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Spatial heterogeneity in the mechanisms contributing to acetylcholine-induced dilatation in the rabbit isolated ear

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1 Using an X-ray microangiographic technique in rabbit isolated perfused ears preconstricted with 5-HT (300 nM) and histamine (300 nM), we investigated the combined actions of N^{ω}-nitro-L-arginine methyl ester (L-NAME) and indomethacin on acetylcholine-induced depressor responses.

2 Under control conditions, acetylcholine (10 nM – 30 μM) induced a concentration-dependent reversal of the pressor response, reaching a maximum of $66.0 \pm 13.6\%$ ($n=6$). In the presence of L-NAME (300 μM) and indomethacin (10 μM), this depressor action was reduced, reaching a maximum of $38.6 \pm 5.9\%$ ($n=6$).

3 The control response was associated with substantial vasodilatation in the central ear artery (G_0), a smaller dilatory action on first generation branch arteries (G_1) and no effect on second generation branch arteries (G_2). In the presence of L-NAME and indomethacin, vasodilatation occurred in G_2 with no effect in G_0 or G_1.

4 Two calcium-activated K^+ channels blockers, charybdotoxin (ChTX; 10 nM) and penitrem A (100 nM), further inhibited, but did not abolish, the L-NAME- and indomethacin-resistant response to acetylcholine (10 nM – 300 μM). Both agents abolished the vasodilatory action of acetylcholine in G_2.

5 In conclusion, L-NAME and indomethacin induced a shift in acetylcholine-induced vasodilatation from G_0 and G_1 to G_2. This is consistent with the suggestion that nitric oxide dominates in larger vessels whilst other mechanisms dominate in smaller vessels. The L-NAME- and indomethacin-resistant component was inhibited by ChTX and penitrem A, suggesting it is mediated, at least in part, by activation of K_{Ca} channels and could therefore involve a hyperpolarising mediator such as endothelium-derived hyperpolarising factor.

Keywords: EDHF; nitric oxide; prostanoids; calcium-activated potassium channels; acetylcholine; microangiography; rabbit ear

Introduction

It is now well established that agonists, such as acetylcholine, can induce the release of relaxing and hyperpolarising factors from vascular endothelium that act on the underlying smooth muscle and modify tissue perfusion. One such factor has been identified as nitric oxide (NO), or a NO-related substance, synthesised from L-arginine (see Moncada *et al*., 1991 for review), whilst prostacyclin, a cyclo-oxygenase product of arachidonic acid, may also be released in some tissues (Weksler *et al*., 1978; Gryglewski *et al*., 1986). In many preparations, however, hyperpolarisation via activation of K^+ channels and relaxation have been observed in the presence of inhibitors of NO and prostacyclin production or action. Although the endothelium and smooth muscle may be electrically coupled (Xia *et al*., 1995), bioassay studies performed under combined blockade of NO and prostacyclin synthesis have demonstrated the release of a diffusible factor from donor endothelial cells and intact perfused arterial segments which can hyperpolarise downstream smooth muscle cells (Popp *et al*., 1996). This supports the proposal that an additional factor may be released which has been termed endothelium-derived hyperpolarising factor (EDHF) (Taylor & Weston, 1988). Recent experiments with specific synthetic peptides that modulate the opening of gap junctions have provided evidence that direct heterocellular communication between the endothelium and the smooth muscle of the vessel wall is also a major contributor to agonist-induced relaxation (Chaytor *et al*., 1998). This could represent the transfer of a low molecular weight factor, and/or electrical coupling, via gap junctions.

At the present time, the identity of the putative EDHF remains a matter of controversy. Many studies have shown that relaxation attributed to EDHF in various tissues can be inhibited by cytochrome P450 inhibitors suggesting that EDHF may be a cytochrome P450-derived arachidonic acid metabolite (Bauersachs *et al*., 1994; Hecker *et al*., 1994; Zygmunt *et al*., 1996; Dong *et al*., 1997). Doubt has however been cast on this theory by the recent discovery that many of the inhibitors employed may merely be acting as K^+ channel blockers and may therefore be inhibiting the action of EDHF rather than its synthesis (Edwards *et al*., 1996). Some recent evidence has been put forward to suggest that EDHF may be anandamide, another derivative of arachidonic acid which is an endogenous cannabinoid (Randall *et al*., 1996). Cohen *et al*. (1997) suggested that in the rabbit carotid artery acetylcholine-dependent relaxation believed to be insensitive to the NO synthase inhibitors, N^{ω}-nitro-L-arginine (L-NOARG) and N^{ω}-nitro-L-arginine methyl ester (L-NAME), may in fact be mediated by NO itself since a combination of 30 μM L-NAME and 300 μM L-NOARG was found not to elicit complete inhibition of NO production.

NO- and prostanoid-independent mechanisms of vaso-relaxation have been suggested to play a more predominant role in small arteries (Garland *et al*., 1995). Hence in this study, we wished to compare the roles of NO and non-NO mediators of acetylcholine-induced dilatation in vessels of three different sizes within the intact arterial bed of the preconstricted rabbit isolated ear. This was achieved using an X-ray microangiographic technique which facilitates visualisation of the resistance network enabling the pattern of vasodilatation

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within the bed to be investigated in the presence and absence of L-NAME and indomethacin. We also investigated the actions of charybdotoxin (ChTX) and penitrem A, two blockers of calcium-activated K^+ channels (K_{Ca} channels), on L-NAME- and indomethacin-resistant responses to acetylcholine.

Methods

Rabbit isolated intact ear preparation

Male New Zealand White rabbits (2.5 kg) were killed by injection with sodium pentobarbitone (120 mg kg^{-1} i.v). Intact ears were then isolated and the central ear artery cannulated using a polypropylene cannula. This enabled perfusion of the ear with oxygenated Holman's buffer (composition in mM: 120 NaCl, 5 KCl, 2.5 $CaCl_2$, 1.3 NaH_2PO_4, 25 $NaHCO_3$, 11 glucose and 10 sucrose at pH 7.2–7.4, 34±2°C) using a Watson Marlow peristaltic pump (Model No. 505U) at a flow rate of 2 ml min^{-1}. An air-filled compliance chamber was connected to the perfusion system via a T piece to dampen pressure fluctuations to within 5% of the mean pressure and the pressure was recorded by way of a pressure transducer connected to a Gould (Windograf) chart recorder. Following a 30 min equilibration period, ears were preconstricted with a combination of 5-HT (300 nM) and histamine (300 nM) which were added directly to the perfusion buffer. Cumulative concentration-response curves to acetylcholine were then constructed in the presence or absence of a combination of L-NAME (300 μM) and indomethacin (10 μM) over the range 10 nM–30 μM. Prior to the addition of the first concentration of acetylcholine and following the generation of a stable pressure response after each subsequent addition of acetylcholine, microangiograms were produced using a 4 μm microfocal X-ray source as previously described (Griffith *et al.*, 1987). Briefly, to eliminate artefactual rises in perfusion pressure, dextran (5%, mol wt. 80,000) was dissolved in the buffer to increase its viscosity to 2.23 mPas. This matched the viscosity of the contrast medium (Iohexol; 300 mg iodine ml^{-1} diluted 3 fold in buffer) with which the ear was perfused immediately prior to each X-ray microangiogram being taken. Iohexol, at the low concentration employed, has previously been shown to have no significant effect on vasomotor tone or vascular responses during the short period of X-ray exposure (Griffith *et al.*, 1988). The ear was mounted vertically to facilitate drainage of the perfusate and to prevent any overflow of the contrast medium across its surface. X-ray microangiograms were produced using a 20 s exposure at 30 kV. Following completion of the concentration-response curve, acetylcholine was washed out and the concentration-response curve was repeated following administration of a combination of L-NAME (300 μM) and indomethacin (10 μM), ChTX (10 nM) or penitrem A (100 nM) according to the experimental protocol being followed. When concentration-response curves to acetylcholine were constructed in the presence of ChTX or penitrem A, these were constructed over the range 100 nM–300 μM.

Rabbit isolated central ear artery rings

Using isolated rabbit ears, a skin flap was dissected to expose the underlying central ear artery. A 1–2 cm section of the artery from the middle portion of the ear was cleaned of adherent fat and connective tissue, severed at both ends and removed to a dish. Two rings 2 mm in width were taken from this section of artery and mounted in a dual-chamber myograph (J.P. Trading) in oxygenated Holman's buffer. Tension was recorded using a Grass Polygraph (Model 79) and a resting tension of 0.5 g was applied to each ring. Following a 30 min equilibration period, L-NAME (300 μM) and indomethacin (10 μM) were added directly to the myograph chambers and were present for the remainder of the experiment. After a further 30 min period, the rings were preconstricted using 5-HT (300 nM) and histamine (300 nM) and concentration-response curves were then constructed to acetylcholine over the range 100 nM–100 μM. After washout, the protocol was repeated three times with the concentrations of 5-HT and histamine both raised firstly to 1 μM, then to 3 μM and subsequently to 10 μM.

Drugs

Acetylcholine chloride, charybdotoxin, dextran, histamine dihydrochloride, 5-hydroxytryptamine (as creatine sulphate complex), indomethacin, N^{ω}-nitro-L-arginine methyl ester hydrochloride and penitrem A were obtained from Sigma, Poole, U.K. Iohexol (Omnipaque) was obtained from Nycomed (U.K.) Ltd., Birmingham, U.K. All drugs were dissolved initially in distilled water and then diluted to the required concentration in the Holman's buffer except Iohexol which was diluted directly in Holman's buffer, indomethacin which was initially dissolved in 5% $NaHCO_3$ and penitrem A which was initially dissolved in dimethyl sulphoxide (DMSO). The final concentration of DMSO in the perfusate did not exceed 0.01%.

Data and statistical analysis

All data are presented as means±s.e.mean. Statistical significance between treatment groups was determined using ANOVA followed by Fisher's test. $P \leqslant 0.05$ was taken to be significant.

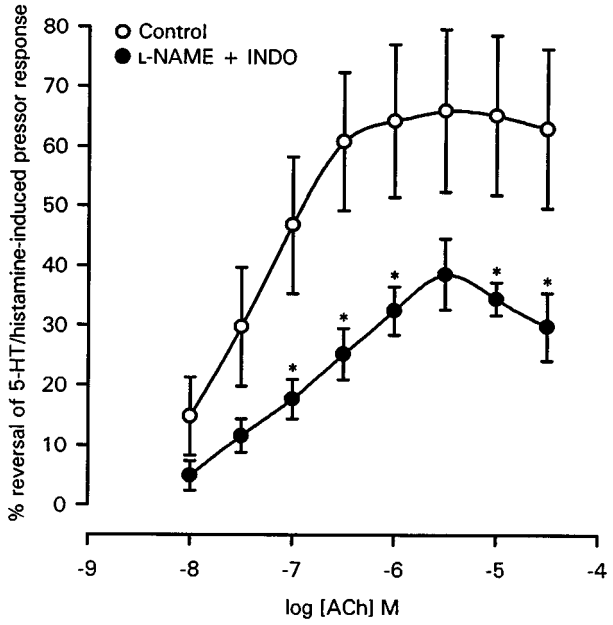

Figure 1 % reversal of developed pressure by acetylcholine (10 μM–30 μM) in preconstricted rabbit isolated ears under control conditions and in the combined presence of 300 μM L-NAME and 10 μM indomethacin (INDO) ($n=6$). Data are shown as means with vertical lines indicating s.e.mean. $^*P<0.05$ indicates a significant difference from control.

Results

Following elevation of pressure in the isolated rabbit ear using a combination of 5-HT (300 nM) and histamine (300 nM) which elicits 69.8±18.9% ($n=4$; data not shown) of the maximum response obtainable from addition of these two agonists, acetylcholine (10 nM – 30 μM) induced a concentration-dependent reversal of the pressor response which reached a maximum of 66.0±13.6% under control conditions (Figure 1; $n=6$). In the combined presence of L-NAME (300 μM) and indomethacin (10 μM), a concentration-dependent depressor response was still observed in response to acetylcholine, but there was a significant reduction in its magnitude, with a maximum being observed at 38.6±5.9%.

Figure 2 shows representative X-ray microangiograms that illustrate the effects of acetylcholine on the central ear artery (G_0), the first generation daughter branch artery (G_1) and the second generation daughter branch artery (G_2) under control conditions and in the combined presence of L-NAME and indomethacin. Mean results from the analysis of the experiments in Figure 1 are shown in Figure 3. Under control conditions, the depressor response induced by acetylcholine was associated with a concentration-dependent increase in the diameter of G_0 vessels, a small increase in the diameter of G_1 vessels, but no significant effect on G_2 vessels ($n=6$). In contrast, the depressor response induced by acetylcholine in the presence of L-NAME and indomethacin was associated with a substantial increase in the diameter of G_2 vessels, but no significant effects on the diameters of G_0 or G_1 vessels.

The effects of ChTX (10 nM; $n=6$) and penitrem A (100 nM; $n=6$) on the depressor response induced by acetylcholine (10 nM – 300 μM) in the presence of L-NAME

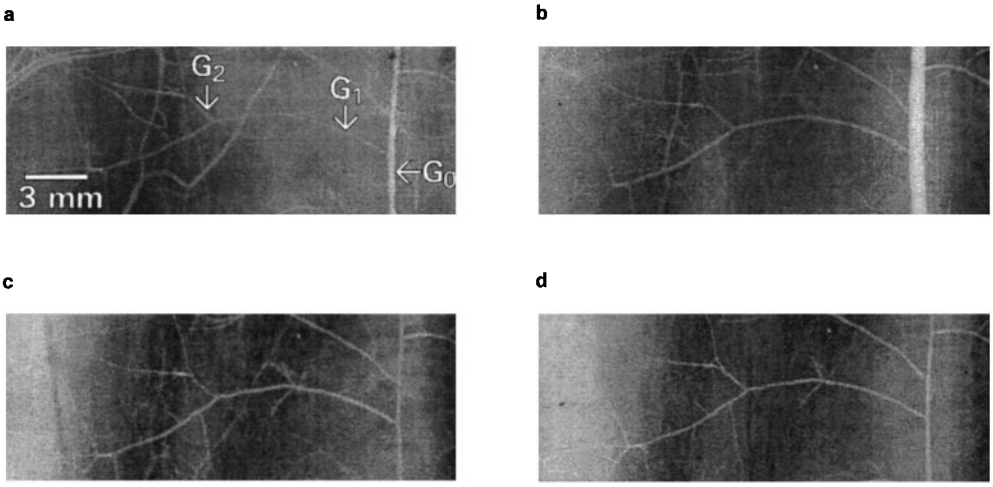

Figure 2 Representative X-ray microangiograms of a preconstricted rabbit isolated ear showing the effects of acetylcholine administration on G_0, G_1 and G_2 vessels in the control situation (a,b) and in the combined presence of 300 μM L-NAME and 10 μM indomethacin (INDO) (c,d). (a) and (c) were produced in the absence of acetylcholine whilst (b) and (d) were produced following administration of 30 μM acetylcholine.

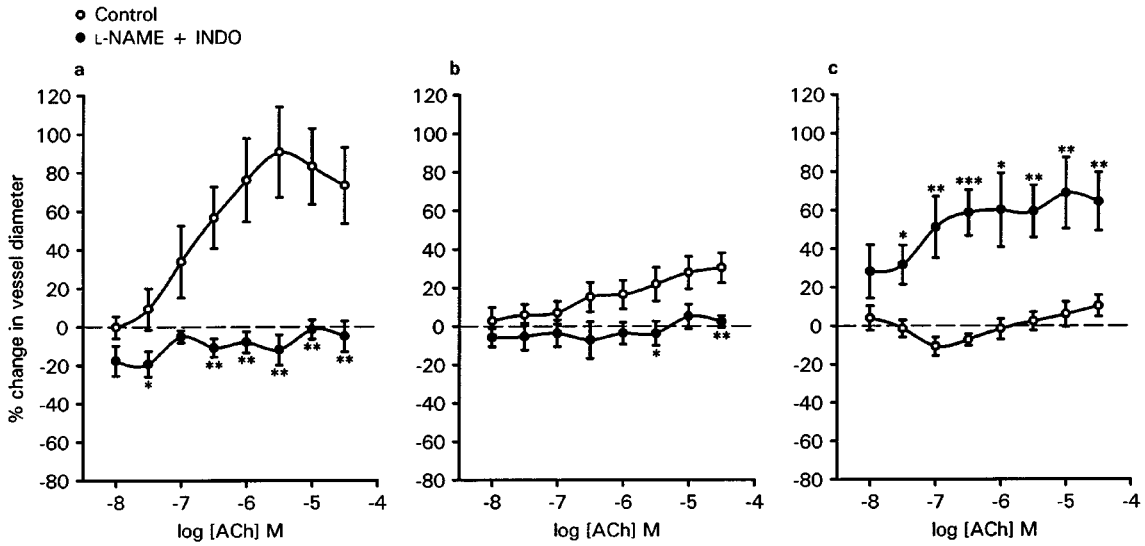

Figure 3 % change in vessel diameter induced by acetylcholine (10 nM – 30 μM) in (a) G_0, (b) G_1 and (c) G_2 arteries of preconstricted rabbit isolated ears determined by microangiography under control conditions and in the combined presence of 300 μM L-NAME and 10 μM indomethacin (INDO) ($n=6$). Data are shown as means with vertical lines indicating s.e.mean. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ indicates a significant difference from control.

and indomethacin are shown in Figure 4a and b respectively. Both agents caused a significant reduction, but not an abolition, of the response to acetylcholine across the concentration ranges investigated although in neither case was a maximal response obtained.

The results of microangiographic analysis of the effects of ChTX ($n=6$) and penitrem A ($n=6$) are shown in Figures 5 and 6. Neither agent altered the lack of effect of acetylcholine on G_0 or G_1 vessels observed in the presence of L-NAME and indomethacin ($n=6$). In G_2 vessels, however, ChTX and penitrem A both significantly inhibited the increase in vessel diameter induced by acetylcholine across the concentration ranges investigated.

Figure 7 shows the mean diameters of G_0, G_1 and G_2 vessels prior to acetylcholine administration determined by microangiography from the same three sets of experiments which are depicted in Figures 1–6. Combined addition of L-NAME and indomethacin caused a significant reduction in the basal diameter of G_0 vessels compared to the control situation but had no significant effect on the diameters of G_1 and G_2 vessels

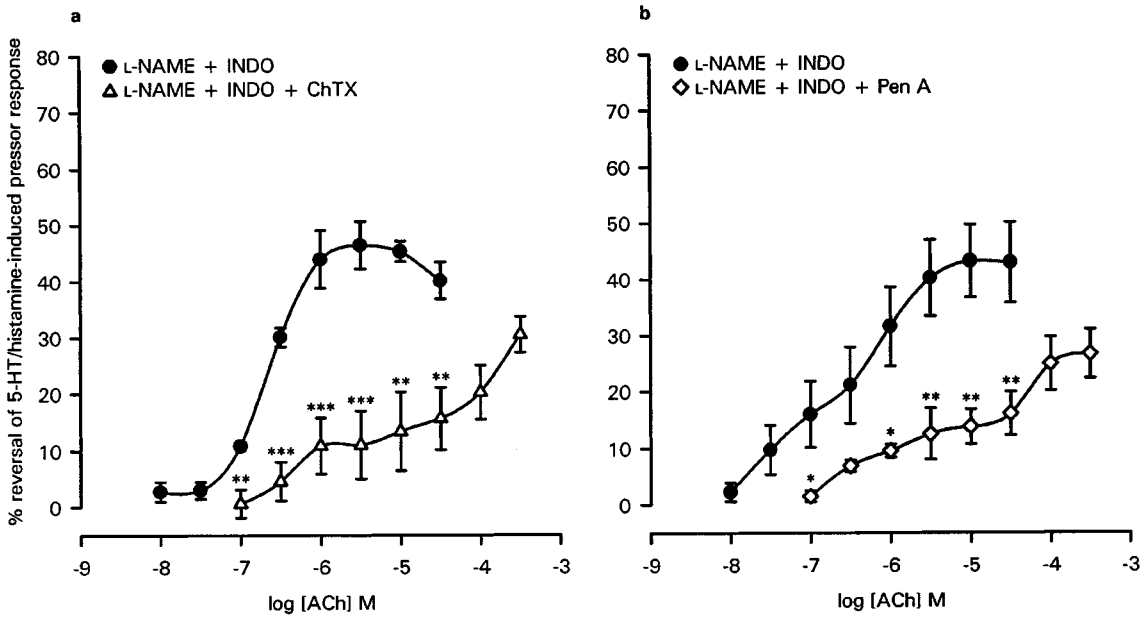

Figure 4 % reversal of developed pressure by acetylcholine (10 nM – 300 μM) in preconstricted rabbit isolated ears in the combined presence of 300 μM L-NAME and 10 μM indomethacin (INDO) and following subsequent addition of (a) 10 nM ChTX ($n=6$) and (b) 100 nM penitrem A (Pen A; $n=6$). Data are shown as means with vertical lines indicating s.e.mean. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ indicates a significant difference from L-NAME and indomethacin alone.

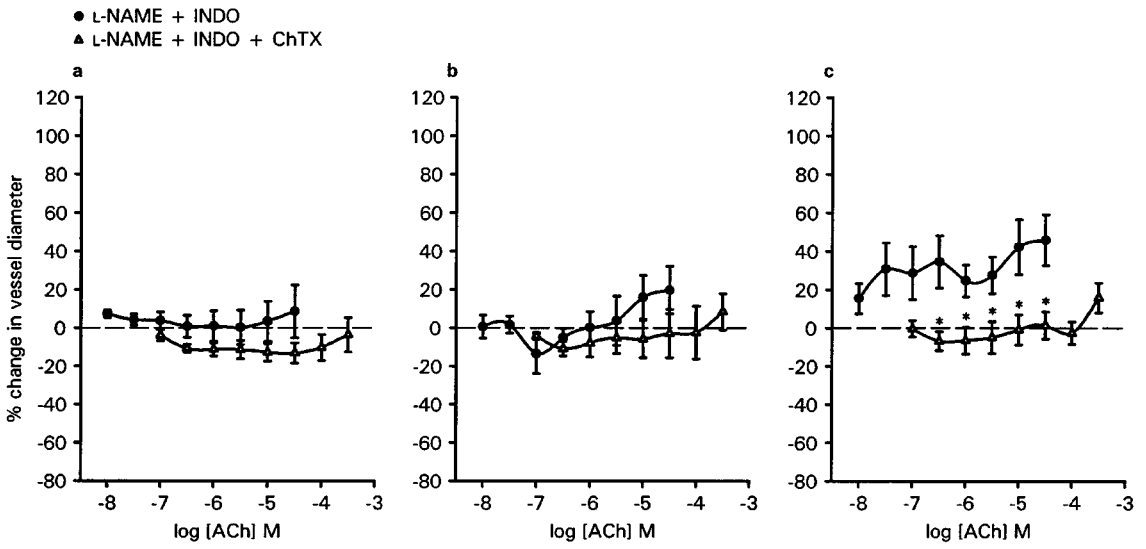

Figure 5 % change in vessel diameter induced by acetylcholine (10 nM – 300 μM) in (a) G_0, (b) G_1 and (c) G_2 arteries of preconstricted rabbit isolated ears determined by microangiography in the combined presence of 300 μM L-NAME and 10 μM indomethacin (INDO) and following subsequent addition of 10 nM ChTX ($n=6$). Data are shown as means with vertical lines indicating s.e.mean. $^{*}P<0.05$ indicates a significant difference from L-NAME and indomethacin alone.

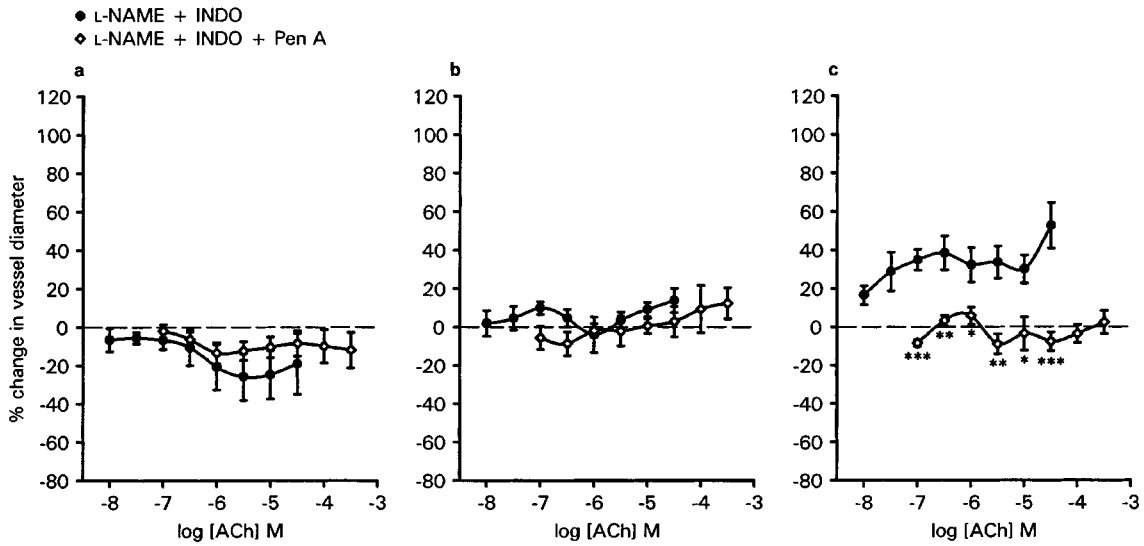

Figure 6 % change in vessel diameter induced by acetylcholine (10 nM – 300 μM) in (a) G_0, (b) G_1 and (c) G_2 arteries of preconstricted rabbit isolated ears determined by microangiography in the combined presence of 300 μM L-NAME and 10 μM indomethacin (INDO) and following subsequent addition of 100 nM penitrem A (Pen A; $n=6$). Data are shown as means with vertical lines indicating s.e.mean. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ indicates a significant difference from L-NAME and indomethacin alone.

(Figure 7a; $n=6$). Addition of either ChTX (Figure 7b; $n=6$) or penitrem A (Figure 7c; $n=6$) subsequent to combined administration of L-NAME and indomethacin was without significant effect on the diameters of all three vessel types.

In isolated G_0 rings, 5-HT and histamine added in increasing equimolar concentrations induced a concentration-dependent increase in tone (Figure 8a; $n=4$). As shown in Figure 8b, acetylcholine (100 nM – 100 μM) induced a small concentration-dependent relaxation of the tone induced by 300 nM 5-HT and 300 nM histamine, reaching a maximum, of $14.7\pm3.3\%$ ($n=4$). Increasing the concentrations of both 5-HT and histamine to 1 μM produced a small but non-significant reduction of the effects of acetylcholine reducing the maximum response to $11.8\pm3.5\%$. Raising the concentrations of both 5-HT and histamine to 3 μM further reduced the maximal response to $7.0\pm0.7\%$, whilst increasing their concentrations to 10 μM reduced it to $5.0\pm1.1\%$.

Discussion

Under control conditions the depressor response evoked by acetylcholine in the isolated rabbit ear potentially involves a variety of diffusible mediators such as NO, prostacyclin and the putative EDHF, as well as direct heterocellular endothelial-smooth muscle communication via gap junctions. Co-administration of L-NAME and indomethacin to inhibit NO and prostacyclin synthesis, respectively, was found to abolish only around a third of the depressor response to acetylcholine, indicating that NO- and prostanoid-independent mechanisms of vasorelaxation are of major importance in this vascular bed.

EDHF has been suggested to play a progressively more important role in the relaxation of smaller arteries (Garland *et al.*, 1995). Some recent studies have investigated the relative contributions played by NO-dependent and independent mechanisms in large and small arteries of the rat mesenteric bed. Hwa *et al.* (1994) showed that acetylcholine-induced relaxation of the rat superior mesenteric artery was abolished both by scavenging NO with haemoglobin or inhibiting its synthesis with N^{ω}-monomethyl-L-arginine. In contrast, acetylcholine-induced relaxation of mesenteric resistance arteries was unaffected by these agents but was inhibited by KCl and by ChTX, suggesting that it is, at least in part, mediated by hyperpolarisation via ChTX-sensitive K^+ channels. Shimokawa *et al.* (1996) compared acetylcholine-induced relaxation in proximal and distal rat mesenteric arteries with aorta and found that L-NAME was a more effective inhibitor of relaxation in aorta than in proximal mesenteric arteries, and least effective in distal arteries. Parsons *et al.* (1994), however, found no significant difference in the extent to which acetylcholine- and A23187-induced relaxations were inhibited by L-NAME in isolated segments of second, third and fourth order branch arteries of the rat mesenteric bed, although no comparison was made with the superior mesenteric artery itself.

Our study is the first to evaluate the relative contributions of NO and non-NO components of the dilator response to acetylcholine in an intact arterial network as opposed to individual isolated artery segments. The X-ray microangiographic technique employed enables visualisation of the intact arterial bed of the rabbit ear, allowing its component vessels to be studied *in situ* whilst remaining part of an integrated network, as in the physiological situation. The results obtained reveal that under control conditions, the depressor response induced by acetylcholine results predominantly from vasodilatation in G_0 vessels, with only a small increase in diameter being observed in G_1 vessels and no effect on G_2 vessels. However, in the presence of L-NAME and indomethacin, a substantial change in the pattern of relaxation was found to occur with no change in diameter being observed in either G_0 or G_1 vessels and significant vasodilatation occurring in G_2 vessels. These results therefore support the hypothesis that NO is a more important mediator of acetylcholine-induced dilatation of larger arteries, whilst other mechanisms may be more predominant in smaller arteries.

The L-NAME- and indomethacin-resistant component of the response to acetylcholine in G_2 vessels was not apparent under control conditions. Within an intact network, however, complex secondary hydraulic factors are also likely to influence the behaviour of individual blood vessels in addition to any effects of pharmacological agents (Griffith *et al*., 1987; Randall *et al*., 1990; Berman & Griffith, 1997). It is therefore conceivable that dilatation in G_2 vessels is masked in the control situation by the more dominant effects of NO on G_0 and G_1 vessels. It is also possible that NO directly inhibits the production of another mediator in the control situation. Indeed, Bauersachs *et al*. (1996) observed that dilatation attributed to EDHF in both rabbit carotid artery and porcine coronary artery was attenuated by exogenous donors of NO.

A recent study (Cohen *et al*, 1997) has suggested that the L-NAME- and indomethacin-resistant component of relaxation induced by acetylcholine in rabbit carotid artery may nevertheless be mediated by NO itself, as NO synthesis was found not to be completely inhibited by a combination of 30 μM L-NAME and 300 μM L-NOARG. The fact that L-NAME and indomethacin not only reduced the magnitude of the depressor response to acetylcholine in the present study, but caused a major change in the pattern of responsiveness within the arterial network would suggest that this is unlikely to be the case in the rabbit ear and that two distinct dilatory mechanisms are involved.

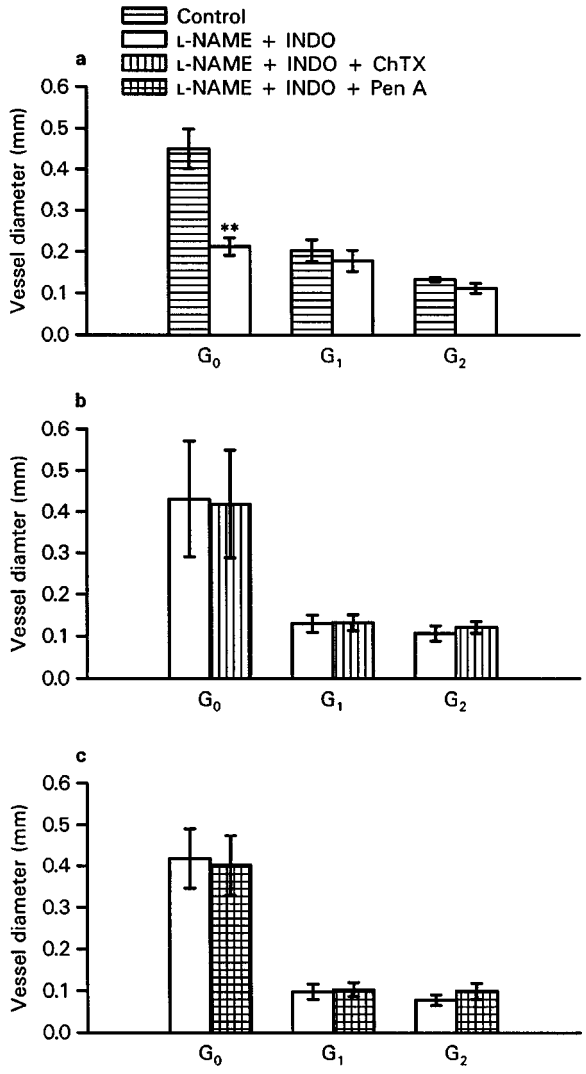

Figure 7 Diameter of G_0, G_1 and G_2 arteries of preconstricted rabbit isolated ears determined by microangiography (a) under control conditions and following subsequent administration of 300 μM L-NAME and 10 μM indomethacin (INDO; $n=6$), (b) in the presence of L-NAME and indomethacin and following subsequent administration of 10 nM ChTX ($n=6$) and (c) in the presence of L-NAME and indomethacin and following subsequent administration of 100 nM penitrem A (Pen A; $n=6$). Data are shown as means with vertical lines indicating s.e.mean. $^{**}P<0.01$ indicates a significant difference from control.

Conflicting evidence has been presented concerning the identity of the K^+ channel which may be activated by EDHF and it would appear to vary from one tissue or species to another under pharmacological blockade. K_{Ca} channels (Bauersachs *et al*., 1994; Hwa *et al*., 1994; García-Pascual *et al*., 1995; Murphy & Brayden, 1995), ATP-sensitive K^+ channels (K_{ATP} channels) (Standen *et al*., 1989) and voltage-dependent K^+ channels (K_V channels) (Petersson *et al*., 1997) have all been suggested to participate in EDHF-mediated relaxation. Some studies have suggested that blockade of more than one K^+ channel subtype is required to obtain complete abolition of EDHF-mediated. relaxation in certain tissues. Dong *et al*. (1997) showed that EDHF-mediated relaxation in rabbit carotid artery involves the opening of at least two types of K_{Ca} channel, whilst Petersson *et al*. (1997) suggested that EDHF-mediated relaxation in guinea-pig cerebral arteries may involve the opening of both K_V and small-conductance K_{Ca} channels.

In the present study, we investigated the actions of ChTX. This K^+ channel blocker caused a significant inhibition but not complete abolition of the depressor response induced by acetylcholine, in the presence of L-NAME and indomethacin, across the concentration range investigated. At the same time, ChTX was found to abolish the vasodilatory response observed to acetylcholine in G_2 vessels without altering the lack of a response to acetylcholine in either G_0 or G_1 vessels. Previous work in this laboratory has shown that in the isolated rabbit ear, ChTX induced an upwards shift in the pressure-flow relationship that was associated with vasoconstriction in G_0 vessels but little effect on G_1 or G_2 vessels, whilst L-NAME induced an upwards shift that was associated with vasoconstriction in all three vessel types (Berman & Griffith, 1997). The lack of effect of ChTX in G_2 vessels in that study contrasts with the ability of ChTX in the present study to abolish the vasodilatory response induced by acetylcholine in G_2 vessels in the presence of L-NAME and indomethacin. This may therefore suggest that while the acetylcholine-mediated response in the isolated rabbit ear may include a component mediated via ChTX-sensitive K^+ channels, the flow-mediated response does not. However, in our earlier study the concentration of ChTX employed was only 1 nM and this may have been too low to induce an effect in G_2 vessels in reponse to an increase in flow.

ChTX is generally regarded as a relatively specific blocker of large-conductance K_{Ca} channels (Nelson & Quayle, 1995) but may also block other K^+ channel subtypes such as K_V channels (Grissmer *et al*., 1994; Sprunger *et al*., 1996), small-conductance K_{Ca} channels (Gebremedhin *et al*., 1996) and K_{ATP} channels (Davies *et al*., 1996). In order to determine, therefore, if the actions of ChTX observed in this study can be attributed to blockade of large-conductance K_{Ca} channels, the effects of another more selective blocker of these channels, penitrem A (Knaus *et al*., 1994), were also evaluated. Similar results were indeed obtained using penitrem A as were obtained with ChTX. Penitrem A inhibited, but did not abolish, the depressor response induced by acetylcholine in the presence of L-NAME and indomethacin, and abolished the

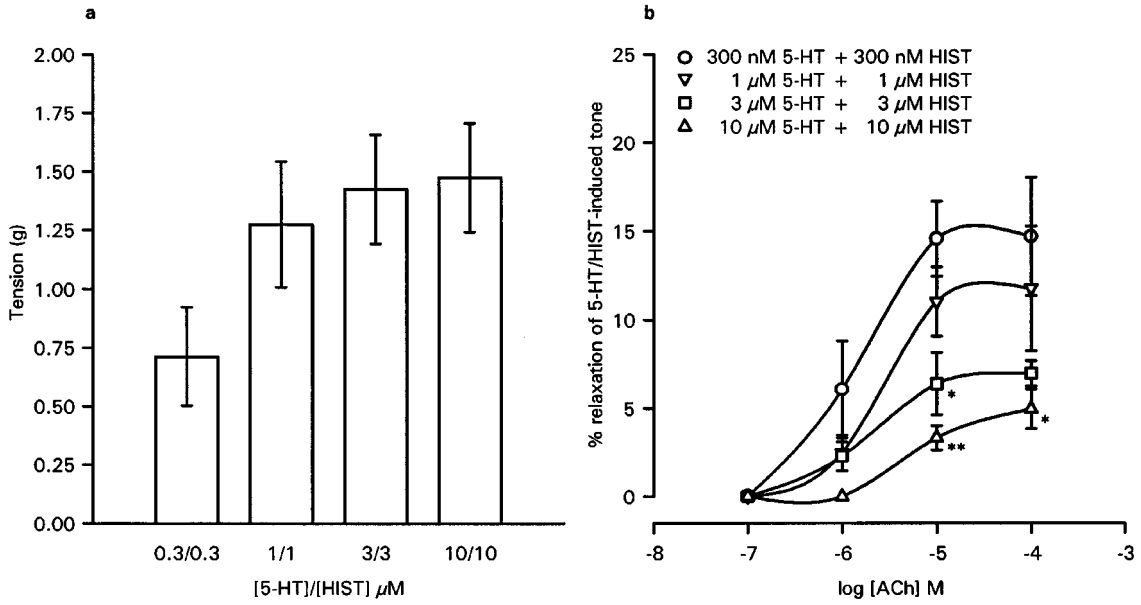

Figure 8 (a) Effects on tone of combined administration of 5-HT and histamine (HIST) in increasing equimolar concentrations to rabbit central ear artery rings ($n=4$). (b) % relaxation of tone by acetylcholine (100 nM – 100 μM) in rabbit central ear artery rings in the combined presence of 300 μM L-NAME and 10 μM indomethacin following preconstriction with 5-HT and histamine (HIST) added in increasing equimolar concentrations ($n=4$). Data are shown as means with vertical lines indicating s.e.mean. $^{*}P<0.05$; $^{**}P<0.01$ indicates a significant difference from 300 nM 5-HT plus 300 nM histamine.

increase in diameter induced by acetylcholine in G_2 vessels without any effect on the lack of response in G_0 or G_1 vessels. Taking these results together, it is therefore highly likely that activation of large-conductance K_{Ca} channels contributes to the L-NAME- and indomethacin-resistant depressor response induced by acetylcholine in the rabbit ear.

In the experiments with ChTX and penitrem A, no significant increases in vessel diameter were observed in any of the three branch arteries studied despite residual concentration-dependent depressor responses to acetylcholine being detected which reached 25 – 30% at the highest concentration of acetylcholine investigated. The fact that a complete block of the depressor response was not observed suggests that L-NAME- and indomethacin-resistant dilatation also occurs further down the arterial tree in branch generations distal to G_2 arteries. Additionally, it would appear that either this dilatory component is mediated by a further distinct mechanism of action or K^+ channel subtype, or that ChTX and penitrem A were not used in sufficient concentrations to elicit complete block of large conductance K_{Ca} channels.

Various groups have shown that the effectiveness of both endothelium-dependent and endothelium-independent relaxants may decrease when the degree of agonist-induced tone is increased (Dainty *et al.*, 1990; Wiener & Thalody, 1993; Stork & Cocks, 1994). Thus, it is possible that the effects of L-NAME and indomethacin and of ChTX or penitrem A, on the responsiveness of acetylcholine observed in this study could be secondary, non-specific effects of these agents on the tone of individual vessels. To determine if such functional antagonism could be occurring, we analysed the effects these agents had induced on the basal diameters of G_0, G_1 and G_2 vessels prior to addition of acetylcholine. The combined addition of L-NAME and indomethacin was found to have caused a significant decrease in the diameter of G_0 vessels compared to the control situation, but had not affected the diameter of G_1 or G_2 vessels. In contrast, addition of ChTX or penitrem A subsequent to L-NAME and indomethacin had been without significant effect on the diameter of any of the three vessel types. On this basis, the actions of ChTX and penitrem A would appear to be due solely to their actions as K^+ channel blockers, but functional antagonism in G_0 vessels cannot be ruled out in contributing to the effects of L-NAME and indomethacin.

To determine if the action of acetylcholine could in fact include a non-NO, non-prostanoid component in G_0 vessels which is being masked by functional antagonism, the actions of acetylcholine in the presence of L-NAME and indomethacin were investigated in isolated rings of G_0 vessels. In contrast to observations in the intact ear, an L-NAME- and indomethacin-resistant relaxation was observed to acetylcholine although it was small in magnitude reaching a maximum of only $\sim$15%. Increasing the extent of preconstriction by raising the concentrations of 5-HT and histamine did attenuate this response, although some 5 – 10% relaxation still persisted even when the extent of contraction was enhanced by $\sim$2 fold. Thus, it is possible that a non-NO, non-prostanoid component which is susceptible to functional antagonism could contribute to the action of acetylcholine in G_0 vessels. However, in the intact ear experiments the diameter increase observed in G_0 was $\sim$90%. Since only a small L-NAME- and indomethacin-insensitive relaxation to acetylcholine was observed in isolated G_0 rings, a non-NO, non prostanoid mediator could at most constitute only a small component of the response in G_0 in the intact ear.

In conclusion, we have shown that inhibition of NO and prostacyclin synthesis in the preconstricted rabbit isolated ear caused a reduction, but not an abolition, of the depressor reponse to acetylcholine. The L-NAME- and indomethacin-resistant response was associated with a change in the pattern of dilatation observed within the arterial network, revealing a shift in dilatation from G_0 and G_1 vessels to G_2 vessels. This is consistent with the hypothesis that NO is the dominant

mediator of dilatation in larger arteries whilst another mediator or mechanism, such as a diffusible EDHF and/or direct endothelial-smooth muscle communication via gap junctions, dominates in smaller arteries. The L-NAME- and indomethacin-resistant dilatation was significantly inhibited by ChTX and penitrem A, suggesting that it involves activation of large-conductance K_{Ca} channels, although other K^+ channel subtypes and mechanisms may additionally be involved.

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