Charybdotoxin and apamin block EDHF in rat mesenteric artery if selectively applied to the endothelium

JOANNE M. DOUGHTY,1 FRANCES PLANE,2 AND PHILIP D. LANGTON1
Departments of 1Physiology and 2Pharmacology, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, United Kingdom

Doughty, Joanne M., Frances Plane, and Philip D. Langton. Charybdotoxin and apamin block EDHF in rat mesenteric artery if selectively applied to the endothelium. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H1107–H1112, 1999.—In rat mesenteric artery, endothelium-derived hyperpolarizing factor (EDHF) is blocked by a combination of apamin and charybdotoxin (ChTX). The site of action of these toxins has not been established. We compared the effects of ChTX and apamin applied selectively to the endothelium and to the smooth muscle. In isometrically mounted arteries, ACh (0.01–10 µm), in the presence of indomethacin (2.8 µM) and N-nitro-L-arginine methyl ester (L-NAME) (100 µM), concentration dependently relaxed phenylephrine (PE)-stimulated tone (EC50 50 nM; n = 10). Apamin (50 nM) and ChTX (50 nM) abolished this relaxation (n = 5). In pressurized arteries, ACh (10 µM), applied intraluminally in the presence of indomethacin (2.8 µM) and L-NAME (100 µM), dilated both PE-stimulated (0.3–0.5 µM; n = 5) and myogenic tone (n = 3). Apamin (50 nM) and ChTX (50 nM) applied intraluminally abolished ACh-induced dilatations. Bath superperfusion of apamin and ChTX did not affect ACh-induced dilatations of either PE-stimulated (n = 5) or myogenic tone (n = 3). This is the first demonstration that ChTX and apamin act selectively on the endothelium to block EDHF-mediated relaxation.

METHODS

Male Wistar rats (wt 200–300 g) were killed by an intraperitoneal injection of pentobarbital sodium (500 mg/kg) or by stunning and cervical dislocation. Third-order superior mesenteric arteries were dissected in physiological saline solution (PSS) containing (in mM) 119 NaCl, 4.7 KCl, 25 NaHCO3, 1.18 K2HPO4, 1.8 or 2.5 CaCl2, 1.2 MgSO4, 11 glucose, and 0.027 EDTA. The pH was 7.4 when gassed with 95% O2-5% CO2.

Wire myography. Arteries (length 2 mm) were mounted in a Mulvany myograph at 37°C under normalized tension for measurement of isometric force, as previously described (23). The tissues were maintained in a static bath in PSS gassed with 95% O2-5% CO2 and containing 2.8 µM indomethacin and 100 µM N-nitro-L-arginine methyl ester (L-NAME). Concentration-effect curves for ACh were constructed by cumulative addition of ACh to arterial segments precontracted with PE (1–3 µM).

Pressure myography. Leak-free segments of artery (length at least 1 mm) were mounted between two glass cannulas in an arteriograph (Living Systems Instrumentation, Burlington, VT) at room temperature (18–21°C) and pressurized to 80 mmHg, under conditions of no luminal flow. A set constant pressure was maintained via a pressure servo-control system (PS200, Living Systems Instrumentation). Pressure transducers at both ends of the artery allowed continual monitoring of intraluminal pressure. Arteries were viewed through a Nikon TMS inverted microscope, and a measurement of the internal diameter was made from a video image using a video dimen...
ACh (0.01–10 µM) applied to isometrically mounted arteries relaxed PE-stimulated tone in a concentration-dependent manner, with an EC50 of 58 nM (n = 10). Neither ChTX (50 nM, n = 4) nor apamin (50 nM, n = 4) significantly affected the relaxation to ACh. However, a combination of apamin and ChTX completely and reversibly abolished the relaxation to ACh (n = 5) (Fig. 2). Pressure myography. On warming from room temperature to 37°C, some arteries developed myogenic tone (n = 3). ACh (10 µM), applied intraluminally, relaxed tone, dilating pressurized rat mesenteric arteries. In the event that myogenic tone failed to develop,
arteries were constricted with PE (0.3–0.5 µM, n = 5). PE was applied cumulatively until the level of tone was similar to the level of tone observed in myogenically active arteries. ACh (10 µM) applied intraluminally also relaxed the PE-induced constriction. Examples of dilatation of a pressurized artery in response to intraluminal application of ACh (10 µM) are shown in Figs. 3 and 4. A transient increase in pressure on both P1 and P2 pressure transducers indicates the period during which the intraluminal solution is exchanged. The ACh dilatation was maintained over a time course of several minutes, after which some desensitization of the response occurred. The dilatation was fully reversible on flushing. Periods of flushing can just be seen in the pressure measurements as a 5-mmHg drop in pressure (see METHODS). ACh-induced dilatations were reproducible, which confirms that flushing of the lumen did not damage the endothelium. Intraluminal application of control PSS did not elicit a dilatation (see Fig. 3, A and B), demonstrating that ACh-induced relaxations could not be explained either by flow or the transient increase in pressure observed during microinjection.

Intraluminal application of ChTX (50 nM) and apamin (50 nM) did not significantly alter vessel diameter but reversibly abolished ACh-induced (NOS and prostacyclin independent) dilatation in both myogenic and

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Fig. 2. ACh-induced relaxation of isometric rings of rat mesenteric artery. Concentration-dependent relaxation of phenylephrine (PE)-constricted (1–3 µM) arterial rings to ACh in presence of 100 µM Nω-nitro-L-arginine methyl ester (L-NAME) and 2.8 µM indomethacin (n = 10). Experiment was repeated with 50 nM apamin (n = 4), 50 nM charybdotoxin (ChTX) (n = 4), and 50 nM apamin plus 50 nM ChTX (n = 5). Plotted points are means ± SE; n = no. of experiments.

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Fig. 3. ACh-induced relaxation of pressurized rat mesenteric artery. A: diameter measurements of artery pressurized at 80 mmHg. P1 and P2, pressure at proximal and distal ends of artery, respectively, throughout experiment. Experiment developed after addition of 0.5 µM PE to superfusate (not shown). Intraluminal perfusion of control buffer did not mediate relaxation. Intraluminal perfusion of 10 µM ACh produced a dilatation. B: summary of paired data for intraluminal perfusion of control buffer (n = 7; 3 arteries). Arterial diameters: 1) initial, 246 ± 6.89 µm; 2) level of tone (pooled from PE-stimulated and myogenic vessels), 176 ± 10.7 µm; 3) intraluminal perfusion of control buffer, 174 ± 10.9 µm. Diameters 2 and 3 are not significantly different. In both B and C, ○ represents mean pressure under each condition. C: summary of paired data for intraluminal perfusion of 10 µM ACh (n = 8; 8 arteries). Arterial diameters: 1) initial arterial diameter, 254 ± 20.7 µm; 2) level of tone (pooled from PE-stimulated and myogenic vessels), 184 ± 17.3 µm; 3) intraluminal perfusion of 10 µM ACh, 253 ± 19.7 µm. *Significantly different from diameter 2.
In several recent reports, relaxation that persists after inhibition of NOS and cyclooxygenase has been ascribed to an unidentified EDHF. In resistance arteries EDHF-mediated hyperpolarization correlates strongly with NO- and prostanoid-independent relaxation, implying a causal relationship (25) (for recent review, see Ref. 6). Whereas no single K<sup>+</sup>-channel blocker has been shown to be completely effective, a cocktail of apamin and ChTX abolishes EDHF-mediated hyperpolarization and relaxation (4, 15, 16, 22, 24). In previous studies the toxins have been applied to isolated vessels by superfusion, such that the endothelium and the smooth muscle are exposed simultaneously and so the site of action is not known. Our data clearly show that apamin and ChTX block EDHF by an action at the endothelial surface and not an action in the smooth muscle.

In the present study we have used L-NAME to inhibit NOS. Kemp and Cocks (10) reported that in human coronary arteries, oxyhemoglobin (HbO<sub>2</sub>), which will scavenge NO, further depressed relaxations to BK in the presence of L-arginine analogs and indomethacin. This implies that NOS-independent NO may contribute to EDHF. It has also been shown in human omental arteries (15) that the relaxation to BK, which was resistant to a combination of indomethacin (10 µM) and either HbO<sub>2</sub> (10 µM) or L-NAME (300 µM), was similar, ruling out NO as a component of EDHF. In both studies the NO- and prostanoid-independent relaxation to BK was abolished by depolarizing concentrations of KCl, which is consistent with an EDHF.

It is clear from our methods that introducing ACh, toxins, or control PSS into the lumen of the arteries involves a pressure change and luminal flow within the artery. This has been shown previously to induce vasodilatation and might be mediated by opening of BK<sub>Ca</sub> channels (18). Figure 3 shows clearly that injection of control PSS resulted in a transient pressure increase that would be associated with flow, but this did not induce dilatation. Therefore, we conclude that neither pressure per se nor flow contributes to the dilatation induced by injection of ACh.
In our experiments, 1–3 µM PE-induced isometric force was abolished by 1–10 µM ACh. ChTX and apamin (each 50 nM) blocked the effect of ACh only when applied in combination. Isobaric myograph experiments were performed using 10 µM ACh (supramaximal in isometric recordings) to dilate both 0.3–0.5 µM PE-constricted and myogenic arteries to their passive diameter. It has been reported that agonist sensitivity is higher in isobaric myography compared with isometric myography (2, 5, 7). PE was used in isobaric experiments at concentrations that gave a level of tone similar to that observed in myogenic arteries, and wall forces were equivalent in the two situations. Falloon et al. (7) have shown that the concentration-effect relationship for vasorelaxation to ACh was not different in rat mesenteric arteries using isometric and isobaric myography. Therefore, it is justifiable to use a single supramaximal concentration of ACh (10 µM). ACh-induced vasorelaxation can be completely abolished by 50 nM ChTX and apamin applied intraluminally either to pressurized arteries or to the PSS superfusing isometric rings.

Several mechanisms have been proposed to explain the effect of apamin and ChTX on EDHF-mediated relaxations. It is unlikely that ChTX (in combination with apamin) blocks EDHF by an action on BKCa channels because neither iberiotoxin, which is selective for BKCa (12), nor tetraethylammonium ions are effective. However, it is well established that ChTX can block voltage-gated K+ (Kv) channels (20), and these channels may be the target of ChTX. Channels that bind and are blocked by apamin are K+ selective, voltage independent, and calcium sensitive, with a small unitary conductance and are referred to as SK channels. Cloned SK channels share little homology with other Kv channels (11), and there have been few reports of an apamin-sensitive, calcium-activated K+ current in either endothelial (19) or arterial muscle cells (9). Given that K+ channels activated by EDHF must pass enough current to substantially hyperpolarize the membrane and that SK current is difficult to find in patch-clamp studies, it seems unlikely that SK channel density is sufficient to account for relaxations to EDHF.

The requirement for apamin and ChTX to block relaxation could be explained if EDHF activates at least two channels, a Kv channel blocked by ChTX and an SK channel blocked by apamin. Alternatively, EDHF-mediated hyperpolarization may be dependent on activation of a channel that shares characteristics of BKCa and Kv (24). Interestingly, Zygmunt and co-workers (24, 25) reported that apamin can significantly enhance ChTX binding. Thus it might be possible for apamin to block EDHF via an allosteric effect that increases ChTX binding, rather than by acting independently to block an apamin-sensitive channel.

In previous reports, it has been assumed that the toxins act on the smooth muscle and do not affect EDHF synthesis or EDHF release from the endothelium. Our data from experiments on pressurized arteries clearly show that apamin and ChTX block EDHF-mediated relaxation by an action at the endothelial surface. It is unlikely for two reasons that toxins applied intraluminally were required to diffuse to the muscle cells to block EDHF-mediated dilatations. First, simultaneous application of the toxins with ACh (i.e., no preaddition of toxins) resulted in complete inhibition of EDHF (data not shown). Second, external application of toxins tended to further constrict pressurized myogenic and PE-stimulated vessels, consistent with the report of Brayden and Nelson (see Ref. 1), whereas intraluminal application did not alter vessel diameter.

Three mechanisms could explain an endothelial target for toxins: 1) EDHF is an as yet unidentified factor whose synthesis or release by the endothelium is blocked in the presence of apamin and ChTX; 2) EDHF is not a diffusible factor but an endothelium-derived hyperpolarizing current transmitted to smooth muscle through gap junctions after hyperpolarization of the endothelium (13); or 3) EDHF is potassium (6). Loss of K+ through endothelial K+ channels might raise the concentration of K+ within the media of the vessel to promote hyperpolarization of smooth muscle by two independent mechanisms. First, increased extracellular K+ concentration would increase current passed by inward rectifier K+ channels, which appear to be expressed preferentially in small arteries (17). Second, elevated extracellular K+ concentration would tend to increase the activity of Na+-K+-ATPase, which is consistent with evidence that EDHF is partially ouabain sensitive (8, 16).

In conclusion, this is the first report to establish that the combination of apamin and ChTX block EDHF-mediated relaxation by an action on the endothelium, contradicting the fundamental assumption that these toxins block K+ conductance(s) in smooth muscle.

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Address for reprint requests: P. Langton, Dept. of Physiology, School of Medical Sciences, Univ. of Bristol, University Walk, Bristol BS8 1TD, UK.

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