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An indirect influence of phenylephrine on the release of endothelium-derived vasodilators in rat small mesenteric artery

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1 The possibility that stimulation of smooth muscle α_1-adrenoceptors modulates contraction *via* the endothelium was examined in rat small mesenteric arteries.

2 N^{ω}-nitro-L-arginine methyl ester, (L-NAME, 100 μM to inhibit NO synthase) increased contraction to single concentrations of phenylephrine (1–3 μM) by approximately 2 fold (from a control level of 14.2 ± 3.0 to $34.1 \pm 4.2\%$ of the maximum contraction of the artery, $n = 20$). The action of L-NAME was abolished by disrupting the endothelium.

3 The subsequent addition of apamin (to inhibit small conductance Ca^{2+}-activated K^+ channels, 50 nM) further augmented phenylephrine contractions, in an endothelium-dependent manner, to more than 3 fold above control ($50.4 \pm 5.3\%$ of the maximum contraction, $n = 11$).

4 Charybdotoxin (non-selective inhibitor of large conductance Ca^{2+}-activated K^+ channels, BK_{Ca}, 50 nM) plus L-NAME augmented the level of phenylephrine contraction to 4–5-fold above control ($64.1 \pm 3.1\%$, $n = 5$), but this effect was independent of the endothelium. The potentiation of contraction by charybdotoxin could be mimicked with the selective BK_{Ca} inhibitor, iberiotoxin,.

5 Apamin together with L-NAME and charybdotoxin further significantly increased the phenylephrine contraction by 5–6-fold, to $79.9 \pm 3.5\%$ of the maximum contraction of the artery ($n = 13$).

6 Phenylephrine failed directly to increase the intracellular Ca^{2+} concentration in endothelial cells freshly isolated from the small mesenteric artery.

7 Stimulation of smooth muscle α_1-adrenoceptors in the mesenteric artery induces contraction that is markedly suppressed by the endothelium. The attenuation of contraction appears to reflect both the release of NO from the endothelium and the efflux of K^+ from both endothelial and smooth muscle cells. This suggests that the release of NO and endothelium-derived hyperpolarizing factor can be evoked indirectly by agents which act only on the smooth muscle cells.

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Abbreviations: ACh, acetylcholine; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; ChTX, charybdotoxin; IbTX, iberiotoxin; L-NAME, N^{ω}-nitro-L-arginine methyl ester; NO, nitric oxide; PE, phenylephrine

Introduction

The release of endothelium-derived relaxing and hyperpolarizing factors (EDRFs, EDHFs) occur in response to an increase in the intracellular calcium concentration ($[Ca^{2+}]_i$) in endothelial cells (Chen & Suzuki, 1990; Garland *et al*., 1995; Plane *et al*., 1995). The increase can follow the application of endothelium-specific agonists such as acetylcholine and bradykinin, or in some cases, an increase in luminal shear stress (Furchgott & Vanhoutte, 1989). In isolated arterioles, an increase in endothelial cell $[Ca^{2+}]_i$ has been shown to follow the addition of the vasoconstrictor phenylephrine, which only stimulates the smooth muscle cells, by acting on α_1-adrenoceptors. The rise in endothelial cell $[Ca^{2+}]_i$ was attributed to either the diffusion of an unknown factor from the smooth muscle to the endothelium, or to the passage of Ca^{2+} through myoendothelial gap junctions. A functional consequence of this indirect increase in endothelial cell Ca^{2+} was an increase in the release of NO which then suppressed the contraction by around 50% (Dora *et al*., 1997). The possibility that the release of other endothelium-derived dilator agents, such as EDHF, could be increased in a similar way was not investigated. However, this seems highly likely as the NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) only reduced relaxation to acetylcholine by approximately half (Dora *et al*., 1997).

In rat small mesenteric arteries, dilatation mediated by the endothelium-dependent agonist acetylcholine is largely due to hyperpolarization of the vascular smooth muscle cells. The prostacyclin synthesis inhibitor, indomethacin, had no effect on responses to acetylcholine (Garland & McPherson, 1992), and L-NAME only slightly shifted the concentration response curve for dilation to acetylcholine while having little effect on repolarization (Garland & McPherson, 1992). However, this NO-independent dilatation and repolarization could be abolished by raising the K^+ concentration to 25 mM (Waldron & Garland, 1994) or by the combined application of the Ca^{2+} activated K^+ channel blockers, apamin (inhibitor of small conductance Ca^{2+}-activated K^+ channels) and charybdotoxin (inhibitor of intermediate and large conductance Ca^{2+}-activated K^+ channels and delayed rectifier K^+ channels) (Plane *et al*., 1997). The ability of this combination of toxins to inhibit the release or action of EDHF has been reported in a number of preparations (Feletou & Vanhoutte, 1999). Recent evidence indicates that they may act to inhibit the release of an EDHF from the endothelium (Edwards *et al*., 1998). Whatever the precise site of action, a characteristic of this blockade is

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that the more selective blocker of large conductance Ca^{2+}-activated K^+ channels, iberiotoxin, cannot be substituted for charybdotoxin (Zygmunt *et al*., 1997).

In the present study, the extent to which the endothelium can be indirectly stimulated to release both NO and EDHF, by stimulating smooth muscle α_1-adrenoceptors, was assessed in the rat mesenteric artery. Smooth muscle contraction evoked by phenylephrine was measured in both endothelium intact and denuded arteries. The possibility that phenylephrine could act directly to stimulate the endothelial cells, was investigated by measurements of endothelial cell $[Ca^{2+}]_i$. Some of these results have been presented in preliminary form to the British Pharmacological Society (Dora & Garland, 1999).

Methods

Tension measurement in small arteries

Male Wistar rats (200–250 g) were stunned and killed by cervical dislocation. The mesenteric arcade was removed and placed in Krebs buffer. Segments (2 mm in length: D_{100} 100–200 μm) of third order branches of the superior mesenteric artery were removed and mounted in a Mulvany-Halpern myograph (model 400 A, J.P. Trading, Denmark, 10 ml) under a normalized tension as previously described (Garland & McPherson, 1992). Tissues were maintained at 37°C in Krebs buffer containing indomethacin (2.8 μM) aerated with 95% O_2:5% CO_2. In some experiments, the endothelium was removed by rubbing the intima with a hair.

After an initial equilibration period of 60 min, the integrity of the endothelium was assessed by pre-contracting the tissues with phenylephrine (1–3 μM) and then adding acetylcholine (1 μM). Tissues in which the acetylcholine reversed the phenylephrine-induced tone by more than 90% were designated as endothelium intact and tissues in which acetylcholine caused less than 10% relaxation were designated as denuded. In some experiments, L-NAME (100 μM), charybdotoxin (50 nM), iberiotoxin (100 nM) and/or apamin (50 nM) were added to the organ bath at least 20 min before addition of phenylephrine. These concentrations were chosen as they have been shown to inhibit EDHF responses in this artery (Plane *et al*., 1997). The toxins were always added in the presence of L-NAME in an effort to mimic EDHF-type responses.

In each experiment, a concentration of phenylephrine (1–3 μM) was chosen to evoke only a small increase in tension under control conditions, and the same concentration was then used throughout the remainder of the experiment. Phenylephrine was added for a period of 5 min. At the end of the experiment, the maximal contractile capability of the artery was determined by adding 10 μM phenylephrine and 65 mM K^+.

$[Ca^{2+}]_i$ in isolated endothelial cells

Endothelial cells were isolated by a procedure developed in this laboratory. Briefly, rat mesenteric small arteries were placed into dissociation medium containing (mM): NaCl 128, KCl 5.4, KH_2PO_4 0.95, Na_2HPO_4 0.35, $NaHCO_3$ 4.16, glucose 10.0, sucrose 2.19, HEPES 10.0 (mg ml^{-1}): dithiothreitol 1.0, papain 1.0, bovine serum albumin (BSA) 1.0; and $CaCl_2$ (100 μM) and incubated for 30 min at 37°C. Samples were then transferred to a second solution of Krebs containing (mg ml^{-1}): collagenase 1.0 (Worthington, type I), elastase 1.0, and BSA 1.0; and then incubated for 10 min at 37°C. After washing with ice-cold Krebs solution, arteries were gently triturated with a fire-polished Pasteur pipette to yield single smooth muscle cells and endothelial cells. Cells were gently pelleted down and washed with sterile phosphate buffered saline, then resuspended in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum containing (mg l^{-1}): L-glutamine 584, and gentamicin 50. Cells were plated onto 22 mm diameter glass coverslips, placed in a petri dish and incubated at 37°C in a humidified atmosphere of 5% CO_2 until use.

Isolated 'cobblestone' endothelial cells were superfused (2 ml min^{-1}) with Krebs aerated with 95% O_2:5% CO_2 and the petri dishes (solution volume 2 ml) were maintained at 37°C with a water-jacketed inflow line and a bipolar temperature controller and open perfusion micro-incubator (Models TC-202 & PDM1-2, Medical Systems Corp, NY, U.S.A.). Individual cells were visualized by fluorescence microscopy (Ushio 100 W Hg lamp with 12% neutral density filter in light path and an Olympus fluo-3 mirror cube, excitation 490 nm, emission 530 nm) at a magnification of $\times$3000 (Olympus 40$\times$, 0.8 NA objective). An adjustable diaphram at the object plane in the microscope (Olympus IX70) limited the field of illumination to a small circle (diam = 100 μm), thereby reducing photodamage and bleach of dye in neighbouring cells. The image of an endothelial cell was produced with a high sensitivity, cooled, digital, intensified CCD camera (Intensified GenIV PentaMAX System, Princeton Instruments) and collected and stored using image acquisition software (MetaFluor, Universal Imaging). Cells were incubated with Krebs containing fluo-3 AM (5 μM) for 30–40 min. Each cell was exposed to both acetylcholine and phenylephrine (2 min recovery period between additions), and the order of addition was rotated between cells. Due to the rapid rate of fluorescence bleaching, Ca^{2+} calibration was not accurate and was not used.

Solutions and drugs

Arteries and isolated endothelial cells were maintained in Krebs buffer of the following composition (mM): NaCl 118.0, $NaHCO_3$ 25.0, KCl 3.6, $MgSO_4.7H_2O$ 1.2, KH_2PO_4 1.2, glucose 11.0 and $CaCl_2$ 2.5.

Drugs used were all from Sigma except for apamin and charybdotoxin (Alamone Labs), foetal calf serum, L-glutamine, amphotericin B and gentamicin (GIBCO) and fluo-3 AM (Molecular Probes).

Analysis of data

Data are expressed as mean $\pm$ s.e.mean; n indicates the number of animals. In the tension experiments, data are expressed as a percentage of the maximal contraction obtained with phenylephrine and K^+; and in fluo-3 studies, as a percentage change in fluorescence intensity.

Results

Tension changes to phenylephrine in small arteries

Under control conditions, the addition of phenylephrine (2.4$\pm$0.3 μM) only caused a small increase in tension (1.7$\pm$0.3 mN, 14.2$\pm$3.0% of the maximal contraction, n = 20) which tended to develop oscillations (Figure 1). The magnitude of this control contraction did not vary with time (Figures 2 and 3). Addition of L-NAME had no effect on basal tone (Table 1), but on average the magnitude of phenylephrine contraction (2.4$\pm$0.3 μM) was now more than doubled

(4.4±0.5 mN, 34.1±4.2% of the maximal contraction, $n=20$), a significant increase from control. Similarly, in the presence of L-NAME the subsequent addition of either apamin or charybdotoxin alone did not affect basal tone (Table 1), but did augment the contraction to phenylephrine significantly (to 50.4±5.3%, $n=11$ and 64.1±3.1% of maximal contraction, $n=5$, respectively). The combination of L-NAME with apamin plus charybdotoxin caused the greatest augmentation of the

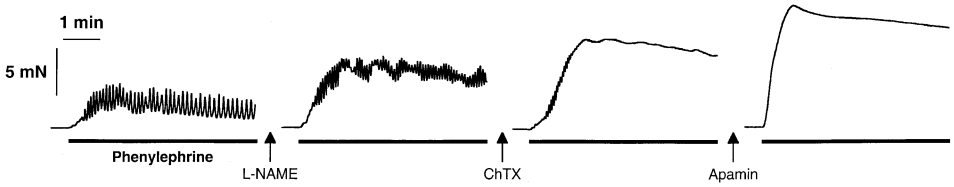

Figure 1 Typical record demonstrating the augmentation of phenylephrine (3 μM) contraction by a blocker of NO synthesis and inhibitors of Ca^{2+} activated K^+ channels in wire mounted rat mesenteric arteries with intact endothelium. Addition of L-NAME, followed by charybdotoxin (ChTX) and apamin each augmented the level of contraction. Note the oscillation of contraction under control conditions, but not after treatment with toxins.

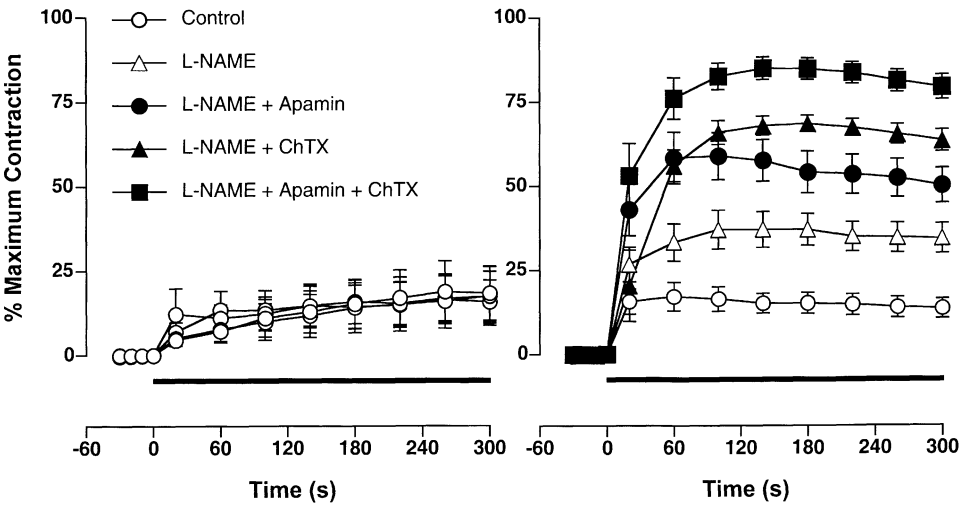

Figure 2 Time course of contraction evoked by phenylephrine in arteries with intact endothelium. The level of phenylephrine contraction did not vary after repeated exposure under control conditions ($n=4$, left). Addition of L-NAME (100 μM, $n=20$) alone or in combination with apamin (50 nM, $n=11$) and/or charybdotoxin (ChTX, 50 nM, $n=5$ alone, $n=13$ with apamin) augmented the level of contraction compared to control conditions ($n=20$). For each treatment, values are means±s.e.mean of 5 s averages. Phenylephrine was added for the period indicated by the bar (5 min).

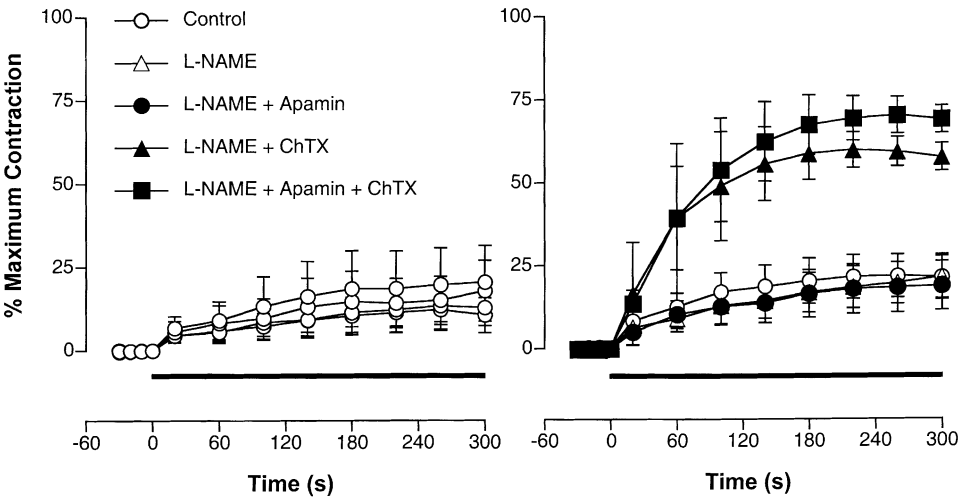

Figure 3 Time course of contraction evoked by phenylephrine in endothelium denuded arteries. The level of phenylephrine contraction did not vary after repeated exposure under control conditions ($n=4$, left). Addition of L-NAME (100 μM) alone ($n=10$) or in combination with apamin (50 nM, $n=4$) did not affect the level of contraction, whereas charybdotoxin (ChTX, 50 nM, $n=3$ both alone and with apamin) did. For each treatment, values are means±s.e.mean of 5 s averages. Phenylephrine was added for the period indicated by the bar (5 min).

contraction to phenylephrine, by more than five times the control (79.9±3.5% of maximum contraction, $n=13$) (Figures 2 and 4). Oscillations in contraction were never observed after the combination of L-NAME and toxins.

In arteries without endothelium, the addition of L-NAME with and without toxins had no effect on basal tone (Table 1). Control responses to phenylephrine (increase of 2.7±0.9 mN, 21.1±6.4% of maximal contraction, $n=10$) did not significantly increase with time (Figure 3). The magnitude of the control contractions to phenylephrine were not different between endothelium intact and denuded arteries (Figure 4). However the concentration of phenylephrine used to evoke these contractions (1.3±0.4 μM) was almost half that used in endothelium-intact arteries. L-NAME alone (20.8±6.6% of maximal contraction, $n=10$) or together with apamin (17.8±14.0%, $n=4$) did not significantly increase the contraction to phenylephrine, whereas the addition of L-NAME plus charybdotoxin (58.2±4.2%, $n=3$) did (Figure 3).

The augmentation of phenylephrine contraction in the presence of charybdotoxin was mimicked with iberiotoxin (Figure 4). L-NAME plus iberiotoxin increased contraction to 66.9±7.1% of the maximum contraction in endothelium intact arteries ($n=3$) and to 66.9±5.4% of the maximum contraction in endothelium denuded segments ($n=5$). The subsequent addition of apamin only caused a small increase in contraction in arteries with endothelium, and had no effect in the denuded arteries (Figure 4).

Table 1 Effects of blocking release of NO and Ca^{2+} activated K^+ channels on basal tension in small mesenteric arteries of the rat

Treatment	*Basal tension* (mN)	*Maximum tension* (mN)	n
Endothelium intact			
Control	1.4±0.2	14.9±1.0	20
L-NAME	1.2±0.1	14.9±1.0	20
L-NAME + Apamin	1.2±0.2	14.5±1.2	11
L-NAME + ChTX	1.0±0.3	16.2±2.2	5
L-NAME + Apamin + ChTX	1.2±0.1	14.5±1.2	13
Endothelium denuded			
Control	1.6±0.2	14.6±1.8	10
L-NAME	1.6±0.2	14.6±1.8	10
L-NAME + Apamin	1.7±0.4	14.2±2.2	4
L-NAME + ChTX	1.3±0.6	13.5±4.8	3
L-NAME + Apamin + ChTX	1.3±0.6	13.5±4.8	3

Data are means±s.e.mean of the number of animals indicated by *n*. There was no statistically significant difference between treatments for either basal tension (before addition of phenylephrine) of for maximum tension (addition of 10 μM PE plus 65 mM K^+) (Mann-Whitney Test).

$[Ca^{2+}]_i$ in isolated endothelial cells

In endothelial cells freshly isolated from small mesenteric arteries, phenylephrine was unable to evoke an increase in fluorescence intensity. However each cell did respond to

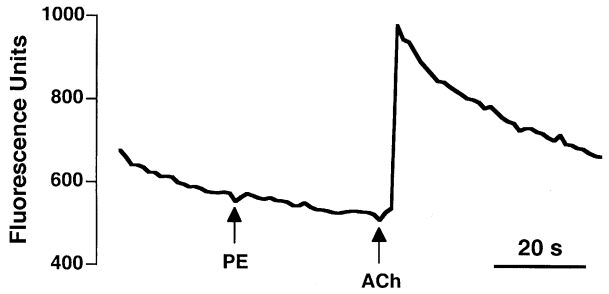

Figure 5 Typical record demonstrating the change in fluo-3 fluorescence intensity in freshly isolated rat mesenteric artery endothelial cells. Phenylephrine (PE, 10 μM bolus application) was unable to cause a change in fluorescence intensity, whereas acetylcholine (ACh, 10 μM bolus application) caused a rapid rise indicating an increase in intracellular Ca^{2+} concentration.

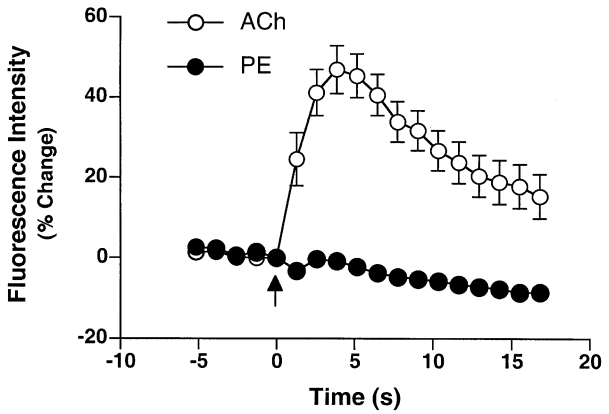

Figure 6 Time course of changes in fluo-3 fluorescence intensity in freshly isolated endothelial cells. Bolus doses of phenylephrine (PE, 10 μM) and acetylcholine (ACh, 10 μM) were added to each cell. Values are means±s.e.mean of 20 cells from four animals.

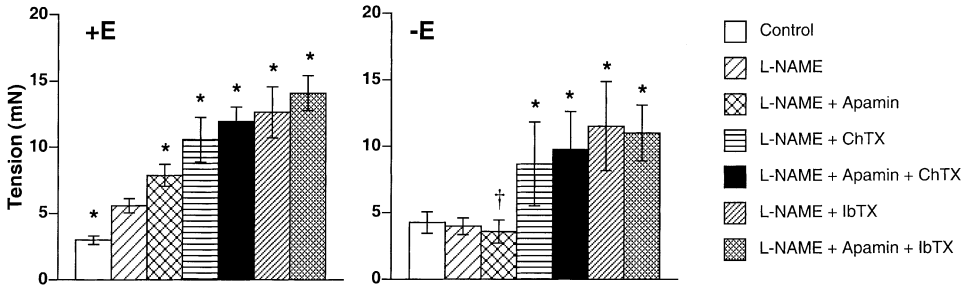

Figure 4 Summary of the effect of blockers on contraction evoked by phenylephrine. Original values from data shown in Figures 2 and 3 were averaged over the final 30 s of phenylephrine application. Iberiotxoin (IbTX, 100 nM) caused the same magnitude of blockade as charybdotoxin (ChTX) when added alone ($n=3,4$ endothelium intact (+E) and denuded (−E), respectively) or in combination with apamin ($n=3,5$ endothelium intact and denuded, respectively). Mean values (±s.e.mean) were analysed non-parametrically with the Mann-Whitney Test. The asterisks indicate statistically significant differences from treatment with L-NAME, $P<0.05$; and the cross indicates a statistically significant difference between endothelium intact and denuded arteries, $P<0.05$. For each treatment, the maximum contraction for the arteries are given in Table 1.

acetylcholine with a large increase in fluo-3 evoked fluorescence (46.9 ± 6.0%, $n = 20$, four animals) (Figures 5 and 6).

Discussion

This study demonstrates that contraction in mesenteric vascular smooth muscle cells is also associated with activation of the endothelial cells to release NO and to activate both endothelial cell and smooth muscle cell Ca^{2+} activated K^+ channels. In the latter, the profile of effective K^+ channel blockade is similar to that observed against the action of EDHF, where the combination of apamin and charybdotoxin blocks the NO-independent responses. This suggests that EDHF is released together with NO during smooth muscle contraction to phenylephrine, as block of NO synthesis, alone or together with EDHF-release, greatly augmented the contraction to phenylephrine. These observations demonstrate that even before the onset of contraction there are already signalling processes occurring between the endothelial and smooth muscle cells which can significantly modulate the contraction.

Under basal conditions, the tension in the small mesenteric artery was unaffected after blockade of NO synthesis and Ca^{2+} activated K^+ channels. However, at this time there was no stimulus for contraction, so simply recording resting tension would not allow an assessment of either NO or EDHF release. Measurements of cyclic GMP formation suggest that there is, in fact, a low basal level of NO release, as a combination of L-NAME and the inhibitor of soluble guanylyl cyclase 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one abolished formation (Plane *et al*., 1998). Although there have been no reports of basal EDHF release in unstimulated preparations, it is known that Ca^{2+} sensitive K^+ channels in smooth muscle cells are activate in myogenically-active cerebral arteries (Nelson & Quayle, 1995) and that EDHF is released basally in perfused mesenteric arteries (Adeagbo & Triggle, 1993).

After supplying a stimulus for contraction by adding phenylephrine, effects attributable to the release of NO and activation of K^+ channels, the latter consistent with the release of EDHF, became apparent. L-NAME significantly increased the contraction to phenylephrine, suggesting that normally NO release suppresses the contraction. A similar observation with NO synthase inhibition has been made in myogenically-active arterioles (Dora *et al*., 1997). In addition, assays of cyclic GMP formation also support this observation. After the addition of phenylephrine to isolated arteries, the rate of cyclic GMP formation increased 2–3-fold in comparison to the basal synthesis (Plane *et al*., 1996). It is unlikely that phenylephrine is causing the formation of cyclic GMP *via* a pathway confined to the smooth muscle cells or by acting *via* the release of NO from non-adrenergic, non-cholinergic nerves (Brave *et al*., 1993), since the effect of L-NAME was abolished after removing the endothelium. Similarly, the significant augmentation of contraction to phenylephrine obtained in the presence of the inhibitors of Ca^{2+} activated K^+ channels is also consistent with an activation of the endothelium. In endothelium-denuded arteries, the increase in contraction in the presence of apamin was abolished, indicating the presence of apamin-sensitive K^+ channels solely on the endothelial cells as suggested in patch-clamp studies (Marchenko & Sage, 1996; Mistry & Garland, 1998). Thus, a signal appears to pass from the smooth muscle cells to the endothelium resulting in the release of NO and the opening of apamin-sensitive K^+ channels. The mechanisms by which the efflux of K^+ and concomitant hyperpolarization of the endothelial cells leads to smooth muscle dilatation could be many-fold. The diffusion of K^+ itself to adjacent smooth muscle cells can cause dilatation (Edwards *et al*., 1998), as could another hyperpolarizing factor (Feletou & Vanhoutte, 1999), or, alternatively, hyperpolarizing current could spread to the smooth muscle layers through myoendothelial gap junctions (Yamamoto *et al*., 1999; Dora *et al*., 1999).

The involvement of Ca^{2+} activated K^+ channels in the processes that regulate smooth muscle tone is not a new observation *per se*. In rat cerebral artery smooth muscle cells, there appears to be a spontaneous Ca^{2+}-dependent activation of outward K^+ currents that decreases artery tone (Nelson *et al*., 1995). A slightly different picture exists in hamster cremaster, in which the modulation of arteriolar tone, both *in situ* and *in vitro*, only occurs after applying an external stimulus for contraction, presumably reflecting the lower Ca^{2+} sensitivity of this channel (Jackson & Blair, 1998). In rat mesenteric artery, the smooth muscle contraction to phenylephrine is associated with depolarization and an increase in smooth muscle $[Ca^{2+}]_i$ (Nilsson *et al*., 1998; Raat *et al*., 1998). In the present study, the magnitude of rise in smooth muscle $[Ca^{2+}]_i$ was sufficient to activate Ca^{2+} activated K^+ channels that are sensitive to both charybdotoxin and iberiotoxin. Indeed, after inhibition of large conductance Ca^{2+}-activated K^+ channels, the magnitude of the phenylephrine contraction approached maximum in both endothelium intact and denuded arteries. However, it is important to note that charybdotoxin-sensitive K^+ channels are additionally present in endothelial cells (Edwards *et al*., 1998; Edwards & Weston, 1998). So like the results with apamin, part of the augmentation of phenylephrine contraction will to be due to blockade of endothelial cell charybdotoxin-sensitive K^+ channels. The extent of this contribution could not be assessed, and hence a direct comparison to blockade of EDHF responses is not possible.

In endothelial cells, both the release of NO and EDHF, the latter assessed as the opening of Ca^{2+} activated K^+ channels, usually depend on an increase in $[Ca^{2+}]_i$ (Chen & Suzuki, 1990; Garland *et al*., 1995; Plane *et al*., 1995). In isolated arterioles, smooth muscle activation with phenylephrine leads to an increase in $[Ca^{2+}]_i$ in both the smooth muscle and adjacent endothelial cells (Dora *et al*., 1997). Since in mesenteric arteries, α-adrenergic stimulation also evokes an increase in smooth muscle cell $[Ca^{2+}]_i$ (Nilsson *et al*., 1998; Raat *et al*., 1998), it is reasonable to predict that this increase will be followed by an elevation of Ca^{2+} within the endothelial cells. This could reflect a passive diffusion of Ca^{2+} or of IP_3 down their concentration gradients, passing to the endothelial cells through myoendothelial gap junctions (Dora *et al*., 1997). These connections allow the transfer of small molecules as well as current between the two cell types (Xia *et al*., 1995; Little *et al*., 1995; Marchenko & Sage, 1994; Yamamoto *et al*., 1998). Although there has not been direct evidence for Ca^2 or IP_3 diffusion through myoendothelial junctions, both molecules can diffuse through homocellular gap junctions in a variety of tissues (Rooney & Thomas, 1993; Sanderson *et al*., 1994). Other mechanisms by which endothelial cell $[Ca^{2+}]_i$ may rise in response to phenylephrine include the diffusion of an undefined factor from the smooth muscle cells or a change in luminal shear stress. An increase in endothelial cell shear stress under isometric conditions without luminal flow may occur due to a change in the orientation of the endothelial cells relative to the smooth muscle and elastic lamina (Fleming *et al*., 1999). This mechanism has been attributed to the Ca^{2+}-independent release of NO after stimulation with phenylephrine in rabbit aorta (Fleming *et al*., 1999). However, a similar

mechanism cannot explain our observations, because the attenuation of contraction under control conditions occurred in the presence of only a minimal change in artery tension. In addition, endothelial cell Ca^{2+} activated K^+ channels were stimulated by phenylephrine, which indicates a significant increase in the endothelial cell $[Ca^{2+}]_i$ was evoked.

Although the expression of α_1-adrenoceptors has not been reported in mesenteric artery endothelial cells, we assessed the possibility that phenylephrine might act directly on the endothelial cells to activate a rise in endothelial cell $[Ca^{2+}]_i$. Although we found that freshly isolated rat mesenteric artery endothelial cells did respond to acetylcholine, an agonist that causes an increase in endothelial cell $[Ca^{2+}]_i$ both *via* a release from intracellular stores and calcium influx mechanisms (Busse *et al*., 1989), the same cells did not respond to phenylephrine. This shows directly that functional α_1-adrenoceptors linked to an increase in $[Ca^{2+}]_i$ are not located on the endothelial cells.

Increases in smooth muscle contraction to α-adrenergic agonists after blockade of nitric oxide synthesis have been observed in multiple arterial beds (Angus *et al*., 1986; Jones *et al*., 1993; Kaley *et al*., 1992; Pohl & de Wit, 1996; Dora *et al*., 1997). Here we show that blockade of Ca^{2+} activated K^+ channels also augments the contraction to phenylephrine, and that a large component of this effect is due to the activation of K^+ channels on the endothelial cells. Thus, under physiological conditions of pressure and flow, the spontaneous and evoked release of noradrenaline from sympathetic nerve terminals could potentially stimulate the release of endothelium-derived dilators. The inhibitory action of these dilators would then dampen the influence of the sympathetic nervous system in arteries and arterioles.

The present findings demonstrate an important stimulus for the release of NO and EDHF which is likely to be of physiological significance. They show that the activation of the endothelium is not confined simply to endothelium-specific vasodilators or to changes in endothelial cell shear stress. Stimulation of smooth muscle cells with an agent acting on α_1-adrenoceptors can lead both to increased NO synthesis and opening of K^+ channels, the latter causing the most pronounced attenuation in contraction. These observations provide further evidence supporting an important role for endothelial cell hyperpolarization in the control of diameter in small arteries.

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