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Differential actions of charybdotoxin on central and daughter branch arteries of the rabbit isolated ear

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1 By use of rabbit isolated perfused intact ears and isolated perfused segments of central and first generation daughter branch ear arteries, we investigated the actions of charybdotoxin (ChTX), a blocker of calcium-activated K^+ channels (K_{Ca} channels), and N^{ω}-nitro-L-arginine methyl ester (L-NAME) on pressure-flow and diameter-flow relationships.

2 ChTX (1 nM) induced an upwards shift in the pressure-flow curve in the rabbit intact isolated ear preconstricted with 5-hydroxytryptamine (5-HT; 100 nM) with subsequent administration of L-NAME (100 μM) inducing a further upwards shift. L-NAME itself induced an upwards shift in the pressure-flow curve, but subsequent administration of ChTX was without significant effect.

3 Microangiographic analysis revealed a tendency of ChTX (1 nM) to decrease vessel diameter in the central ear artery (G_0) with little effect on the first two generations of daughter branch arteries (G_1 and G_2) in the intact ear. Subsequent addition of L-NAME (100 μM) did not significantly further decrease vessel diameter in G_0, but did decrease vessel diameter in G_1 and G_2. L-NAME itself showed a tendency to decrease vessel diameter in G_0, G_1 and G_2 vessels with subsequent addition of ChTX being without significant effect.

4 In an isolated G_0 preparation which was preconstricted with 5-HT (100 nM), ChTX (1 nM) caused an upwards shift in the pressure-flow curve which was augmented by subsequent addition of L-NAME (100 μM). L-NAME (100 μM) itself caused an upwards shift in the pressure-flow curve but subsequent addition of ChTX (1 nM) had no significant effect.

5 In comparison, in an isolated G_1 preparation which was preconstricted with 5-HT (100 nM), ChTX (1 nM) had no significant effect on the pressure-flow curve relative to control, but subsequent addition of L-NAME (100 μM) caused an upwards shift. L-NAME (100 μM) itself induced an upwards shift in the pressure-flow curve with subsequent addition of ChTX (1 nM) being without significant effect.

6 ChTX (10 pM – 10 nM) caused a concentration-dependent increase in perfusion pressure in isolated G_0 and G_1 preparations at fixed flow rates of 2 ml min^{-1} and 0.5 ml min^{-1}, respectively. These responses were enhanced in the presence of L-NAME (100 μM) in G_1 but not G_0 preparations.

7 We conclude that at 1 nM, ChTX exhibits differential actions on central and daughter branch arteries of the intact ear of the rabbit, which are also apparent in the corresponding arteries when studied in isolation. The action of 1 nM ChTX in G_0 vessels may reflect inhibition of either the release or action of nitric oxide as it was blocked in the presence of L-NAME. At higher concentrations of ChTX, there would appear to be a direct constrictor effect on vascular smooth muscle which is apparent in both G_0 and G_1 vessels. This observed heterogeneity could reflect different distributions of K_{Ca} channels between central and daughter branch arteries at either the endothelial or smooth muscle levels, or both.

Keywords: Charybdotoxin; calcium-activated potassium channels; nitric oxide; rabbit ear; microangiography; flow; shear stress

Introduction

K^+ channels may modulate vascular tone through both direct and indirect mechanisms. In vascular smooth muscle cells, they play a key role in the regulation of the membrane potential (see Nelson & Quayle, 1995 for review). When opened they cause an efflux of K^+ resulting in hyperpolarization which leads in turn to the closure of voltage-dependent Ca^{2+} channels, decreased Ca^{2+} entry and vasodilatation. Ca^{2+}-activated K^+ channels (K_{Ca} channels) are activated both by membrane depolarisation and by intracellular calcium and therefore are activated during vasoconstriction. In certain artery types, they are also believed to play a role in the maintenance of resting vascular myogenic tone (Asano *et al*., 1993).

K^+ channels are also present in vascular endothelial cells and have been suggested to play a regulatory role in both flow- and agonist-induced release of endothelium-derived relaxing factor (EDRF) which has now been identified as nitric oxide, or a closely related substance (see Moncada *et al*., 1991 for review). Hyperpolarization has been shown to be induced by flow and shear stress in a variety of endothelial cells types through activation of an inward-rectifying K^+ current (Nakache & Gaub, 1988; Olesen *et al*., 1988; Jacobs *et al*., 1995). Evidence that the K_{Ca} channel subtype may also play a regulatory role in flow-induced release of nitric oxide has been provided by cascade bioassay studies in which the K_{Ca} channel blockers, tetraethylammonium (TEA) and charybdotoxin (ChTX), have been shown to inhibit nitric oxide release in response to flow (Cooke *et al*., 1991; Hutcheson & Griffith, 1994). However, in contrast Wellman & Bevan (1995) found that ChTX and TEA were without effect on flow-induced nitric oxide release, whilst Busse *et al*. (1991) found that ChTX had no effect on shear stress-induced vasodilatation. Studies with bovine aortic endothelial cells have shown that TEA can inhibit shear stress-induced acute increases in the production of guanosine 3′:5′-cyclic monophosphate (cyclic GMP) (Ohno *et al*., 1993) and longer term expression of nitric oxide synthase mRNA (Uematsu *et al*., 1995).

In addition to modulating the release of nitric oxide, K_{Ca} channels may also mediate part of its relaxant activity in some types of vascular smooth muscle. Nitric oxide released from endothelium in response to acetylcholine has been shown to

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induce hyperpolarization in vascular smooth muscle (Feletou & Vanhoutte, 1988), whilst patch clamp studies have shown that K_{Ca} channels may be activated by exogenously applied nitric oxide, cyclic GMP analogues and cyclic GMP-dependent protein kinase in a variety of vascular smooth muscle types (Robertson *et al*., 1993; George & Shibata, 1995; Koh *et al*., 1995). Furthermore, K_{Ca} channel blockers have been shown to inhibit relaxations of endothelium-denuded arterial rings induced by exogenous nitric oxide, nitric oxide donors and (S_P)-guanosine cyclic 3′,5′-phosphotothioate, a cyclic GMP-dependent protein kinase activator (Archer *et al*., 1995; Bialecki & Stinson-Fisher, 1995). K_{Ca} channel activation in smooth muscle by nitric oxide has been suggested to occur both through a pathway involving cyclic GMP production as well as by an independent direct action (Koh *et al*., 1995). Indeed, Bolotina *et al*. (1994) found that when guanylate cyclase activity was inhibited by methylene blue, exogenous nitric oxide could directly activate K_{Ca} channels in excised membrane patches from rabbit aortic smooth muscle cells and induce ChTX-sensitive relaxations in isolated aortic rings.

In the present study we have utilized an X-ray microangiographic technique, which enables the visualization of an intact resistance bed, to investigate the role of K_{Ca} channels in resistance arteries by studying the actions of ChTX on vessel diameter of central and daughter branch arteries of the rabbit isolated ear. In addition, we have investigated the effect of ChTX on pressure-flow relationships in the intact ear and in isolated segments of both central ear arteries and first generation daughter branch arteries. Experiments were conducted across a range of flow rates since K_{Ca} channel activation may be determined by shear stress. To help differentiate any direct smooth muscle effects of ChTX from those related to nitric oxide production or activity, the actions of N^{ω}-nitro-L-arginine methyl ester (L-NAME) were also investigated both alone and in combination with ChTX.

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 facilitated perfusion of the ear with oxygenated Holman's buffer (composition in mM: NaCl 120, KCl 5, $CaCl_2$ 2.5 NaH_2PO_4 1.3, $NaHCO_3$ 25, glucose 11 and sucrose 10 at pH 7.2–7.4, 34±2°C) with a Watson Marlow peristaltic pump (Model No. 505U) at an initial 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 half-hour equilibration period, ears were initially preconstricted with 5-hydroxytryptamine (5-HT; 100 nM). Subsequently, in the presence and absence of ChTX (1 nM), L-NAME (100 μM) or a combination of these, pressure-flow curves were constructed by varying the flow rate across a range from 0.5–5 ml min^{-1}. Following the generation of a stable pressure response at each flow rate, microangiograms were produced with 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 in order to match that of the contrast medium (Iohexol; 300 mg iodine ml^{-1} diluted 3 fold in buffer) with which the ear was perfused immediately before each X-ray microangiogram was taken. Iohexol has previously been shown to have no significant effect on vasomotor tone or vascular responses during the period of X-ray exposure (Griffith *et al*., 1988). The ear was mounted vertically to facilitate drainage of the perfusate away from the ear and thus prevent any overflow of the contrast medium across the ear's surface. X-ray microangiograms were produced by a 20 s exposure at 30 kV. Following the completion of the pressure-flow curve, subsequent curves were constructed following administration of ChTX (1 nM), L-NAME (100 μM) or a combination of these according to the experimental protocol being followed.

Rabbit isolated central ear artery preparation

Isolated central ear artery preparations were prepared *in situ* by use of rabbit intact isolated ear preparations from which a skin flap was dissected to expose the underlying central ear artery. The artery was then cannulated and all first generation daughter branch arteries which originated along a length of approximately 3–4 cm distal to the cannula were identified and ligated close to their junctions with the central ear artery. The artery was then severed immediately beyond the most distal of the ligated branch arteries, and the preparation was perfused with oxygenated Holman's buffer with a peristaltic pump at an initial flow rate of 2 ml min^{-1}. The skin flap was replaced over the cannulated vessel to prevent its adventitial surface from drying out during the period of the study. Artery perfusion pressure was recorded as before via a pressure transducer connected to a chart recorder. Following a half-hour equilibration period, the vessel was preconstricted with 5-hydroxytryptamine (5-HT, 100 nM) and one of two basic protocols followed depending on the nature of the experiment being conducted. Under the first protocol, pressure-flow curves were constructed in the presence and absence of ChTX (1 nM), L-NAME (100 μM) and a combination of these two agents, across a range of flow rates from 0.5–5 ml min^{-1}. Under the second protocol, cumulative concentration-response curves were constructed to ChTX (10 pM–10 nM) in either the presence or absence of L-NAME (100 μM).

Rabbit isolated first generation daughter branch artery preparation

Isolated first generation branch arteries were prepared *in situ* in a similar manner to the isolated central ear artery preparations. A skin flap was first dissected and a cannula inserted into the central ear artery, positioned so that its tip was immediately proximal to the origin of an identified daughter branch artery. The cannula was then tied to the central ear artery and the central ear artery ligated immediately distal to its junction with the daughter branch artery. The daughter branch artery was severed approximately 1–1.5 cm distal to its origin and then perfused with oxygenated Holman's buffer by a peristaltic pump at an initial flow rate of 0.5 ml min^{-1}. The skin flap was again replaced over the cannulated vessel to prevent its adventitial surface from drying out and perfusion pressure was again recorded with a pressure transducer connected to a chart recorder. As with the isolated central ear artery preparations, following a half-hour equilibration period the vessel was preconstricted with 5-HT (100 nM) and one of two basic protocols followed depending on the nature of the experiment being conducted. Under the first protocol, pressure-flow curves were constructed in the presence and absence of ChTX (1 nM), L-NAME (100 μM) and a combination of these two agents, across a range of flow rates from 0.25–1 ml min^{-1}. Under the second protocol, cumulative concentration-response curves were constructed to ChTX (100 pM–10 nM) in either the presence or absence of L-NAME (100 μM).

Drugs

Dextran, 5-hydroxytryptamine (as creatine sulphate complex) and N^{ω}-nitro-L-arginine methyl ester hydrochloride were obtained from Sigma (Poole, U.K.). Charybdotoxin was obtained from Alomone Labs (Jerusalem, Israel). 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).

Data and statistical analysis

All data are presented as means ± s.e.mean. Statistical significance between treatment groups was determined by ANOVA followed by Bonferroni's test. A probability of 0.05 or less was taken to be significant.

Results

Isolated intact ear experiments

Pressure-flow curves were constructed across a range of flow rates from 0.5–5.0 ml min^{-1} in intact ear preparations which had been preconstricted with 5-HT (100 nM). Figure 1a shows that ChTX (1 nM) induced an upwards shift in the pressure-flow curve relative to control levels, although the observed pressure increases were only significant at flow rates of 3 ml min^{-1} and above ($n=9$). Subsequent administration of L-NAME (100 μM) resulted in a further upwards shift. L-NAME was itself observed to induce an upwards shift in the pressure-flow curve relative to control levels, but when ChTX was administered subsequently, it did not cause a further amplification of the response on the pressure-flow curve (Figure 1b; $n=7$).

Microangiographic analysis revealed a tendency of ChTX to cause constriction in the central ear artery (G_0), although the observed decrease in vessel diameter was only significant at 3 ml min^{-1} and was less apparent at lower or higher flow rates (Figure 2a; $n=9$). Subsequent addition of L-NAME slightly further decreased diameter at the lower flow rates studied but not significantly. In contrast, at the higher flow rates tested, it caused a reversal of the decrease induced by ChTX. Figure 2b shows that at the level of the first generation daughter branch artery (G_1), ChTX had no significant effect on vessel diameter indicating the lack of a constrictor response, but subsequent administration of L-NAME caused a reduction in vessel diameter that was significant at 3–4 ml min^{-1} ($n=9$). The actions of ChTX on second generation branch arteries (G_2) are shown in Figure 2c ($n=9$). As in G_1 vessels, ChTX had no significant effect on vessel diameter but subsequent administration of L-NAME induced a decrease which was most apparent at 3–4 ml min^{-1}. L-NAME itself decreased vessel diameter in G_0 although this was not found to be statistically significant and at 5 ml min^{-1}, the highest flow rate tested, there was again a 'paradoxical' reversal of this vasoconstrictor response (Figure 2d; $n=7$). Subsequent addition of ChTX was without significant effect. Figure 2e shows that L-NAME induced a significant decrease in the diameter of G_1 vessels which was more apparent at higher flow rates and which was not significantly affected by subsequent administration of ChTX ($n=7$). A similar pattern of responses was found in G_2 vessels, although the decrease in diameter induced by L-NAME was not as great as in G_1 vessels and was not found to be statistically significant (Figure 2f; $n=7$). Consistently, in both series of experiments, the final vessel diameters obtained in each artery type in the presence of the combination of ChTX and L-NAME were the same regardless of the order in which they were administered.

Isolated central ear artery experiments

In isolated segments of central ear artery preconstricted with 5-HT (100 nM), pressure-flow curves were constructed across a range of flow rates from 0.5–5 ml min^{-1}. Figure 3a shows that, compared to control, ChTX (1 nM) induced an upwards shift in the pressure-flow curve which was significant at the two highest flow rates studied ($n=6$). A further upwards shift was observed on subsequent administration of L-NAME (100 μM).

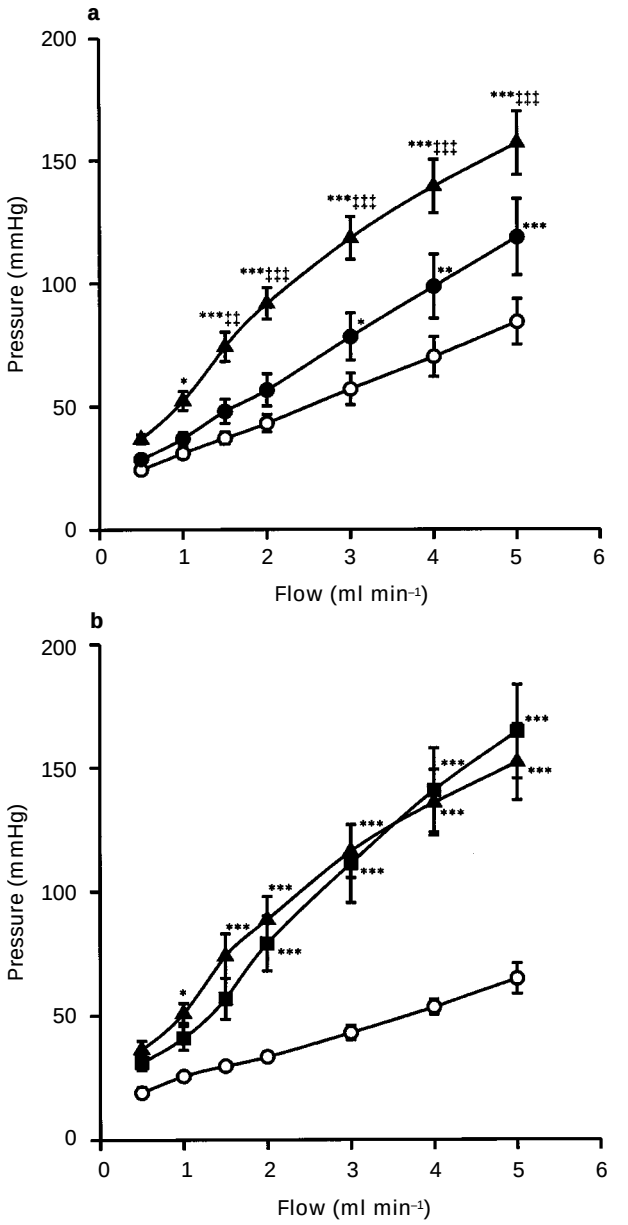

Figure 1 Pressure-flow curves obtained under control conditions (○) and in the presence of 1 nM ChTX (●), 100 μM L-NAME (■) and a combination of these two agents (▲) in rabbit intact ears. In (a), ChTX was administered before the addition of L-NAME ($n=9$), whilst in (b), L-NAME was administered before the addition of ChTX ($n=7$). Data are shown as means and vertical lines indicate s.e.mean. $^{*}P<0.05$; $^{**}P<0.01$, $^{***}P<0.001$ indicates a significant difference from control. ‡‡$P<0.01$; ‡‡‡$P<0.001$ indicates a significant difference from ChTX.

When L-NAME was administered first (Figure 3b), it was found itself to induce an upwards shift in the pressure-flow curve compared to control levels, but subsequent addition of ChTX was without effect ($n=6$).

At a fixed flow rate of 2 ml min^{-1}, concentration-response curves to ChTX (10 pM–10 nM) were constructed in the presence and absence of L-NAME (100 μM) and compared after correcting for the difference in baseline pressure induced by L-NAME. Figure 5a shows that ChTX induced a concentration-dependent increase in perfusion pressure which did not reach a maximal response within the concentration range tested ($n=6$). When concentration-response curves were constructed in the presence of L-NAME ($n=6$), there were no significant differences observed in the pressure increases induced by ChTX administration.

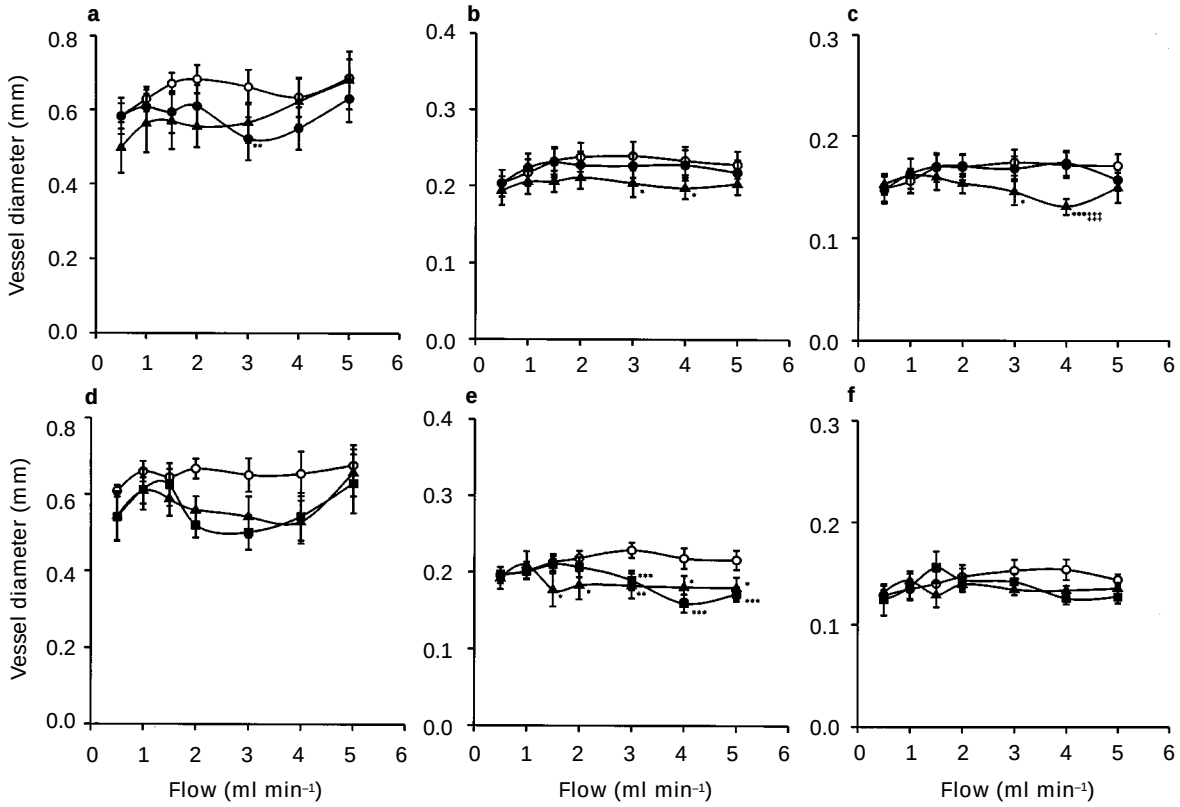

Figure 2 Diameter-flow relationships in (a,d) G_0, (b,e) G_1 and (c,f) G_2 vessels of rabbit intact, isolated ears determined by microangiography under control conditions (○) and in the presence of 1 nM ChTX (●), 100 μM L-NAME (■) and a combination of these two agents (▲). Experiments were performed under conditions corresponding to those of experiments shown in Figure 1. In (a), (b) and (c), ChTX was administered before the addition of L-NAME ($n=9$), whilst in (d), (e) and (f), L-NAME was administered before the addition of ChTX ($n=7$). 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. ‡‡‡$P<0.001$ indicates a significant difference from ChTX.

Isolated first generation daughter branch artery experiments

Pressure-flow curves across the range 0.25 – 1.0 ml min^{-1} were constructed in isolated segments of G_1 vessels which were preconstricted with 5-HT (100 nM). As shown in Figure 4a, ChTX (1 nM) had no effect on the pressure-flow curve compared to control levels, but subsequent addition of L-NAME (100 μM) induced a significant upwards shift ($n=6$). Figure 4b shows that when L-NAME was administered first, an upwards shift in the pressure-flow curve was observed although the increase in pressure was only found to be statistically significant at 1 ml min^{-1}, the highest flow rate studied ($n=6$). Subsequent addition of ChTX was observed to be have no significant effect.

At a fixed flow rate of 0.5 ml min^{-1}, concentration-response curves to ChTX (100 pM – 10 nM) were constructed in the presence and absence of L-NAME (100 μM) and compared after correcting for the difference in baseline pressure induced by L-NAME. Figure 5b shows that ChTX induced a concentration-dependent increase in perfusion pressure which did not reach a maximal response within the concentration range tested ($n=6$). When concentration-response curves were constructed in the presence of L-NAME ($n=7$), the responses to ChTX at the top end of the concentration range tested were found to be augmented although, again, a maximal response was not reached within the concentration range investigated.

Discussion

The major finding of this study is that at a concentration of 1 nM, ChTX, a blocker of high- and medium-conductance K_{Ca} channels (see Cook & Quast, 1990 for review), exhibited a differential action on central and daughter branch arteries of the rabbit isolated ear in terms of its effect on pressure-flow and diameter-flow relationships. In the intact isolated ear, ChTX induced an upwards shift in the pressure-flow curve, relative to control levels, which was associated with a decrease in vessel diameter in G_0 vessels but not in G_1 or G_2 vessels. This was in comparison to the action of L-NAME which was observed to induce an upwards shift in the pressure-flow curve in the intact ear that was attributable to a decrease in vessel diameter in G_0, G_1 and G_2 vessels.

The behaviour of individual arteries within an intact network will inevitably be influenced by secondary hydraulic factors in addition to the direct effects of pharmacological and physiological agents. For instance, earlier work in this laboratory, in which the same microangiographic technique employed in this study was utilised, revealed that in the rabbit unconstricted ear, scavenging basal nitric oxide with haemoglobin caused constriction in G_1, G_2 and the third generation of daughter branch arteries, G_3, but paradoxically caused dilatation in G_0 (Griffith *et al*., 1987). This dilator effect was presumed to result as a consequence of the upstream rise in pressure which was induced by downstream constriction. That observed pattern of responsiveness was not fully reflected in the present study by the actions of L-NAME, which induced constriction in G_0 as well as in G_1 and G_2 vessels in the intact ear. This is most likely due to the fact that in this study the ear was preconstricted with 5-HT, altering the pattern of vascular reactivity. However, at the highest flow rates studied, the constrictor response to L-NAME in G_0 vessels was lost, and it is possible this reflects the onset of a paradoxical dilator mechanism.

In order to determine if the observed differential action of

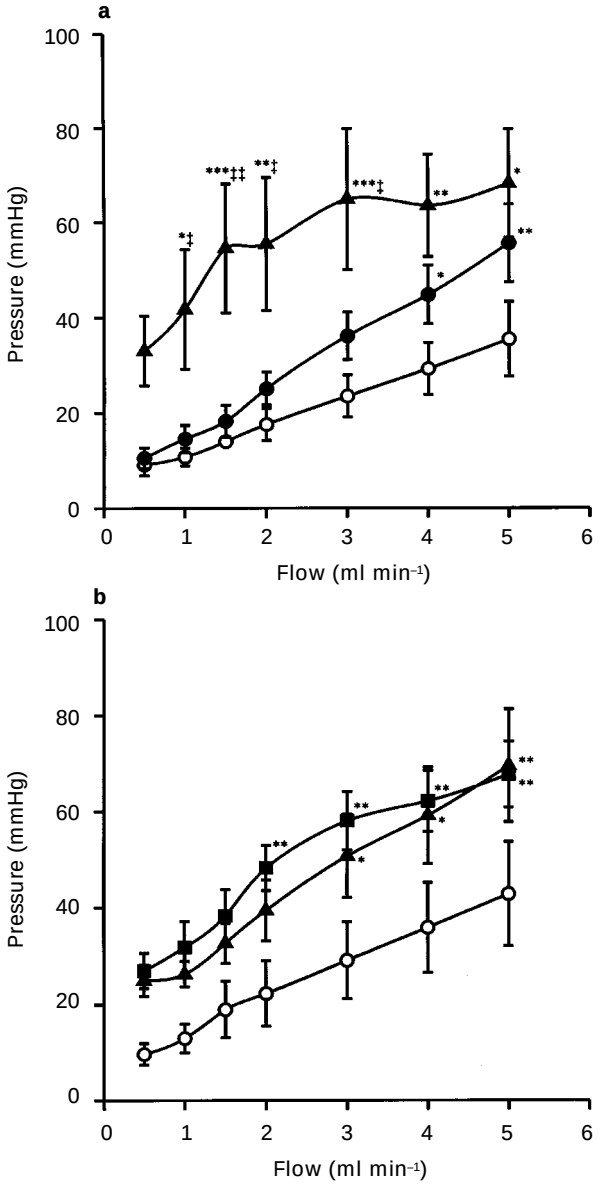

Figure 3 Pressure-flow curves obtained under control conditions (○) and in the presence of 1 nM ChTX (●), 100 μM L-NAME (■) and a combination of these two agents (▲) in isolated segments of G_0 vessels. In (a), ChTX was administered before the addition of L-NAME ($n=6$) whilst in (b), L-NAME was administered before the addition of ChTX ($n=6$). 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. ‡$P<0.05$; ‡‡$P<0.01$ indicates a significant difference from ChTX.

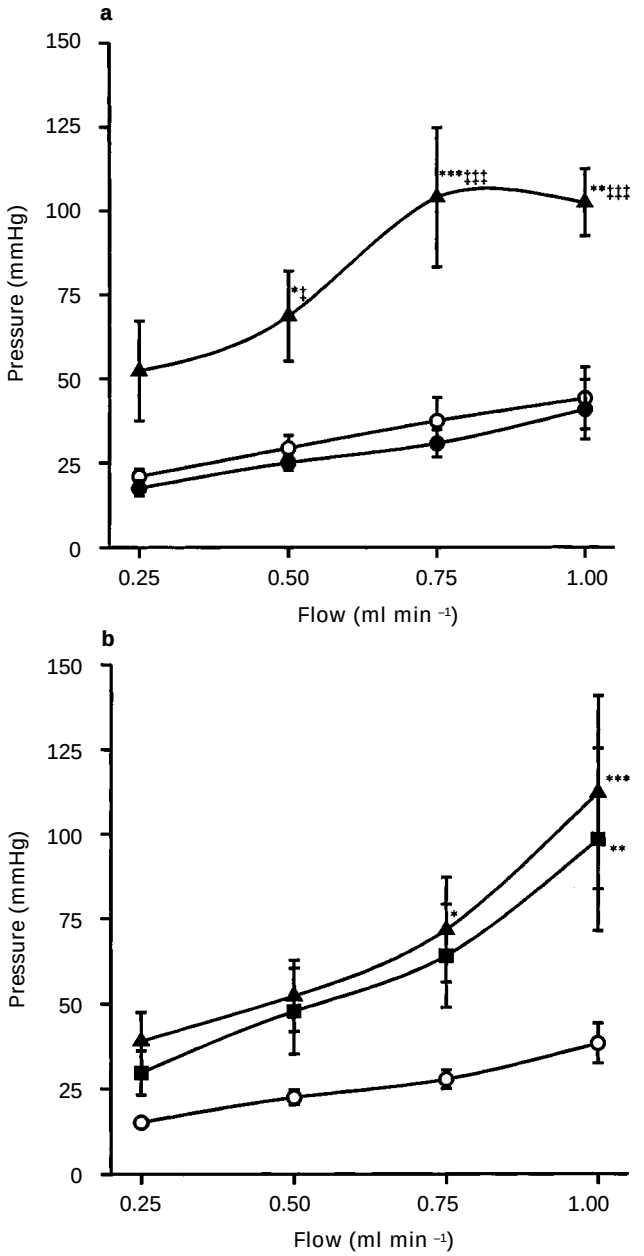

Figure 4 Pressure-flow curves obtained under control conditions (○) and in the presence of 1 nM ChTX (●), 100 μM L-NAME (■) and a combination of these two agents (▲) in isolated segments of G_1 vessels. In (a), ChTX was administered before the addition of L-NAME ($n=6$), whilst in (b), L-NAME was administered before the addition of ChTX ($n=6$). Data are shown as means and vertical lines indicate s.e.mean. $*P<0.05$; $**P<0.01$; $***P<0.001$ indicates a significant difference from control. ‡$P<0.05$; ‡‡‡$P<0.001$ indicates a significant difference from ChTX.

ChTX on central and daughter branch arteries in the intact ear arose merely as a result of integrated network behaviour, as opposed to differences in the sensitivity of the individual vessels to the K_{Ca} channel blocker, pressure-flow curves were also constructed in isolated segments of G_0 and G_1 vessels. In G_1 vessels, the pressure-flow curves were obtained over a lower range of flow rates than those in the intact ear or in isolated G_0 vessels since, in the intact ear, the perfusate entering the G_0 vessel becomes divided amongst its branch vessels in which flow levels would therefore be proportionately lower. The results of these experiments revealed that the differential action of 1 nM ChTX, observed between G_0 and G_1 vessels in the intact ear, was paralleled in the isolated vessels. Thus, ChTX induced an upwards shift in the pressure-flow curve in isolated G_0 vessels, but had no effect on the pressure-flow curve in isolated G_1 vessels. Consequently, the differential action of ChTX on central and daughter branch arteries appears to be principally due to a differential sensitivity between these artery types, and not merely the result of network behaviour.

These findings may reflect differences in the distribution of K_{Ca} channels between central and daughter branch arteries. Indeed, other studies have revealed that there is a heterogenous distribution of K^+ channel subtypes in cardiac muscle in different regions of the heart in rat (Dixon & McKinnon, 1994) and in ferret (Brahmajothi *et al.*, 1996). Moreover, two recent studies have revealed a different distribution of K^+ channel subtypes between the vascular smooth muscle of large and small vessels in the rat pulmonary arterial tree. Albarwani *et al.* (1995) showed that in main pulmonary artery, one main

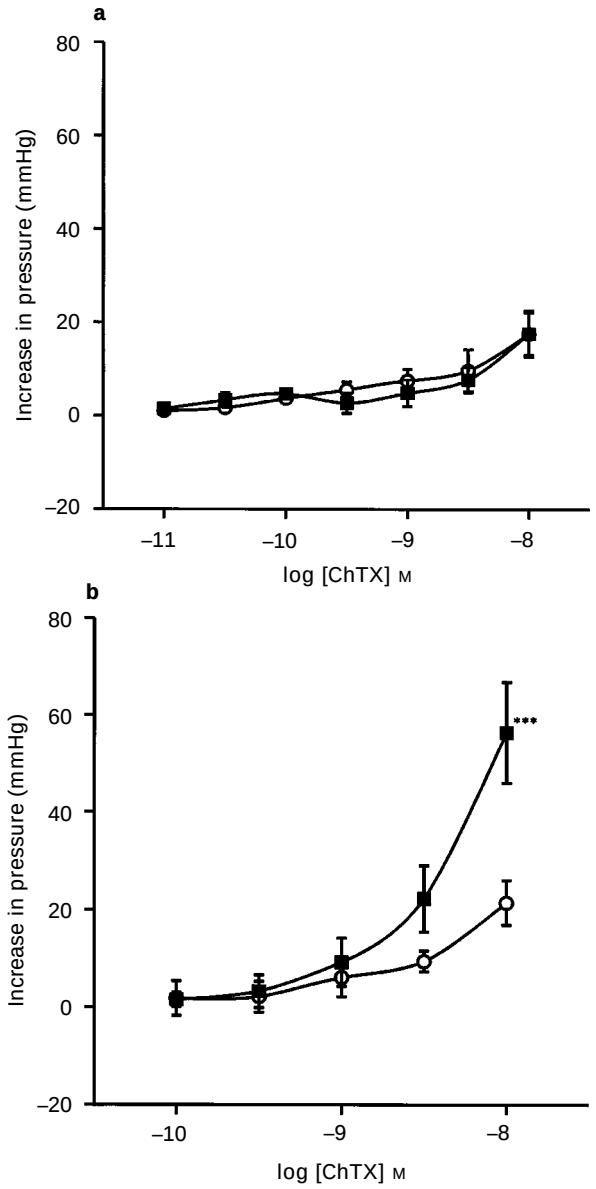

Figure 5 (a) Increases in perfusion pressure induced by addition of 10 pM – 10 nM ChTX in isolated segments of G_0 vessels preconstricted with 100 nM 5-HT at a fixed flow rate of 2 ml min^{-1}. In some preparations, experiments were conducted under control conditions (○; $n=6$) whilst in others, experiments were performed in the presence of 100 μM L-NAME (■; $n=6$). (b) Increases in perfusion pressure induced by addition of 100 pM – 10 nM ChTX in isolated segments of G_1 vessels preconstricted with 100 nM 5-HT at a fixed flow rate of 0.5 ml min^{-1}. In some preparations, experiments were conducted under control conditions (○; $n=6$) whilst in others, experiments were performed in the presence of 100 μM L-NAME (■; $n=7$). Data are shown as means with vertical lines indicating s.e.mean. ***$P<0.001$ indicates a significant difference from control.

type of K^+ channel predominates which is activated by both Ca^{2+} and ATP. This channel was also present in smaller branch arteries, but there was also another, equally abundant, K^+ channel found in these vessels which was insensitive to Ca^{2+} or ATP. Archer *et al.* (1996) showed that in larger conduit arteries, smooth muscle cells containing a TEA- and ChTX-sensitive K_{Ca} channel predominated, whilst in smaller resistance arteries, smooth muscle cells predominately containing a 4-aminopyridine-sensitive delayed rectifier K^+ channel (K_{DR} channel) or a combination of K_{Ca} and K_{DR} channels were mostly observed. It is therefore conceivable that in the rabbit ear K_{Ca} channels are more prevalent in either the endothelium or the smooth muscle in G_0 vessels than in smaller branch arteries, thus providing a possible explanation for the differential action of ChTX observed between these vessels.

The techniques employed in this study do not enable us to determine whether the action of ChTX on G_0 vessels is at the level of the vascular smooth muscle or the endothelium. As discussed in the Introduction, in addition to a possible direct constrictor action on the vascular smooth muscle, K_{Ca} channel blockade could potentially inhibit flow-induced release of nitric oxide from the endothelium (Cooke *et al.*, 1991; Hutcheson & Griffith, 1994) or inhibit the relaxant action of nitric oxide on the vascular smooth muscle (Robertson *et al.*, 1993; Bolotina *et al.*, 1994; George & Shibata, 1995; Koh *et al.*, 1995; Archer *et al.*, 1995). From the results obtained, it is also difficult to assess the flow-dependency of the actions of ChTX in the intact ear and in isolated G_0 vessels, although in both cases pressure-increases were more significant at the higher flow rates investigated, suggesting that at least a component of the ChTX response could be flow-dependent. Furthermore, the upwards shifts in the pressure-flow curves induced by ChTX were not seen in the presence of L-NAME. This might indicate that the action of ChTX in G_0 vessels is dependent on the presence of endothelium-derived nitric oxide, and it is therefore conceivable that ChTX causes these upwards shifts by inhibiting nitric oxide release or action. Indeed it has previously been shown in this laboratory by cascade bioassay in rabbit aorta that, at 1 nM, ChTX can substantially inhibit flow-induced release of nitric oxide (Hutcheson & Griffith, 1994). It is unlikely that the reason ChTX had no effect on the pressure-flow curves in the presence of L-NAME was because L-NAME had already resulted in maximal constriction, thereby masking any further increased in tone. When concentration-response curves were subsequently performed in G_0 vessels, ChTX was still able to induce constriction in the presence of L-NAME when it was added at concentrations above 1 nM.

The finding that when L-NAME was added subsequently to ChTX, it was able to induce further upwards shifts in the pressure-flow curves in both the intact ear and in G_0, nevertheless indicates that if ChTX was inhibiting either the action or the release of nitric oxide from the endothelium, it was doing so submaximally. This could be either because nitric oxide release or action is only partially dependent on K_{Ca} channel activity, or merely because the concentration of ChTX employed, 1 nM, was less than maximal in terms of its ability to block K_{Ca} channels. This is likely to be the case since previous studies have suggested that half-maximal block of K_{Ca} channels by ChTX occurs at a concentration of $\sim$10 nM (Miller *et al.*, 1985; Smirnov & Aaronson, 1992).

Concentration-response curves obtained at fixed flow rates in both G_0 and G_1 vessels revealed that at higher concentrations, ChTX was able to constrict both artery types and that these constrictor responses were not inhibited in the presence of L-NAME. Indeed in G_1, but not G_0 vessels, the response to 10 nM ChTX was enhanced by L-NAME, although the reason for this selective enhancement was unclear. These results would, nevertheless, suggest that the response to ChTX in the rabbit ear comprises more than one component, one of which is L-NAME-sensitive and only apparent in G_0 vessels, and one evident at higher concentrations which is L-NAME-insensitive and which is apparent in both G_0 and G_1 vessels. Since this second component is L-NAME-insensitive, it is therefore attributable to a direct smooth muscle action of ChTX. Indeed, it is possible that the L-NAME-sensitive and insensitive components occur at different sites and that the L-NAME-sensitive component in G_0 vessels occurs at the level of the endothelium. Thus, the difference in sensitivity of the two components might be because K_{Ca} channels are less abundant in the smooth muscle of G_0 and G_1 vessels than in the endothelium of G_0 vessels. It is also possible that the L-NAME-insensitive component results from the blockade of K^+ channel subtypes other than K_{Ca} channels. However, this is unlikely since ChTX is regarded as being a highly specific blocker of high- and

medium-conductance K_{Ca} channels (Cook & Quast, 1990) with no effect having been observed, for example, on voltage-dependent (Beech & Bolton, 1989), inward rectifier (Quayle *et al*., 1993) or ATP-sensitive K^+ channels (Nelson & Quayle, 1995). However, recently, Marchenko & Sage (1996) identified a medium-conductance K_{Ca} channel in the endothelium of rat aorta which was around a hundred fold less sensitive to ChTX than has previously been shown, and suggested this channel may represent a newly-identified class of K_{Ca} channels. The L-NAME-insensitive component of the action of ChTX seen at concentrations $\geqslant$3 nM could therefore result from the blockade of such a K_{Ca} channel, with different populations of K_{Ca} channel subtypes existing within the rabbit eat vasculature. Another potential explanation for the L-NAME-insensitivity of this component is that it could be an inhibition of the action of endothelium-derived hyperpolarising factor (EDHF), an endothelium-derived factor distinct from nitric oxide which can be released by a number of agonists and which can hyperpolarize vascular smooth muscle cells through a mechanism sensitive to K^+ channel blockade (Mombouli *et al*., 1996).

In summary, at a concentration of 1 nM, ChTX induces an upwards shift in the pressure-flow curve of the rabbit intact, isolated ear which is associated with constriction in G_0, but not G_1 or G_2 vessels. This differential action between G_0 and G_1 vessels occurs as a result of a differential sensitivity of these vessel types to ChTX, and not due to the effects of network behaviour of these vessels within the arterial bed, and may well reflect differences in the distribution of K_{Ca} channels between these vessel types. This action of ChTX is blocked in the presence of L-NAME, suggesting that it may be caused by inhibition of either the release or action of nitric oxide from the endothelium. At higher concentrations, an L-NAME-insensitive constrictor action of ChTX is apparent in both G_0 and G_1 vessels and this may be due to a direct action on the vascular smooth muscle.

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