

## SPECIAL REPORT

# Inhibition of EDHF by two new combinations of K<sup>+</sup>-channel inhibitors in rat isolated mesenteric arteries

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It is widely established that in rat mesenteric arteries, endothelium-derived hyperpolarizing factor (EDHF)-mediated relaxation evoked by acetylcholine is abolished by a combination of charybdotoxin plus apamin. 4-Aminopyridine, an inhibitor of voltage-gated (K<sub>v</sub>) K<sup>+</sup>-channels, in combination with apamin had moderate effects on the EDHF-mediated relaxation. Maurotoxin (MTX), an inhibitor of K<sub>v</sub> and intermediate-conductance Ca<sup>2+</sup>-activated K<sup>+</sup>-channels (IK), had no effect on EDHF-mediated relaxation. However, MTX in combination with apamin completely abolished EDHF-mediated relaxation and endothelial cell hyperpolarization. The selective IK inhibitor 2-(2-chlorophenyl)-2,2-diphenyl acetonitrile (TRAM-39) had no significant effect on EDHF-mediated relaxation. EDHF-mediated vasorelaxation and hyperpolarization was abolished by a combination of TRAM-39 and apamin. These data demonstrate two new combinations of K<sup>+</sup>-channel inhibitors for the investigation of EDHF. Furthermore, by using TRAM-39, a potent selective inhibitor of IK channels, we provide the first *direct* evidence that abolition of EDHF requires the simultaneous presence of intermediate- and small-conductance Ca<sup>2+</sup>-activated K<sup>+</sup>-channel inhibitors.

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**Keywords:** EDHF; endothelium; Ca<sup>2+</sup>-activated K<sup>+</sup>-channels; charybdotoxin; maurotoxin; apamin; TRAM-39

**Abbreviations:** 4-AP, 4-aminopyridine; BK, large-conductance Ca<sup>2+</sup>-activated K<sup>+</sup>-channel; ChTX, charybdotoxin; CYP, cytochrome P<sub>450</sub>; 1-EBIO, 1-ethyl-2-benzimidazolone; EDHF, endothelium-derived hyperpolarizing factor; IK, intermediate-conductance Ca<sup>2+</sup>-activated K<sup>+</sup>-channel; K<sub>v</sub>, voltage-gated K<sup>+</sup>-channel; MTX, maurotoxin; SK, small-conductance Ca<sup>2+</sup>-activated K<sup>+</sup>-channel; TRAM-39, 2-(2-chlorophenyl)-2,2-diphenyl acetonitrile

**Introduction** In addition to nitric oxide (NO) and prostacyclin, endothelium-derived hyperpolarizing factor (EDHF) is an important mediator of vasodilatation in many vascular beds, including the rat mesenteric artery. EDHF-mediated relaxation of these arteries is inhibited by the simultaneous presence of apamin, an inhibitor of small-conductance Ca<sup>2+</sup>-activated K<sup>+</sup>-channels (SK), and charybdotoxin (ChTX), an inhibitor of large- (BK) and intermediate- (IK) conductance Ca<sup>2+</sup>-activated K<sup>+</sup>-channels, but not by either toxin alone (Waldron & Garland, 1994). Subsequent work demonstrated that the site of action of apamin and ChTX was on the endothelium, not the smooth muscle (Doughty *et al.*, 1999).

The inhibitory action of ChTX may suggest that EDHF mediates relaxation by activating BK channels. However, iberiotoxin, a selective inhibitor of BK, cannot substitute for ChTX (Waldron & Garland, 1994). Voltage-dependent K<sup>+</sup>-channels (K<sub>v</sub>) have been proposed to be involved in EDHF-mediated responses, as ChTX inhibits K<sub>v1.2</sub> and K<sub>v1.3</sub> (Garcia *et al.*, 1995). Although K<sub>v</sub> channels are expressed in endothelial cells from several arteries (Nilius & Droogmans, 2001), a role in EDHF-mediated relaxation remains unclear.

The present study uses the scorpion toxin, maurotoxin (MTX) (Castle *et al.*, 2003), a potent, synthetic, selective inhibitor of intermediate-conductance Ca<sup>2+</sup>-activated K<sup>+</sup>-channels, 2-(2-chlorophenyl)-2,2-diphenyl acetonitrile (TRAM-39) (Wulff *et al.*, 2000), and a nonselective inhibitor of K<sub>v</sub>, 4-aminopyridine (4-AP), to investigate their effects on

endothelium-dependent relaxation and hyperpolarization. Preliminary data have been presented to The Physiological Society (Hinton & Langton, 2001) and The Biophysical Society (Hinton *et al.*, 2002).

**Methods** Male Wistar rats (225–250 g) were killed by stunning and cervical dislocation. Third-order mesenteric arteries were dissected free in physiological saline solution (PSS) gassed with 95% O<sub>2</sub>/5% CO<sub>2</sub> containing (mM) NaCl 119, KCl 4.7, NaHCO<sub>3</sub> 25, KH<sub>2</sub>PO<sub>4</sub> 1.18, CaCl<sub>2</sub> 1.8, MgSO<sub>4</sub> 1.2, glucose 11 and EDTA 0.027. All experiments were performed in the presence of N<sup>G</sup>-nitro-L-arginine (100 μM) and indomethacin (2.8 μM), inhibitors of NO synthase and cyclooxygenase to suppress synthesis of NO and prostanoids, respectively.

**Wire myography** Segments of mesenteric artery were mounted in a Mulvany – Halpern wire myograph under normalized tension for isometric recording. Arterial segments were initially contracted with phenylephrine (PE, 10 μM) to give a maximal contraction. Subsequently, PE was applied to give approximately 70% of maximal force (0.5–2 μM). Data are expressed as normalized force as a percentage of the 70% maximal response (100%). Sequential, cumulative concentration effect curves for acetylcholine (ACh) were performed, firstly, in the absence of any inhibitors, then each inhibitor alone, and finally in the presence of the inhibitor combinations.

**Microelectrode experiments** Intact mesenteric vessels were opened longitudinally, pinned to the base of a bath, luminal

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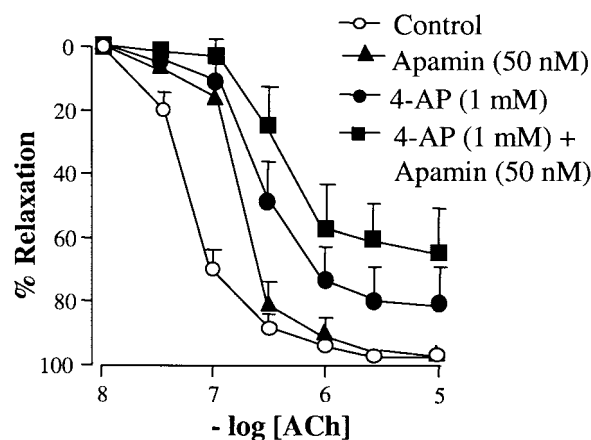
side upward and superfused ( $2.5 \text{ ml min}^{-1}$ ) with PSS. Individual endothelial cells were impaled using microelectrodes filled with 3 M KCl (resistance 35–80 M $\Omega$ ). Electrical responses at the tip of the electrode were monitored by injecting current pulses (0.1 nA, duration 40 ms). The electrode was advanced toward the endothelial cells at an angle of approximately  $40^\circ$ . Successful impalements were seen as a sudden change in voltage to a stable, negative level. If electrodes were advanced through the endothelial cell, the elastic membrane separating endothelial cells and smooth muscle cells, the internal elastic lamina, caused the electrode to bend and break. Owing to the long fine taper of the electrode tip and the shallow angle of approach advancing the electrode still further did not lead to impalement of smooth muscle cells. Furthermore, removal of the endothelium by gentle agitation did not give rise to impalement of either smooth muscle or endothelial cells. ACh, toxins and inhibitors were added to a gassed reservoir of PSS.

**Calculations and statistics** Results are mean values  $\pm$  s.e. mean of  $n$  animals or cells impaled. Statistical analysis was performed using Mann–Whitney test;  $P < 0.05$  was considered to be statistically significant.

**Chemicals and drugs** Synthetic ChTX and apamin were purchased from Calbiochem, 1-ethyl-2-benzimidazolinone (1-EBIO) was purchased from Aldrich and levcromakalin was supplied by SmithKline Beecham. All other drugs, except where stated, were from Sigma, U.K. ChTX was dissolved in 0.9% saline, and indomethacin was dissolved in ethanol. All other drugs were made up as stock solutions in distilled water and diluted in PSS. Stock solutions of 1-EBIO (0.1 M) and TRAM-39 (1 mM) were dissolved in dimethylsulfoxide (DMSO), final concentration 0.1%. Control experiments indicated that 0.1% DMSO had no direct effect on either relaxation or hyperpolarization at this concentration. All other drugs were made up as stock solutions in distilled water and diluted with PSS.

**Results** *Effects of inhibitors of Kv channels* In isometrically mounted arteries contracted with PE (0.5–2  $\mu\text{M}$ ), ACh (0.01–10  $\mu\text{M}$ ) elicited concentration-dependent relaxation ( $E_{\text{max}}$  97.5  $\pm$  2.2%;  $EC_{50}$  59  $\pm$  0.1 nM). Apamin (50 nM) alone had no significant effect on the maximal EDHF-mediated relaxation to increasing concentrations of ACh ( $E_{\text{max}}$  98.1  $\pm$  1.8%;  $EC_{50}$  180  $\pm$  0.2 nM;  $n = 6$ ). The nonselective Kv inhibitor 4-AP (1 mM) produced a significant rightward shift in the concentration–response curve and reduced the maximal relaxation to ACh ( $E_{\text{max}}$  78.9  $\pm$  12.1%;  $EC_{50}$  350  $\pm$  0.4 nM;  $n = 6$ ). 4-AP and apamin in combination produced a further rightward shift in the concentration–response curve and reduced the maximal relaxation ( $E_{\text{max}}$  64.8  $\pm$  7.5%;  $EC_{50}$  660  $\pm$  0.3 nM;  $n = 6$ ; Figure 1).

*Effects of inhibitors of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$ -channels* Charybdotoxin (50 nM), an inhibitor of BK, IK, Kv1.2 and Kv1.3 channels (see Garcia *et al.*, 1995), was without effect on vasorelaxation ( $E_{\text{max}}$  98.2  $\pm$  1.1%;  $EC_{50}$  228  $\pm$  0.2 nM;  $P > 0.05$ ;  $n = 6$ ). However, in combination with apamin (50 nM) ChTX abolished EDHF-mediated relaxation of rat mesenteric arteries ( $P < 0.05$ ;  $n = 6$ ; Figure 2a).



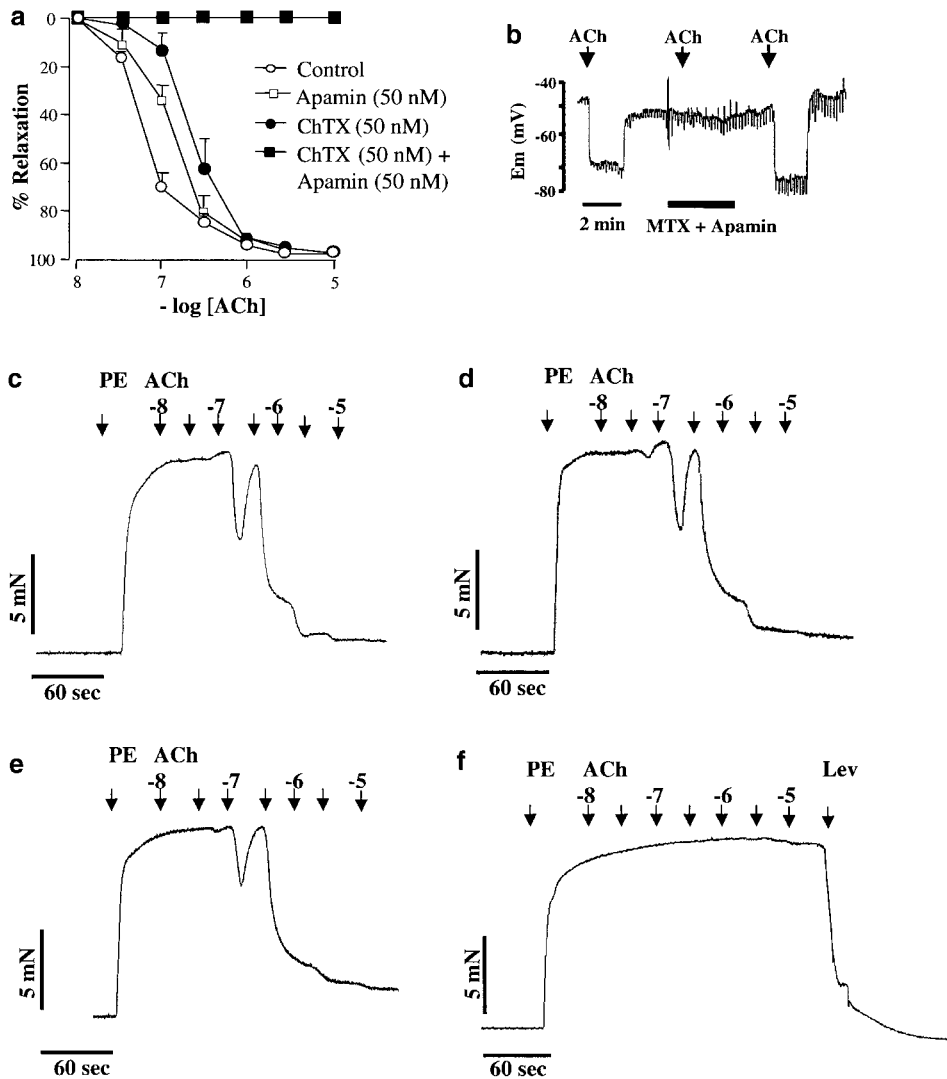
**Figure 1** Concentration–response curve for ACh-induced relaxation in rat isolated mesenteric arteries precontracted with phenylephrine (PE). ACh-evoked relaxation (0.01–10  $\mu\text{M}$ ) was not significantly affected by 4-AP (1 mM;  $n = 6$ ) or apamin (50 nM;  $n = 6$ ). The combination of 4-AP and apamin (1 and 50 nM, respectively;  $n = 6$ ) shifted the curve to the right and reduced the maximal relaxation. Data are means and vertical lines s.e. mean.

MTX (50 nM), a peptide toxin known to block cloned Kv1.2 and Kv1.3 channels and IK channels (Kharrat *et al.*, 1997; Castle *et al.*, 2003), was without effect on vasorelaxation to ACh ( $E_{\text{max}}$  99.4  $\pm$  2.1%;  $EC_{50}$  210  $\pm$  0.2 nM;  $P > 0.05$ ;  $n = 6$ ; Figure 2e). However, the combination of this toxin and apamin (each 50 nM) completely abolished EDHF-mediated relaxation of these arteries ( $P < 0.05$ ;  $n = 6$ ). Maximal relaxation in the presence of the toxin combination was achieved by application of 10  $\mu\text{M}$  levcromakalin (Figure 2f).

In the presence of  $N^G$ -nitro-L-arginine and indomethacin (100 and 2.8  $\mu\text{M}$ , respectively), the resting membrane potential of endothelial cells was  $-51.3 \pm 2.7$  mV ( $n = 6$ ). Application of ACh (10  $\mu\text{M}$ ) induced a rapid, reversible hyperpolarization of the endothelial cells,  $-23.5 \pm 2.8$  mV ( $n = 6$ ). In the presence of apamin and MTX in combination, hyperpolarization to application of ACh (10  $\mu\text{M}$ ) was completely and reversibly abolished ( $n = 3$ ; Figure 2b).

TRAM-39, a potent, selective inhibitor of  $\text{IK}_{\text{Ca}}$  channels ( $K_d$  60 nM; Wulff *et al.*, 2000), alone had no effect on relaxation to ACh ( $E_{\text{max}}$  98.8  $\pm$  1.1%;  $EC_{50}$  165  $\pm$  0.1 nM;  $P > 0.05$ ;  $n = 6$ ; Figure 3a). A combination of TRAM-39 (100 nM) and apamin (50 nM) abolished EDHF-mediated relaxation of the rat isolated mediated relaxation ( $P > 0.05$ ;  $n = 6$ ; Figure 3a); however, in the presence of the toxin combination, smooth muscle relaxation was elicited by levcromakalin (10  $\mu\text{M}$ ). The combination of TRAM-39 (100 nM) and apamin (50 nM) evoked a small, transient depolarization of endothelial cells  $\sim 8$  mV. Hyperpolarization to application of ACh (10  $\mu\text{M}$ ;  $n = 3$ ) was completely abolished in the presence of these inhibitors ( $n = 3$ ); hyperpolarization to ACh was restored after the  $\text{K}^+$ -channel inhibitors had washed off (Figure 3b).

*Effects of 1-EBIO: an activator of  $\text{K}^+$ -channels* 1-EBIO, an activator of IK and SK channels (Wickenden *et al.*, 2000), evoked concentration-dependent vasorelaxation (1–1000  $\mu\text{M}$ ;  $E_{\text{max}}$  98.4  $\pm$  1.3%;  $EC_{50}$  10.5  $\pm$  0.4  $\mu\text{M}$ ;  $n = 18$ ; Figure 4), in rat mesenteric arteries. However, this relaxation was unaffected by apamin in combination with either ChTX, MTX or TRAM-39

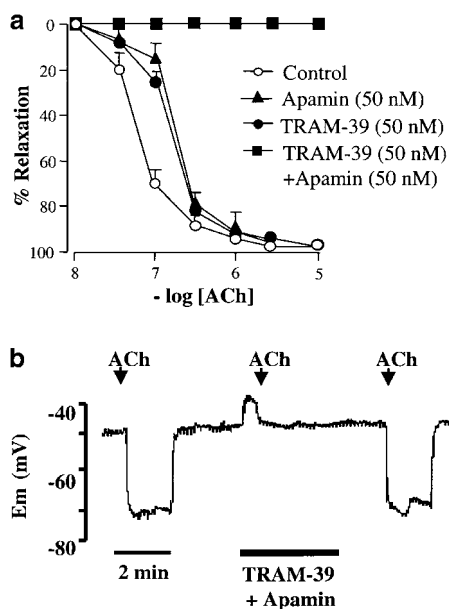


**Figure 2** (a) Effects of apamin and ChTX on EDHF relaxations induced by ACh in arteries precontracted with phenylephrine (PE). ChTX alone (50 nM;  $n = 6$ ) and apamin alone (50 nM;  $n = 6$ ) were without significant effect on EDHF relaxations. ChTX and apamin in combination (each 50 nM;  $n = 6$ ) abolished EDHF-mediated relaxation. Data are means  $\pm$  s.e.mean. (b) Continuous recording of endothelial cell membrane potential ( $E_m$ ). Apamin and MTX in combination completely, and reversibly, abolished the hyperpolarization to ACh ( $10 \mu\text{M}$ ). (c) Representative trace to demonstrate concentration-dependent relaxation to ACh ( $0.01$ – $10 \mu\text{M}$ ) in precontracted arteries. (d) and (e) Representative traces demonstrating concentration-dependent relaxation to ACh ( $0.01$ – $10 \mu\text{M}$ ) in the presence of apamin (50 nM) and MTX (50 nM), respectively. (f) Representative record to demonstrate abolition of relaxation in the presence of apamin and MTX. The ability of the smooth muscle to relax in the presence of the toxin combination was demonstrated by maximal relaxation to levcromakalin (lev;  $10 \mu\text{M}$ ). As evidenced on original traces, relaxations to lower concentrations of ACh were transient. For the construction of concentration–effect curves, the maximal relaxations to each applied dose of ACh were recorded.

( $P > 0.05$ ; each  $n = 6$ ; Figure 4). In preparations without endothelium, as evidenced by the lack of response to ACh, there was no change in response to those arteries with an intact endothelium ( $P > 0.05$ ; each  $n = 6$ ) (data not shown in Figure 4, as overlapping curves obscured the clarity of the figure).

**Discussion** It is widely established that inhibition of EDHF-mediated relaxation of rat isolated mesenteric arteries requires the simultaneous presence of apamin and ChTX. This study identifies two new combinations of  $\text{K}^+$ -channel inhibitors that abolish both the relaxation and endothelial cell hyperpolarization evoked by EDHF in this artery.

**Inhibitors of Kv channels** ChTX is a potent inhibitor of Kv1.2. and Kv1.3 channels (see Garcia *et al.*, 1995). Some studies have speculated that inhibition of Kv channels may underlie the action of ChTX. 4-AP, a non-specific Kv channel blocker, inhibits ACh-induced endothelium-dependent hyperpolarization of guinea pig coronary artery (Eckman *et al.*, 1998); however, this is not observed in all arteries. Agonist-induced hyperpolarization of endothelial cells is dependent upon the influx of extracellular  $\text{Ca}^{2+}$  and the release of  $\text{Ca}^{2+}$  from intracellular pools. It has been proposed that 4-AP may inhibit intracellular  $\text{Ca}^{2+}$  release evoked by ACh in endothelial cells (Ghisal & Morel, 2001). Interpretation of these data is further complicated by the ability of micromolar concentrations of 4-AP to block cloned SK3 channels (Grunnet *et al.*,

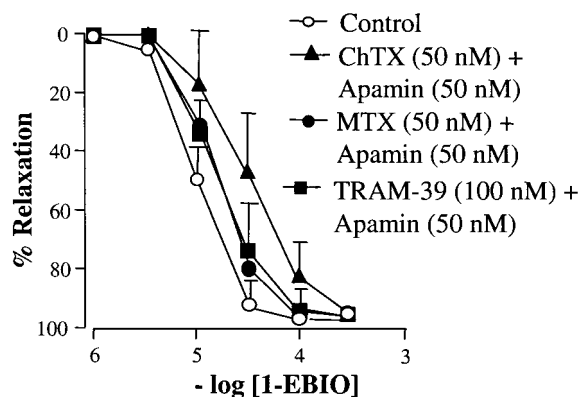


**Figure 3** (a) Effects of apamin and TRAM-39 on EDHF relaxations induced by ACh in arteries precontracted with phenylephrine (PE). TRAM-39 alone (50 nM;  $n=6$ ) and apamin alone (50 nM;  $n=6$ ) were without effect on EDHF relaxations evoked by increasing concentrations of ACh. In contrast, when TRAM-39 and apamin were combined (each 50 nM) these inhibitors abolished EDHF-mediated relaxation. Data are means and vertical lines s.e. mean. (b) Endothelial cell membrane potential ( $E_m$ ) hyperpolarization to ACh. Representative data trace illustrates hyperpolarization of  $E_m$  to application of ACh. A combination of apamin and TRAM-39 completely, and reversibly, abolished ACh hyperpolarization.

2001). Although Kv-channels are expressed in endothelial cells (see Nilius & Droogmans, 2001), it is unclear if the effects of Kv-inhibitors can be attributed to block of channels expressed in endothelial or smooth muscle cells.

**Inhibitors of  $Ca^{2+}$ -activated  $K^+$ -channels** The scorpion toxins MTX and ChTX share significant similarities in secondary structure and the profile of channels they block. Both toxins are potent inhibitors of IK and Kv-channels (Garcia *et al.*, 1995; Kharrat *et al.*, 1997; Castle *et al.*, 2003). Each of these toxins, MTX and ChTX, in combination with apamin abolish both EDHF-mediated relaxation and endothelial cell hyperpolarization. However, unlike ChTX, MTX has no effect on BK channels (Castle *et al.*, 2003), thus providing further evidence that the ability of ChTX to abolish EDHF-mediated effects in combination with apamin is not due to an effect on endothelial or smooth muscle cell BK channels.

The inability of the Kv-inhibitor, 4-AP, in combination with apamin to abolish EDHF-mediated relaxation led us to investigate the IK channel as the target for ChTX and MTX. Several studies investigating IK channels have used clotrimazole, which in addition to directly blocking IK channels also inhibits mammalian cytochrome  $P_{450}$  enzymes (CYP). However, metabolites of CYP have been suggested as candidates for the identity of EDHF (Fisslthaler *et al.*, 1999). To investigate the contribution of the IK channel, we used TRAM-39, which is structurally related to clotrimazole, but without an activity at CYP (Wulff *et al.*, 2000). The



**Figure 4** Concentration–response curves for 1-EBIO-induced relaxation in arteries precontracted with phenylephrine (PE). In endothelium-intact arteries, 1-EBIO evoked concentration-dependent relaxation that was unaffected by combinations of ChTX and apamin (each 50 nM), MTX and apamin (each 50 nM), and TRAM-39 and apamin (100 and 50 nM, respectively). Removal of the endothelium had no significant effect on the relaxation to 1-EBIO (data not shown, as overlapping curves obscured the clarity of the figure).

combination of TRAM-39 and apamin inhibited both EDHF-mediated relaxation and endothelial cell hyperpolarization, thus providing the strongest evidence to date that endothelial cell IK channels are directly involved in EDHF-mediated responses.

**1-EBIO: an activator of  $Ca^{2+}$ -activated  $K^+$ -channels** To mimic the action of EDHF in these arteries, we used 1-EBIO, an activator of both IK and SK channels. In precontracted arteries, EBIO-1 evoked concentration-dependent relaxation. Relaxation to 1-EBIO was unaffected by removal of the endothelium or raising extracellular  $K^+$  concentration to 25 mM, previously shown to abolish EDHF-mediated relaxation and hyperpolarization (Waldron & Garland, 1994) (data not shown). Therefore, the relaxations evoked by 1-EBIO are by a mechanism that is not dependent on endothelial cell  $K^+$ -channel activation. This apparent lack of specificity for  $K^+$ -channels questions the usefulness of 1-EBIO for studies using intact arteries. However, 1-EBIO has been used to activate  $K^+$ -currents in cultured (Edwards *et al.*, 1999) and acutely isolated endothelial cells (Walker *et al.*, 2001). In these studies, 1-EBIO induced a ChTX-sensitive, iberiotoxin-insensitive current. Most interestingly, the 1-EBIO-activated  $K^+$ -current was totally abolished by ChTX. Although 1-EBIO opens IK and SK channels with equal potency (Wickenden *et al.*, 2000), apamin in combination with ChTX was not required to abolish this current, despite the apamin-sensitivity of EDHF responses. These data suggest that apamin-sensitive SK channels, if expressed in endothelial cells, may not share the same characteristics as cloned, expressed SK channels. The characterization and identification of apamin-sensitive SK channels remains to be established in these arteries.

**Conclusions** These data demonstrate two novel combinations of  $K^+$  channel inhibitors that abolish EDHF-mediated relaxation in the rat isolated mesenteric artery. Furthermore, using a potent, selective inhibitor of IK channels we provide the first *direct* evidence that endothelial cell IK channels are crucial for EDHF-mediated responses in these blood vessels.

Scorpion toxins, such as ChTX, are poorly selective, inhibiting BK, IK and Kv channels in both the smooth muscle and endothelial cells. The apparent selectivity of nonpeptide inhibitors, such as TRAM-39, provides a useful pharmacological tool for further defining the physiological role of IK channels and their role in EDHF-mediated responses.

Although the distribution of SK and IK channels in the vascular wall and their precise role in EDHF remain to be established, these data provide the strongest evidence to date that channels displaying the pharmacological characteristics of

SK and IK are involved in EDHF responses of the rat mesenteric artery.

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