and the responses were compared. Liver plasma membranes were also prepared from both the control and PTX-treated rats for the assessment of the degree of ADP ribosylation resulting from the PTX treatment.

Drugs

The following drugs were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A.: noradrenaline bitartrate, acetylcholine bromide, arginine vasopressin, histamine dihydrochloride, apamin (from bee venom), procaine hydrochloride, crude scorpion venom (*Leiurus quinquestraiatus hebraeus*), tetraethylammonium, *p*-bromophenacyl bromide (*p*-BPB), quinidine hydrochloride, nifedipine, methylene blue, sodium nitroprusside and ouabain. Calcium ionophore A23187 was purchased from Aldrich Chem. Co, Milwaukee, WI, U.S.A., while cromakalim (BRL 34915), diltiazem and nitrendipine were obtained *gratis* from Beecham Laboratories, Bristol, TN, Marion Pharmaceutical Co, Kansas City, U.S.A., and Dr Chris Triggle respectively. NA stock solution (10^{-2} M) was prepared in 0.1 M hydrochloric acid and diluted with Krebs solution for infusion. The stock solutions of A23187, *p*-BPB, nifedipine, nitrendipine and cromakalim were prepared in ethanol, while all other compounds were dissolved in saline or Krebs solution.

Data analysis

Changes in perfusion pressure were calculated as percentage of the pressure before the administration of a vasodilator agent in the absence and during the infusion of various inhibitors and expressed as means ± s.e.mean. Differences between the mean values were determined by Student's *t* test. The differences between means were considered significant when $P < 0.05$.

Results

Effect of ACh, A23187, histamine and BRL 34915 on NA-induced arterial tone

The basal perfusion pressure in mesenteric arteries perfused with Krebs solution was 20.0 ± 2.0 mmHg ($n = 35$). In arteries with intact endothelium, infusion of NA (2–5 μM) caused a sustained elevation of arterial perfusion pressure (60.0 ± 8.0 mmHg, $n = 30$); ACh (0.003–1 nmol), histamine

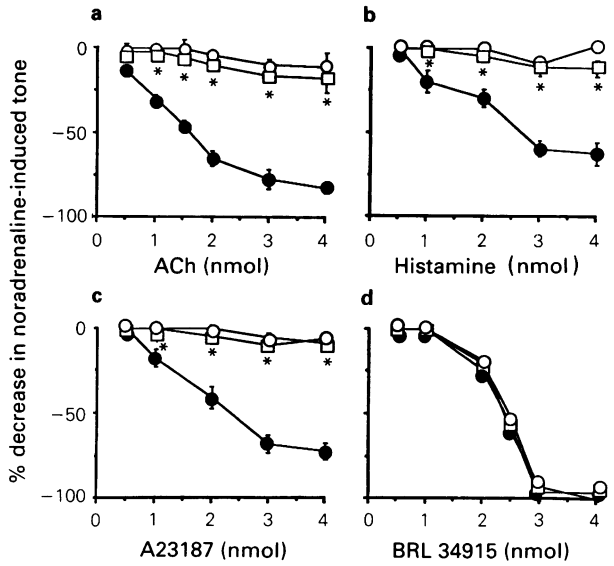

Figure 1 Dose-response curves for the vasodilatation induced by (a) acetylcholine (ACh), (b) histamine, (c) A23187 and (d) BRL 34915 in rat isolated perfused mesenteric arteries. (●) Arteries with intact endothelium; (□) and (○) respectively, represent the responses of arteries after treatment for 10 min with distilled water and *p*-bromophenacyl bromide (*p*-BPB). Each point on the graphs represents the mean and vertical lines show s.e.mean. ($n = 8$).* Denotes values obtained from arteries treated with distilled water and *p*-BPB significantly different from the corresponding values obtained in the control, untreated group ($P < 0.0005$). The doses of each agent shown are on a log scale.

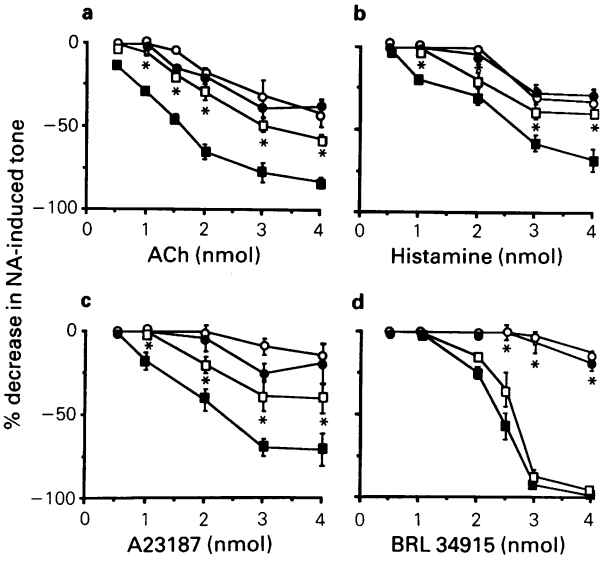

Figure 2 Effects of ouabain and of variations in Krebs solution K^+ on the vasodilatation induced by (a) acetylcholine (ACh), (b) histamine, (c) A23187 and (d) BRL 34915 in rat isolated perfused mesenteric arteries. (■) Control responses, (□) responses of arteries perfused with excess K^+ (50 mM) Krebs solution. (○) and (●) Represnt the responses obtained in K^+-free Krebs solution and ouabain infusion, respectively. Arterial perfusion pressure was elevated by an infusion of noradrenaline (NA, 2 μM), and for each point on the graph, $n = 6$. * Denotes value in the presence of ouabain, O-K^+ and 50 mM K^+ significantly different from the corresponding value obtained in the control group in the presence of 4.7 mM K^+ ($P < 0.05$).

(0.01–10 nmol), A23187 (0.01–1 nmol) and BRL 34915 (0.1–1 nmol) each caused a dose-dependent decrease in NA-induced tone. At the highest dose used, only BRL 34915 completely reversed NA-induced tone, while ACh, histamine and A23187 respectively caused 77.4 ± 5.7, 72.6 ± 6.9 and 72.4 ± 5.1% decrease in NA-induced arterial tone. In some experiments, arterial tone was raised by the infusion of AVP (1 nM). The vasodilator responses to ACh, histamine and A23187 in such arteries did not differ in magnitude from those obtained in arteries where NA infusion was used to raise arterial tone. When endothelium was removed by treatment with either distilled water or *p*-BPB (10 μM), infusion of 2–3 μM NA produced similar levels of arteriolar tone as in endothelium-intact arteries. However, both treatment procedures caused complete abolition of ACh-, histamine- or A23187-induced fall in perfusion pressure, while the fall in perfusion pressure caused by BRL 34915 was unaffected by either of the treatment procedures (Figure 1). Methylene blue (MB) did not alter the effect of any of the vasodilators at 10 μM but at 50 μM, reduced by 44.3 ± 6.4%, 36.1 ± 4.9% and 59.4 ± 5.8% respectively, the effect of ACh, histamine and A23187. MB had no effect on BRL 34915-induced vasodilatation.

Effect of vasodilators during alteration of K^+ concentration or ouabain infusion

In some experiments, arterial tone was elevated by infusion of 50 mM K^+-depolarizing Krebs solution to which prazosin (10 nM) was added to block the effect of possible indirectly-released NA from the adrenergic nerve terminals. In these conditions, the vasodilator effect of ACh, histamine, A23187 was reduced, while that of BRL 34915 was nearly abolished. A similar reduction in the effect of all the vasodilators was observed in arteries perfused with K^+-free Krebs solution (Figure 2), even though under this latter condition, the increase in perfusion pressure induced by NA was significantly greater than in normal Krebs solution. Ouabain infusion completely inhibited KCl-induced vasodilatation (Figure 3), reduced the vasodilator effects of ACh, histamine, A23187 but was ineffective against BRL 34915-induced vasodilatation.

Effect of K^+ channel blockers

The inability of ouabain or K^+ depletion (two conditions that interfere with Na^+/K^+ transport) to abolish the vasodilator effects of ACh, histamine and A23187 suggested a possible involvement of an additional mechanism in the effects of these compounds. We therefore tested the effect of five structurally distinct K^+ channel blockers (apamin, procaine, tetraethylammonium, quinidine and crude scorpion venom) on the

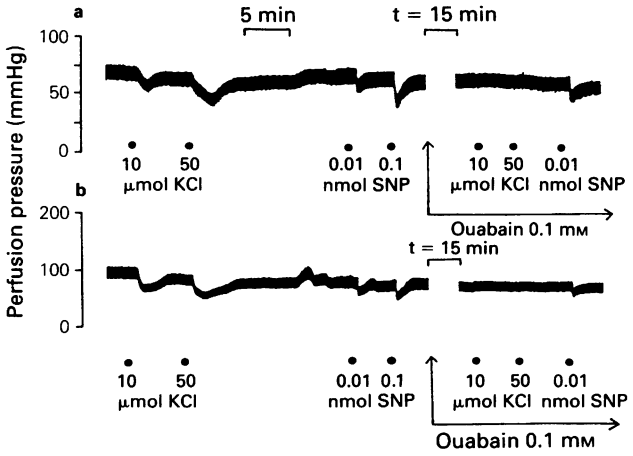

Figure 3 Representative tracings of the effect of ouabain on KCl-and sodium nitroprusside (SNP)-induced vasodilatation in mesenteric arteries perfused with K^+-free Krebs solution containing noradrenaline (2 μM), to elevate arterial tone. In (a), the endothelium was intact; while in (b) the mesenteric artery was previously infused with distilled water for 10 min to remove the endothelium.

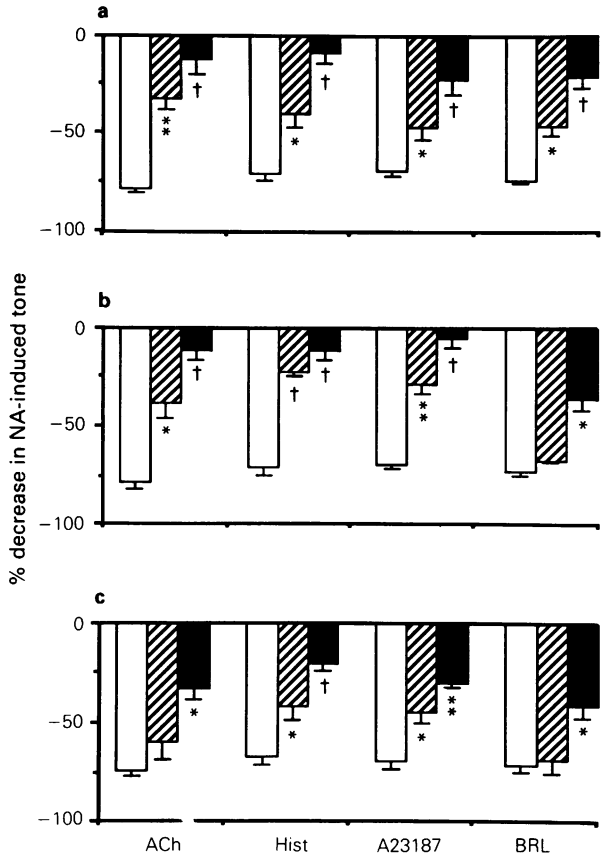

Figure 4 Effects of K^+ channel blockers, (a) procaine 0.1 μM (hatched columns) or 1 mM (solid columns), (b) quinidine 0.5 (hatched columns or 5 (solid columns) μM, and (c) tetraethylammonium 5 (hatched columns) or 10 (solid columns) mM on the vasodilator responses to acetylcholine (ACh), histamine (Hist), A23187 and BRL 34915 in perfused mesenteric arteries of the rat. In (a), (b) and (c), open columns represent vehicle-treated (control) groups. Arterial perfusion pressure was elevated by an infusion of noradrenaline (NA), and each column represents the mean response with bars showing s.e.mean. (n = 6). Statistical differences from corresponding controls are denoted by *P < 0.05, **P < 0.005 and †P < 0.0005.

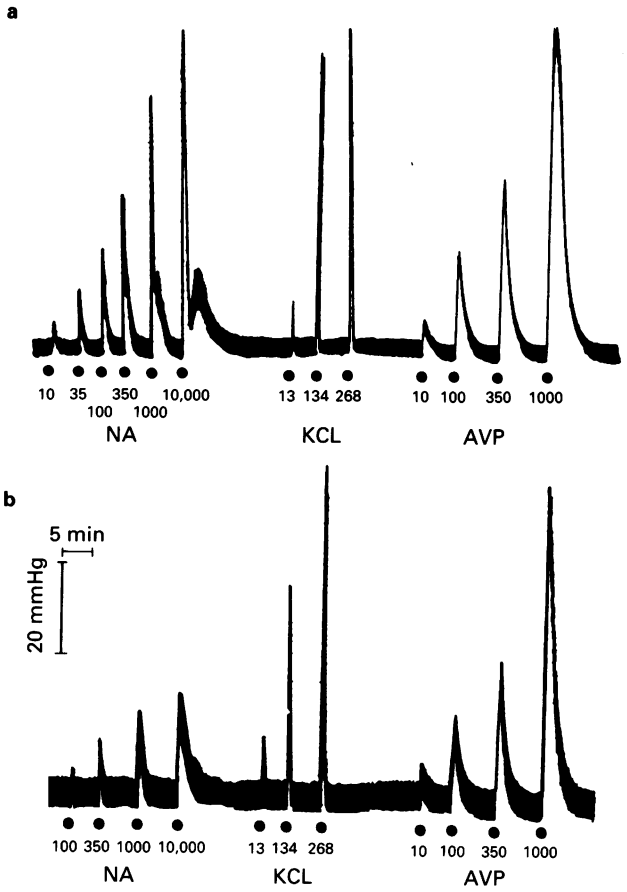

Figure 5 Representative tracings of the vasoconstrictor effects of noradrenaline (NA), arginine vasopressin (AVP) and KCl in mesenteric arteries isolated from saline- (a) and pertussis toxin- (b) treated rats. Doses for NA and AVP are indicated in pmol, while those for KCl are in μmol.

responses to the vasodilators. Procaine (0.1 or 1 mM), quinidine (0.5 or 5 μM) and tetraethylammonium (TEA, 5 or 10 mM) inhibited the vasodilator effect of ACh, histamine and A23187. The decrease in perfusion pressure induced by BRL 34915 was also attenuated by procaine, quinidine and the higher concentration of TEA (Figure 4). Two additional inhibitors, apamin and crude scorpion venom were tested on the vasodilator responses. Apamin (0.1 or 0.5 μM) and crude scorpion venom (2.0 $\mu g\,ml^{-1}$) also blocked the effects of ACh, histamine and A23187; but not that of BRL 34915 (Table 1).

Effect of ACh, histamine and A23187 in pertussis toxin-treated rats

Mesenteric arteries isolated from rats after 72 h of treatment with PTX exhibited markedly depressed responsiveness to NA (0.01–10 nmol) compared to control tissues, whereas responses to arginine vasopressin (AVP, 0.001–1 nmol) and KCl (10–300 μmol) were apparently unaffected by this treatment (Figure 5). The effect of PTX treatment was less pronounced after 24 h. For this reason, arterial tone was elevated with AVP (1 nM) infusion in situations requiring assessment of vasodilator activity. When infused, AVP (1 nM) caused a sustained increase in perfusion pressure: 48.0 ± 4.00 mmHg, n = 5 and 46.0 ± 2.0 mmHg, n = 5 respectively in saline- and PTX-treated rats. ACh (0.01 or 0.1 nmol), histamine (0.1 or

Table 1 Effect of apamin and crude scorpion (*Leiurus quinquestriatus*) venom on the vasodilator responses to acetylcholine (ACh), A23187 and BRL 34915 in isolated perfused mesenteric arteries of the rat

	% decrease in noradrenaline-induced tone			
		Apamin		*Scorpion venom*
Vasodilator	*Control*	*0.1 μM*	*0.5 μM*	*2.5 $\mu g\,ml^{-1}$*
ACh, 0.1 nmol	77.8 ± 4.3	25.6 ± 3.4*	6.2 ± 2.0†	
	80.7 ± 3.7	—	—	8.4 ± 3.9†
A23187, 0.1 nmol	75.0 ± 2.6	Not tested	Not tested	26.9 ± 3.8*
BRL 34915, 1 nmol	94.0 ± 2.2	95.8 ± 1.9	91.8 ± 1.6	
	97.1 ± 3.1	—	—	96.5 ± 2.0

Data represent the means ± s.e.mean. (n = 4). * and † denote statistically significant differences (P < 0.005 and P < 0.0005, respectively) from corresponding control values.

Table 2 Comparison of the endothelium-dependent vasodilatation induced by acetylcholine (ACh), histamine and A23187 in perfused mesenteric arteries of saline- (control) and pertussis toxin- (PTX) treated rats

Vasodilator	*Dose* (nmol)	*% decrease in AVP-induced tone* *Control*	*PTX-treated*
ACh	0.01	29.1 ± 1.7	32.6 ± 4.9
	0.1	64.9 ± 4.1	66.0 ± 6.7
Histamine	0.1	29.2 ± 4.9	30.1 ± 6.8
	1.0	58.5 ± 4.7	63.1 ± 9.5
A23187	0.03	27.7 ± 5.6	25.8 ± 6.2
	0.3	69.5 ± 4.1	67.5 ± 2.8

Data represent the mean ± s.e.mean. ($n = 5$). Statistical comparison (analysis of variance) of the control and PTX-treated revealed no significant difference. Arterial tone was elevated by an infusion of arginine vasopressin (AVP, 1 nM).

1 nmol) and A23187 (0.03 or 0.3 nmol) produced consistent, and dose-dependent vasodilator responses of similar magnitudes in saline- (control) and PTX-treated rats (Table 2).

Effect of calcium channel antagonists

Nifedipine (10 nM) did not alter NA-induced arteriolar tone, but attenuated the effects of all the vasodilator compounds (Figure 6); an effect that was mimicked by diltiazem (0.1 μM) and nitrendipine (1 nM) (see Table 3). All calcium channel antagonists tested produced a less marked effect against BRL 34915-induced vasodilatation. However nifedipine 0.1 μM or diltiazem 1 μM markedly reduced BRL 34915-induced vasodilatation, even though under these conditions, a higher NA (10 μM) concentration was required to sustain arteriolar tone (data not shown).

Table 3 Effects of diltiazem (Dilt) and nitrendipine (Nit) on the vasodilator responses of acetylcholine (ACh, 0.1 nmol), histamine (1 nmol), A23187 (0.1 nmol) and BRL 34915 (1 nmol) in isolated perfused mesenteric arteries of the rat

Inhibitor	*% decrease in noradrenaline-induced tone* *ACh*	*Histamine*	*A23187*	*BRL34915*
Dilt	20.7 ± 3.3†	24.5 ± 3.1†	9.4 ± 1.8†	39.7 ± 5.0*
0.1 μM	(72.5 ± 4.6)	(64.0 ± 2.8)	(74.4 ± 2.9)	(69.0 ± 3.0)
Nit	10.9 ± 4.0†	8.3 ± 2.2†	4.8 ± 0.9†	29.1 ± 3.6*
1 nM	(68.7 ± 2.8)	(60.2 ± 2.9)	(76.2 ± 5.0)	(70.2 ± 2.1)

Data represent the means ± s.e.mean. ($n = 6$). Statistically significant differences from corresponding control values in parentheses are denoted by $*P < 0.005$ and $\dagger P < 0.0005$. In all experiments, arterial tone was elevated by infusion of noradrenaline (2 μM).

Discussion

The contribution of EDRF to the vasodilator effects of ACh, histamine and A23187 has been documented in resistance (Byfield *et al.*, 1986; Furchgott *et al.*, 1987; Randall & Hiley, 1988; Bhardwaj & Moore, 1988) and non-resistance (Van De Voorde & Leusen, 1983; Spokas & Folco, 1984) blood vessels of the rat. This is further confirmed by the results of the present study. In addition, data from the present study also show that the endothelium-dependent responses by these agents are very sensitive to blockade by calcium channel antagonists and appear to involve activation of both Na^+/K^+-ATPase and K^+ channels which are insensitive to PTX.

Perfusion of the mesenteric arteries with either distilled water or *p*-BPB for 10 min abolished the vasodilator effect of ACh, histamine and A23187, while neither of the treatments affected the response to the K^+ channel activator, BRL 34915 suggesting that the integrity of the underlying vascular smooth muscle (VSM) cells was unaffected. This view is further supported by the observation that the responsiveness to NA infusion was not smaller (as would be expected if the VSM cells were damaged) than that in arteries with intact endothelium. Furthermore, previous work has shown short-term (10 min) perfusion with distilled water removes the endothelium from the blood vessels of the mesentery and transmission electron microscopy after such a procedure revealed no damage to the smooth muscle cells (Criscione *et al.*, 1984). Treatment with distilled water presumably caused endothelial cell (EC) loss and consequently affected both the production and release of EDRF; conversely, attenuation of endothelium-dependent vasodilatation by *p*-BPB may be related either to EC damage (Furchgott, 1983; Busse *et al.*, 1985) or to actions related or unrelated to smooth muscle cyclic GMP production (Johns & Peach, 1988). However, the failure of the guanylate cyclase inhibitor, methylene blue to attenuate the endothelium-dependent vasodilator responses suggests (Khan *et al.*, 1986) that activation of guanylate cyclase might not be an important factor in endothelium-dependent vasodilatation in rat mesenteric arteries.

The vasodilator effect of ACh before the discovery of EDRF was attributed to activation of the Na^+, K^+ pump (Haddy, 1978; Webb & Bohr, 1978; De Mey & Vanhoutte, 1980). The involvement of this pump in the vasodilator action of ACh, histamine and A23187 is evident in the present study, based

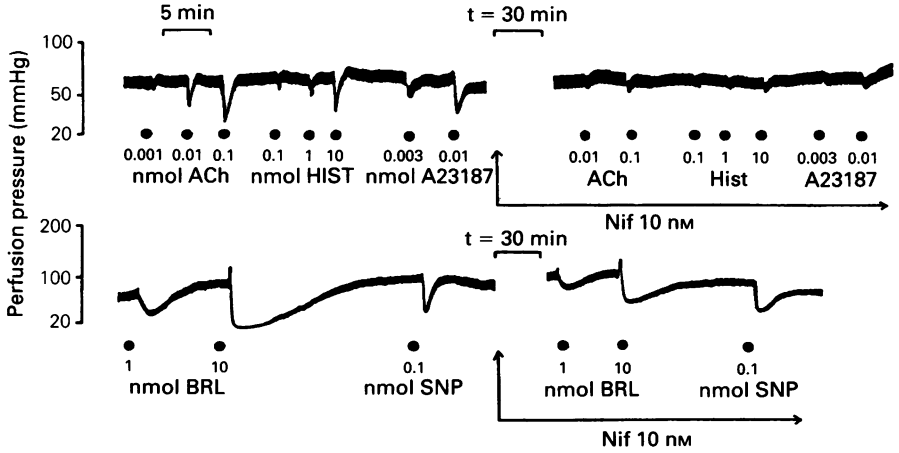

Figure 6 Representative tracings of the effects of nifedipine (Nif, 10 nM) on the vasodilator responses to acetylcholine (ACh), histamine (Hist), A23187, BRL 34915 and sodium nitroprusside (SNP, 0.1 nmol) in rat isolated perfused mesenteric arteries. Arterial tone was induced by noradrenaline (2 μM). Both the perfusion chamber and solution of nifedipine were protected from light by covering them with aluminum foil.

on the observed partial reduction in the effect of these compounds during procedures known to modify Na^+/K^+-ATPase activity *viz*: infusion of ouabain or K^+-depleted Krebs solution (Blaustein, 1977; Haddy, 1978; Webb & Bohr, 1978; Rapoport *et al.*, 1985; Feletou & Vanhoutte, 1988). The selectivity of ouabain for Na^+/K^+-ATPase activity in the present study was indicated by its ability to abolish KCl-induced vasodilatation during infusion of K^+-free Krebs solution. KCl caused vasodilatation in mesenteric arteries with or without endothelium and ouabain abolished this action under either condition, indicating that the Na^+/K^+-ATPase inhibited by ouabain is located on the VSM cells rather than the endothelial cells. That ouabain did not affect K^+ conductance directly was indicated by its failure, but not of extracellular K^+ depletion, to attenuate the vasodilator effect of the K^+ channel activator BRL 34915. Therefore, since neither ouabain nor external K^+ depletion abolished the vasodilator response to ACh, histamine or A23187, it may be concluded that an additional mechanism, possibly increase in K^+ conductance through membrane K^+ channels, contributes to the vasodilator action of these agents. This conclusion was supported by the finding that the vasodilator effect of these agents, like that of BRL 34915, was markedly reduced during elevation of arterial tone with excess K^+ (50 mM) Krebs solution.

The importance of potassium efflux in the endothelium-dependent action of vasodilator agents was suggested by Bolton *et al.* (1983). These authors concluded that EDRF increases potassium permeability in several blood vessels including guinea-pig anterior mesenteric arteries. The loss of potassium from cells occurs through membrane K^+ channels (Lattore & Miller, 1983; Peterson & Maruyama, 1984), which are known to be blocked by compounds such as apamin, procaine, quinidine, tetraethylammonium (Romey *et al.*, 1984; Cook & Haylett, 1985; Cook, 1988) and scorpion venom (Abia *et al.*, 1986; Castle & Strong, 1986). Our finding that ACh-, histamine- and A23187-induced endothelium-dependent vasodilatation was attenuated by K^+ channel blockers further supports the view that these agents increase K^+ conductance. Similar conclusions have been reached in the rabbit superior mesenteric artery (Kuriyama & Suzuki, 1978) and aortic strips (Gebremedhin *et al.*, 1987), and in bovine aortic endothelial cells (Olesen *et al.*, 1988). The vasodilator effect of BRL 34915 is endothelium-independent (present study) and has been attributed by previous studies to be due to activation of an apamin-insensitive type of K^+ channel (Allen *et al.*, 1986; Weir & Weston, 1986). Since procaine, quinidine and TEA also blocked BRL 34915-induced vasodilatation, vascular smooth muscle cells rather than the endothelium would appear to be the site of action of these K^+ channel blockers.

Many recent studies have implicated the guanine nucleotide binding proteins (G-proteins) as mediators of signal transduction of muscarinic receptor activated responses in several tissues. These include inhibition of adenylate cyclase (Jakobs *et al.*, 1979), opening of cardiac potassium channels (Pfaffinger *et al.*, 1985; Codina *et al.*, 1987), activation of phospholipase C (Sasaguri *et al.*, 1985), and opening of calcium-activated channels in lacrimal cells (Evans & Marty, 1986). In the present study, endothelium-dependent responses induced by ACh, histamine or A23187 were not altered in arteries isolated from rats treated with pertussis toxin. Since this toxin is generally used as a tool to study the role of various G proteins in membrane transduction mechanisms, our data suggest that K^+ channels associated with endothelium-dependent responses of these vasodilators are not coupled to a pertussis toxin-sensitive GTP binding protein. The finding, with respect to A23187 is in agreement with that of Flavahan *et al.* (1989) in porcine coronary arteries, but contradicts the suggestion by Sasaki & Sato (1987) that a GTP-binding protein such as G_i regulates the opening of K^+ channels coupled to receptors for dopamine, acetylcholine and histamine in the abdominal ganglion cells of the sea slug *Aplysia*. The latter authors had observed that PTX irreversibly blocked all K^+-dependent responses to these agonists. The insensitivity of the endothelium-dependent vasodilators employed in the present study to PTX is unlikely to be due to the ineffectiveness of the PTX treatment regimen since, *in vitro*, ADP-ribosylation of the liver plasma membranes isolated from the PTX-treated rats revealed no significant incorporation of radioactivity from [^{32}P]-NAD into a 41-kDa peptide as compared to hepatic plasma membranes obtained from vehicle-treated (control) rats (Bipin & Patel, personal communication).

Perhaps the most striking observation in the present study was the profound inhibitory effect of the calcium channel blockers on the vasodilator action of ACh, histamine, A23187 and BRL 34915; indicating that calcium may play a more crucial role than pertussis-toxin sensitive G-proteins in the vasodilator action of these agents in mesenteric arteries. Although the release of EDRF has been shown to be calcium-dependent (Furchgott, 1983; Singer & Peach, 1982; Long & Stone, 1985), the effectiveness of calcium channel antagonists on this process is debatable. For instance, nifedipine and verapamil have been shown to have a modest inhibitory effect in rabbit aorta (Singer & Peach, 1985), but endothelium-dependent vasodilatation in rat aorta is somewhat insensitive to calcium channel inhibition (Miller *et al.*, 1985) and activation (Spedding *et al.*, 1986). Our results, therefore, imply differences in the mechanism of endothelium-dependent vasodilatation in resistance and non-resistance vessels.

Recently, Adams *et al.* (1989) have demonstrated the existence of at least three ion transport pathways through which extracellular calcium can enter the endothelial cell: receptor-mediated calcium influx, the calcium leakage pathway and the stretch-activated calcium pathway. In the present study, calcium channel blockers attenuated the effect of both receptor- (ACh, histamine) and non-receptor (A23187, BRL 34915) activating agents, which indicates that receptor-mediated calcium influx is not of prime importance. On the other hand the electrophysiological data of Ryan *et al.* (1988) have indicated that endothelial cells lack voltage-dependent calcium channels. Therefore the VSM, rather than the endothelium would appear to be the site of action of the calcium channel blockers. This conclusion is supported by our observation that the endothelium-independent vasodilator action of BRL 34915 was also attenuated by calcium channel blockers. Moreover, Bean *et al.* (1986) have shown the presence of dihydropyridine-sensitive calcium channels in smooth muscle cells isolated from rat mesenteric arteries.

Another important finding in the present study pertains to the differential effect of PTX treatment on NA-, AVP- and KCl-induced vasoconstrictor responses. NA-, but not AVP- or KCl-induced vasoconstriction was suppressed in PTX-treated rats compared with control rats. Even though these three agents are known to require Ca^{2+} for their vasoconstrictor action, they differ remarkably in their dependence on extracellular Ca^{2+} for this effect; AVP- and KCl-induced vasoconstriction was abolished in the absence of extracellular calcium, while NA-induced vasoconstriction persisted (present study, data not shown). NA, which acts on α_1-adrenoceptors (Lynch *et al.*, 1985), and AVP on V_1-receptors (Fox *et al.*, 1987; Nabika *et al.*, 1985) and KCl, which acts directly on smooth muscle cells (Underwood *et al.*, 1988) have all been shown to be capable of inducing the breakdown of polyphosphoinositides to myoinositol trisphosphate and diacylglycerol. Therefore, the differential effects of pertussis toxin on these Ca^{2+}-mobilizing processes activated by these agents suggest that either the events associated with each of the agents are coupled to different G-proteins or only α_1-receptors, but not V_1-receptors or voltage-dependent calcium channels are coupled to PTX-sensitive G-proteins in rat mesenteric arteries.

In conclusion, this study demonstrates that endothelium-dependent vasodilator effects to ACh, histamine and A23187 are mediated via activation of K^+ efflux notably through K^+ channels and Na^+/K^+-ATPase which are regulated by calcium, and not by a PTX-sensitive G-protein. Guanylate

cyclase possibly plays a less important role in the endothelium-dependent vasodilator effects of these agents in this vascular bed.

This work was supported by U.S. Public Health Service Grant No. GRHL 19134-13 to K.U.M. and Fogarty International Fellowship Grant No. 1 F05 TW03931-01 to A.S.O.A. The authors wish to thank Bristol Laboratories and Marion Pharmaceutical Co for the generous gifts of BRL 34915 and diltiazem respectively. We also thank Anne Estes for skilful technical assistance, and Dr Chris Triggle for allowing A.S.O.A. to use the facilities of his laboratory for the revision of the manuscript.

References

ABIA, A., LOBATON, C.D., MORENO, A. & GARCIA-SANCHO, J. (1986). *Leiurus quinquestriatus* venom inhibits different kinds of Ca^{2+}-dependent K^+ channels. *Biochim. Biophys. Acta*, **856**, 403–407.

ADAMS, D.J., BARAKEH, J., LASKEY, R. & VAN BREEMEN, C. (1989). Ion channels and regulation of intracellular calcium in vascular endothelial cells. *FASEB J.*, **3**, 2389–2400.

ALLEN, S.L., BOYLE, J.P., CORTIJO, J., FOSTER, R.W., MORGAN, G.P. & SMALL, R.C. (1986). Electrical and mechanical effects of BRL 34915 in guinea-pig isolated trachealis. *Br. J. Pharmacol.*, **89**, 395–405.

ANDRADE, R., MALENKA, R.C. & NICOLL, R.A. (1986). A G protein couples serotonin and $GABA_B$ receptors to the same channels in hippocampus. *Science*, **234**, 1261–1265.

BEAN, B.P., STUREK, M., PUGA, A. & HERMSMEYER, K. (1986). Calcium channels in muscle cells isolated from rat mesenteric arteries: Modulation by dihydropyridine drugs *Circ. Res.*, **59**, 229–235.

BHARDWAJ, R. & MOORE, P.K. (1988). Endothelium-derived relaxing factor and the effects of acetylcholine and histamine on resistance blood vessels. *Br. J. Pharmacol.*, **95**, 835–843.

BLAUSTEIN, M.P. (1977). Sodium ions, calcium ions, blood pressure regulation, and hypertension: a reassessment and a hypothesis. *Am. J. Physiol.*, **232**, C165–C173.

BOLTON, T.B., LANG, R.J. & CLAPP, L.H. (1983). Effects of activation of muscarinic and adrenoceptors on membrane properties of mammalian arteries studied by electrophysiological and radioactive tracer flux techniques. *Blood Vessels*, **20**, 187.

BURDET, R., CRISCIONE, L., POWELL, J. & SIPPOLA, L. (1986). Role of the endothelium in the vasodilator effects of acetylcholine, histamine, hydralazine and trypsin in resistance beds. *Br. J. Pharmacol.*, **88**, 266P.

BUSSE, R., TROGISCH, G. & BASSENGE, E. (1985). The role of endothelium in the control of vascular tone. *Basic Res. Cardiol.*, **80**, 475–490.

BYFIELD, R.A., SWAYNE, G.T.J. & WARNER, T.J. (1986). A method for the study of endothelial derived relaxant factor (EDRF) in the perfused rat mesentery. *Br. J. Pharmacol.*, **88**, 438P.

CASTLE, N.A. & STRONG, P.N. (1986). Identification of two toxins from scorpion (*Leiurus quinquestriatus*) venom which block distinct classes of calcium-activated potassium channel. *FEBS Lett.*, **209**, 117–121.

CHEN, G., SUZUKI, H. & WESTON, A.H. (1988). Acetylcholine releases endothelium-derived hyperpolarizing factor and EDRF from rat blood vessels. *Br. J. Pharmacol.*, **95**, 1165–1174.

CODINA, J., YATANI, A., GRENET, D., BROWN, A.M. & BIRNBAUMER, L. (1987). The α subunit of the GTP binding protein G_k opens atrial potassium channels. *Science*, **236**, 442–445.

COOK, N.S. (1988). The pharmacology of potassium channels and their therapeutic potential. *Trends Pharmacol. Sci.*, **9**, 21–28.

COOK, N.S. & HAYLETT, D.G. (1985). Effects of apamin, quinine and neuromuscular blockers on calcium-activated potassium channels in guinea-pig hepatocytes. *J. Physiol.*, **358**, 373–394.

CRISCIONE, L., MULLER, K. & PRESCOTT, M.F. (1984). Endothelial cell loss enhances the pressor responses in resistance vessels. *J. Hypertension*, **2**, 441–444.

DE MEY, J.G. & VANHOUTTE, P.M. (1980). Interaction between Na^+, K^+ exchanges and the direct inhibitory effect of acetylcholine on canine femoral arteries. *Circ. Res.*, **46**, 826–836.

EVANS, M.G. & MARTY, A. (1986). Potentiation of muscarinic and α-adrenergic responses by an analog of guanosine 5'-triphosphate. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 4099–4103.

FELETOU, M. & VANHOUTTE, P.M. (1988). Endothelium-dependent hyperpolarization of canine coronary smooth muscle. *Br. J. Pharmacol.*, **93**, 515–524.

FLAVAHAN, N.A., SHIMOKAWA, H. & VANHOUTTE, P.M. (1989). Pertussis toxin inhibits endothelium-dependent relaxations to certain agonists in porcine coronary arteries. *J. Physiol.*, **408**, 549–560.

FOX, A.W., FRIEDMAN, P.A. & ABEL, P.W. (1987). Vasopressin receptor mediated contraction and [^{3}H]-inositol metabolism in rat tail artery. *Eur. J. Pharmacol.*, **135**, 1–10.

FURCHGOTT, R.F. (1983). Role of endothelium in responses of vascular smooth muscle. *Circ. Res.*, **53**, 557–573.

FURCHGOTT, R.F., CARVALHO, M.H., KHAN, M.T. & MATSUNAGA, K. (1987). Evidence for endothelium-dependent vasodilation of resistance vessels by acetylcholine. *Blood Vessels*, **24**, 145–149.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288**, 373–376.

GEBREMEDHIN, D., HADHAZY, P. & MAGYAR, K. (1987). Inhibition by quinine of endothelium-dependent relaxation of rabbit aortic strips. *Br. J. Pharmacol.*, **92**, 835–841.

GILMAN, A.G. (1987). G-proteins: Transducers of receptor-generated signals. *Ann. Rev. Biochem.*, **56**, 615–649.

GORDON, J.L. & MARTIN, W. (1983). Endothelium-dependent relaxation of the pig aorta: Relationship to stimulation of ^{86}Rb from isolated endothelial cells. *Br. J. Pharmacol.*, **79**, 531–541.

HADDY, J.F. (1978). The mechanism of potassium vasodilatation. In *Mechanisms of Vasodilatation*, pp. 200–205, Basel: Karger.

JAKOBS, K.H., AKTORIES, K. & SCHULTZ, G. (1979). GTP-dependent inhibition of cardiac adenylate cyclase by muscarinic cholinergic agonists. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **310**, 113–119.

JOHNS, R.A. & PEACH, M.J. (1988). *Para*-bromophenacyl bromide inhibits endothelium-dependent arterial relaxation and cyclic GMP accumulation by effects produced exclusively in the smooth muscle. *J. Pharmacol. Exp. Ther.*, **244**, 859–865.

KHAN, M.T., FURCHGOTT, R.F., HUNG, I-L. & JOTHIANANDAN, D. (1986). Endothelium-dependent and independent vasodilation in rat aorta and mesenteric vasculature. *Fedn. Proc.*, **45**, 425.

KURIYAMA, H. & SUZUKI, H. (1978). The effects of acetylcholine on the membrane and the contractile properties of smooth muscle cells of the rabbit superior mesenteric artery. *Br. J. Pharmacol.*, **64**, 493–501.

LATTORE, R. & MILLER, C. (1983). Conduction and selectivity in potassium channels. *J. Membr. Biol.*, **71**, 11–30.

LONG, C.J. & STONE, T.W. (1985). The release of endothelium-derived relaxant factor is calcium dependent. *Blood Vessels*, **22**, 205–208.

LYNCH, C.J., BLACKMORE, P.F., CHAREST, R. & EXTON, J.H. (1985). The relationships between receptor binding capacity for norepinephrine, angiotensin II, and vasopressin and release of inositol triphosphate, calcium mobilization, and phosphorylase activation in rat liver. *Mol. Pharmacol.*, **28**, 93–99.

LYNCH, C.J., PRPIC, V., BLACKMORE, P.F. & EXTON, J.H. (1986). Effect of islet-activating pertussis toxin on the binding characteristics of Ca^{2+}-mobilizing hormones and on agonist activation of phosphorylase in hepatocytes. *Mol. Pharmacol.*, **29**, 196–203.

MARTIN, W., VILLANI, G.M., JOTHIANANDIAN, D. & FURCHGOTT, R.F. (1985). Selective blockade of endothelium-dependent and glyceryl trinitrate induced relaxation by hemoglobin and by methylene blue in the rabbit aorta. *J. Pharmacol. Exp. Ther.*, **232**, 708–716.

McGREGOR, D.D. (1965). The effect of sympathetic nerve stimulation on vasoconstrictor responses in perfused mesenteric blood vessels of the rat. *J. Physiol.*, **177**, 21–30.

MILLER, R.C., SCHOEFFTER, P. & STOCLET, J.C. (1985). Insensitivity of calcium-dependent endothelial stimulation in rat isolated aorta to the calcium entry blocker, flunarizine. *Br. J. Pharmacol.*, **85**, 481–487.

MONCADA, S., RADOMSKI, M.W. & PALMER, R.M.J. (1988). Endothelium-derived relaxing factor: Identification as nitric oxide and role in the control of vascular tone and platelet function. *Biochem. Pharmacol.*, **37**, 2495–2501.

MURAD, F., MITTEL, C.K., ARNOLD, W.P., KATSUKI, S. & KIMURA, H. (1978). Guanylate cyclase activation by azide, nitro compounds, nitric oxide, and hydroxy radicals and inhibition by hemoglobin and myoglobin. *Adv. Cyclic Nucleotide Res.*, **9**, 145–158.

NABIKA, T., VELLETRI, P.A., LOVENBURG, W. & BEAVEN, M.A. (1985). Increase in cytosolic calcium and phosphoinositide metabolism induced by angiotension II and arg-vasopressin in vascular muscle cells. *J. Biol. Chem.*, **260**, 4661–4670.

NAKAJIMA, Y., NAKAJIMA, S. & INOUE, M. (1988). Pertussis toxin-insensitive G protein mediates substance P-induced inhibition of

potassium channels in brain neurons. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 3643–3647.

OLESEN, S-P., DAVIES, P.F. & CLAPHAM, D.E. (1988). Muscarinic-activated K^+ current in bovine aortic endothelial cells. *Circ. Res.*, **62**, 1059–1064.

PETERSON, O.H. & MARUYAMA, Y. (1984). Calcium-activated potassium channels and their role in secretion. *Nature*, **307**, 693–696.

PFAFFINGER, P., MARTINS, J.M., HUNTER, D.D., NATHANSON, N.M. & HILLE, B, (1985). GTP-binding proteins couple cardiac muscarinic receptors to a K^+-channel. *Nature*, **317**, 536-538.

RANDALL, M.D. & HILEY, C.R. (1988). Effect of phenobarbitone pretreatment upon endothelium-dependent relaxation to acetylcholine in rat superior mesenteric arterial bed. *Br. J. Pharmacol.*, **94**, 977–983.

RAPOPORT, R.M., SCHWARTZ, K. & MURAD, F. (1985). Effect of Na^+, K^+-pump inhibitors and membrane depolarizing agents on acetylcholine-induced endothelium-dependent relaxation and cyclic GMP accumulation in rat aorta. *Eur. J. Pharmacol.*, **110**, 203–209.

ROMEY, G., HUGUES, M., SCHMID-ANTOMARCHI, H. & LAZDUNSKI, M. (1984). Apamin: a specific toxin to study a class of Ca^{2+}-dependent K^+ channels. *J. Physiol. (Paris)*, **79**, 259–264.

RUBANYI, G.M. & VANHOUTTE, P.M. (1985). Ouabain inhibits endothelium-dependent relaxations to arachidonic acid in canine coronary arteries. *J. Pharmacol. Exp. Ther.*, **235**, 81–86.

RYAN, U.S., JOHNS, A. & VAN BREEMEN, C. (1988). Role of calcium in receptor-mediated endothelial cell responses. *Chest*, **93**, 105S–109S.

SASAGURI, T., HIRATA, M. & KURIYAMA, H. (1985). Dependence on Ca^{2+} of the activities of phosphatidylinositol 4,5-biphosphate phosphodiesterase and inositol 1,4,5-triphosphate phosphatase in smooth muscles of the porcine coronary artery. *J. Biochem.*, **231**, 497–503.

SASAKI, K. & SATO, M. (1987). A single GTP-binding protein regulates K^+-channels coupled with dopamine, histamine and acetylcholine receptors. *Nature*, **325**, 259–262.

SINGER, H.A. & PEACH, M.J. (1982). Calcium- and endothelial-mediated vascular smooth muscle relaxation in rabbit aorta. *Hypertension*, **4** (suppl II), 19–25.

SPEDDING, M., SCHINI, V., SCHOEFFTER, P. & MILLER, R.C. (1986). Calcium channel activation does not increase release of endothelial derived relaxant factors (EDRF) in rat aorta although tonic release of EDRF may modulate calcium channel activity in smooth muscle. *J. Cardiovasc. Pharmacol.*, **8**, 1130–1137.

SPOKAS, E.G. & FOLCO, G.C. (1984). Intima-related vasodilatation of the perfused rat caudal artery. *Eur. J. Pharmacol.*, **100**, 211–217.

STRYER, L. & BOURNE, H.R. (1986). G proteins: a family of signal transducers. *Ann. Rev. Cell. Biol.*, **2**, 391–419.

UNDERWOOD, R.H., GREELY, R., GLENNON, E.T., MENACHERY, A.I., BRALEY, L.M. & WILLIAMS, G.H. (1988). Mass determination of polyphosphoinositides and inositol triphoshphate in rat adrenal glomerulosa cells with a microspectrophotometric method. *Endocrinology*, **123**, 211–219.

VAN DE VOORDE, J. & LEUSEN, I. (1983). Role of the endothelium in the vasodilator response of rat thoracic aorta to histamine. *Eur. J. Pharmacol.*, **87**, 113–120.

WEBB, R.C. & BOHR, D.F. (1978). Potassium-induced relaxation as an indicator of Na^+, K^+-ATPase activity in vascular smooth muscle. *Blood Vessels*, **15**, 198–207.

WEIR, S.W. & WESTON, A.H. (1986). Effect of apamin on responses to BRL 34915, nicorandil and other relaxants in the guinea pig taenia caeci. *Br. J. Pharmacol.*, **88**, 113–120.

YAMASHITA, N., KOJIMA, I., SHIBUYA, N. & OGATA, E. (1987). Pertussis toxin inhibits somatostatin-induced K^+ conductance in human pituitary tumor cells. *Am. J. Physiol.*, **253**, E28–E32.

(Received August 25, 1989
Revised January 29, 1990
Accepted February 14, 1990)