British Journal of Pharmacology (1997) 121, 141–149

Involvement of voltage-dependent potassium channels in the EDHF-mediated relaxation of rat hepatic artery

Peter M. Zygmunt, Gillian Edwards, Arthur H. Weston, *Bengt Larsson & *,[1]Edward D. Högestätt

School of Biological Sciences, G38 Stopford Building, University of Manchester, Manchester M13 9PT, U.K. and *Department of Clinical Pharmacology, Institute of Laboratory Medicine, Lund University Hospital, S-221 85 Lund, Sweden

1 In the rat hepatic artery, the acetylcholine-induced relaxation mediated by endothelium-derived hyperpolarizing factor (EDHF) is abolished by a combination of apamin and charybdotoxin, inhibitors of small (SK_{Ca}) and large (BK_{Ca}) conductance calcium-sensitive potassium (K)-channels, respectively, but not by each toxin alone. The selective BK_{Ca} inhibitor iberiotoxin cannot replace charybdotoxin in this combination. Since delayed rectifier K-channels (K_V) represent another target for charybdotoxin, we explored the possible involvement of K_V in EDHF-mediated relaxation in this artery.

2 The K_V inhibitors, agitoxin-2 (0.3 μM), kaliotoxin (0.3 μM), β-dendrotoxin (0.3 μM), dofetilide (1 μM) and terikalant (10 μM), each in combination with apamin (0.3 μM) had no effect on the EDHF-mediated relaxation induced by acetylcholine in the presence of N^{ω}-nitro-L-arginine (0.3 mM) and indomethacin (10 μM), inhibitors of nitric oxide (NO) synthase and cyclo-oxygenase, respectively ($n=2-3$). Although the K_V inhibitor margatoxin (0.3 μM) was also without effect ($n=5$), the combination of margatoxin and apamin produced a small inhibition of the response (pEC_{50} and E_{max} values were 7.5 ± 0.0 and $95\pm1\%$ in the absence and 7.0 ± 0.1 and $81\pm6\%$ in the presence of margatoxin plus apamin, respectively; $n=6$; $P<0.05$).

3 Ciclazindol (10 μM) partially inhibited the EDHF-mediated relaxation by shifting the acetylcholine-concentration-response curve 12 fold to the right ($n=6$; $P<0.05$) and abolished the response when combined with apamin (0.3 μM; $n=6$). This combination did not inhibit acetylcholine-induced relaxations mediated by endothelium-derived NO ($n=5$).

4 A 4-aminopyridine-sensitive delayed rectifier current ($I_{K(V)}$) was identified in freshly-isolated single smooth muscle cells from rat hepatic artery. None of the cells displayed a rapidly-activating and -inactivating A-type current. Neither charybdotoxin (0.3 μM; $n=3$) nor ciclazindol (10 μM; $n=5$), alone or in combination with apamin (0.3 μM; $n=4-5$), had an effect on $I_{K(V)}$. A tenfold higher concentration of ciclazindol (0.1 mM, $n=4$) markedly inhibited $I_{K(V)}$, but this effect was not increased in the additional presence of apamin (0.3 μM; $n=2$).

5 By use of membranes prepared from rat brain cortex, [^{125}I]-charybdotoxin binding was consistent with an interaction at a single site with a K_D of approximately 25 pM. [^{125}I]-charybdotoxin binding was unaffected by iberiotoxin (0.1 μM, $n=6$), but was increased by apamin in a concentration-dependent manner (E_{max} $43\pm10\%$, $P<0.05$ and pEC_{50} 7.1 ± 0.2; $n=7-8$). Agitoxin-2 (10 nM) displaced [^{125}I]-charybdotoxin binding by $91\pm3\%$ ($n=6$) and prevented the effect of apamin (1 μM; $n=6$).

6 It is concluded that the EDHF-mediated relaxation in the rat hepatic artery is not mediated by the opening of either K_V or BK_{Ca}. Instead, the target K-channels for EDHF seem to be structurally related to both K_V and BK_{Ca}. The possibility that a subtype of SK_{Ca} may be the target for EDHF is discussed.

Keywords: Potassium channels; apamin; charybdotoxin; ciclazindol; terikalant; hyperpolarization; dofetilide; whole-cell patch clamp; radioligand-binding; relaxation; vascular endothelium

Introduction

Evidence for the existence of an endothelium-derived hyperpolarizing factor (EDHF), which is distinct from endothelium-derived relaxing factor (EDRF), was obtained by Chen *et al*. (1988) and by Félétou and Vanhoutte (1988). At that time, it was believed that the critical features which distinguished the two factors were the membrane hyperpolarization and increased K^+ efflux associated with the action of EDHF (see Taylor & Weston, 1988). However, although it now seems certain that the EDRF, identified as nitric oxide (NO), can also generate an increase in membrane potential in certain tissues, the continuing development of endothelium-dependent hyperpolarizations when EDRF production has been blocked by NO synthase inhibitors confirms the existence of EDHF as an entity distinct from EDRF (Zygmunt *et al*., 1994a,b; Petersson *et al*., 1995; for review see Garland *et al*., 1995).

In spite of much effort, there is no consensus view on the identity of the K-channel opened by EDHF (Garland *et al*., 1995; Zygmunt & Högestätt, 1996). In some tissues, such as the rabbit abdominal aorta and carotid artery, EDHF-mediated relaxations are inhibited by charybdotoxin (Cowan *et al*., 1993; Lischke *et al*., 1995), whereas in the rat and rabbit mesenteric, guinea-pig coronary and bovine oviductal arteries, the effects of EDHF have been shown to be apamin-sensitive (Adeagbo & Triggle, 1993; Hecker *et al*., 1994; García-Pascual *et al*., 1995; Murphy & Brayden, 1995; Parsons *et al*., 1996). From these studies, it was concluded that either large (BK_{Ca}) or small (SK_{Ca}) conductance, calcium-sensitive K-channels are opened by EDHF.

In other tissues, the action of EDHF can only be abolished by mixtures of charybdotoxin plus apamin. This finding, first briefly described by Waldron and Garland (1994) in the rat mesenteric artery, has now been comprehensively described in the rat hepatic artery (Zygmunt, 1995; Zygmunt & Högestätt, 1996) and recently confirmed by Corriu *et al*. (1996) in guinea-pig carotid arteries. In the study by Zygmunt and Högestätt (1996), iberiotoxin, the selective inhibitor of BK_{Ca} (Giangiacomo *et al*., 1992) was an ineffective substitute for char-

[1] Author for correspondence.

ybdotoxin, making it highly unlikely that BK_{Ca} is indeed the target K-channel for EDHF. Since charybdotoxin also inhibits delayed rectifier channels (K_V, see Kaczorowski *et al*., 1996), the data of Zygmunt and Högestätt (1996) would be consistent with the involvement of K_V in the action of EDHF.

The objective of the present investigation was thus to extend the findings of Zygmunt and Högestätt (1996) in the rat hepatic artery and to examine the role of K_V in EDHF-mediated responses. By use of the whole-cell configuration of the patch-clamp technique combined with radioligand-binding and tension studies, it was hoped to obtain more information about the K-channels which form the target for EDHF in this tissue. Some of these results have been presented to the British Pharmacological Society (Zygmunt *et al*., 1996).

Methods

Tissue bath experiments

Female Sprague-Dawley rats (250–300 g) were killed by CO_2 asphyxia followed by exsanguination. The hepatic artery was removed and cut into ring segments, 1–2 mm long, and suspended between two metal pins in organ baths, containing physiological salt solution (PSS) of the following composition (mM): NaCl 119, $NaHCO_3$ 15, KCl 4.6, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.5 and (+)-glucose 6.0. The solution was continuously bubbled with a mixture of 95% O_2 and 5% CO_2 at a temperature of 37°C, resulting in a pH of 7.4. During an equilibration period of about 1 h, the vessels were repeatedly stretched until a stable resting tension of approximately 2 mN mm^{-1} vessel length was obtained. Isometric tension was measured as previously described (Högestätt *et al*., 1983).

Relaxations were studied in preparations contracted by phenylephrine. The concentration of phenylephrine was titrated for each vascular segment to give a contraction amounting to 50–70% of a response to 60 mM K^+. When stable contractions were obtained, acetylcholine was added cumulatively to determine the concentration-response relationship. The pre-incubation time with K-channel inhibitors, N^{ω}-nitro-L-arginine (L-NOARG) and indomethacin was 30 min. Control experiments with vehicle were performed in the same manner. Unless otherwise stated, L-NOARG was present in the PSS.

Single-cell electrophysiology

Production of isolated cells Hepatic arteries were removed from male Sprague-Dawley rats (200–300 g) into a 'Ca^{2+}-free' PSS and carefully cleaned of fat and connective tissue with fine scissors in conjunction with a dissecting microscope. The artery was opened along its longitudinal axis and cut into four segments. Cells were dispersed with a collagenase/pronase enzyme solution originally described by Klöckner & Isenberg (1985). Segments of hepatic arteries were agitated in enzyme solution at 37°C for 30 min. They were then washed in the same solution free of enzyme and subsequently triturated with a wide bore, smooth-tipped pipette before a further 5 min agitation at 37°C in (pre-warmed) enzyme solution. The partially-digested tissue was then washed and triturated in Kraftbrühe (KB-medium; Klöckner & Isenberg, 1985). Cells were stored at 8°C in KB-medium and used within 9 h of separation.

Whole-cell current recordings The whole-cell configuration of the patch-clamp technique (Hamill *et al*., 1981) was employed with an Axopatch-1C amplifier (Axon Instruments). The settling time of this system was less than 500 μs. Patch pipettes were pulled from Pyrex glass (H15/10, Jencons, UK) and had resistances of 3–4 MΩ when filled with the internal (intracellular) solution. Voltage commands and data acquisition were performed on-line with an AT-compatible computer equipped with an appropriate interface, the sampling frequency of which was 15 kHz (Axon TL-1, Axon Instruments, U.S.A.). For cell stimulation and for recording and analysing data, the pCLAMP 5.5 programme was used (Axon Instruments). Data were stored on a digital audio tape recorder (Sony; cut-off frequency 20 kHz) and the evoked membrane currents were monitored on a Gould Windograf recorder (−3 dB cut-off frequency 460 Hz).

Amphotericin B was used to produce 'perforated' patches (Horn & Marty, 1988; Rae *et al*., 1991). A fresh amphotericin B stock solution (30 mg ml^{-1}) was made each day by dissolving the drug in dimethylsulphoxide (DMSO). Immediately before experimentation, an amphotericin B-containing pipette solution (300 μg ml^{-1}) was prepared and used for up to 90 min. Pipette tips were briefly dipped into pipette solution and then back-filled with the amphotericin B-containing solution. After formation of giga-seals, 15–25 min were allowed for amphotericin B to diffuse into the cell membrane.

The effects of the compounds were investigated by adding the appropriate amount of each agent to the main reservoir containing the external solution to ensure that responses were obtained under steady-state conditions. The bath (1 ml) was continuously perfused (1 ml min^{-1}) with fresh external solution by a pump (Microperpex, Pharmacia LKB, Freiburg, Germany); a second identical pump was used to remove excess solution from the recording chamber. All experiments were performed at room temperature (22°C–24°C).

Solutions The enzyme solution for separation of hepatic arterial cells comprised (mM): KOH 130, $CaCl_2$ 0.05, taurine 20, pyruvate 5, creatine 5, HEPES 10, buffered with methanesulphonic acid to pH 7.4, collagenase (Type VIII) 1.0 mg ml^{-1}, pronase (Calbiochem) 0.2 mg ml^{-1} and fatty acid free albumin 1 mg ml^{-1}. KB-medium comprised (mM): KCl 85, KH_2PO_4 30, $MgSO_4$ 5, Na_2ATP 5, K-pyruvate 5, creatine 5, taurine 20, β-OH-butyrate 5, fatty acid free albumin 1 mg ml^{-1}, pH adjusted to 7.20 at 6°C with KOH. The composition of the 'Ca^{2+}-free' bath (external) solution was (mM): NaCl 125, KCl 4.8, $MgCl_2$ 3.7, KH_2PO_4 1.2, (+)-glucose 6.0, HEPES 10. The bath solutions were buffered with NaOH to pH 7.30 and aerated with O_2. The 'Ca^{2+}-free' pipette (internal) solution comprised (mM): NaCl 5, KCl 120, $MgCl_2$ 1.2, K_2HPO_4 1.2, (+)-glucose 6.0, HEPES 10, oxalacetic acid 5, sodium pyruvate 2, sodium succinate 5, buffered with KOH to pH 7.30 at 24°C. Unless otherwise stated, all reagents and compounds were obtained from Sigma.

Radioligand-binding studies

Membrane preparation Rat cortical brain tissue from female Sprague-Dawley rats (250–300 g) was homogenized with a Polytron PT 10/35 homogenizer (Kinematica) for 3×10 s (setting 6) in ice-cold 50 mM Tris-HCl buffer (pH 7.5 at room temperature). The homogenate was centrifuged at 48 000 $\times g$ for 10 min at 4°C. The pellet obtained was suspended in fresh buffer and recentrifuged. This pellet was resuspended in fresh buffer containing 0.1% BSA and used in the binding experiments.

Binding assay Incubation mixtures consisted of 160 μl membranes (containing approximately 6 μg protein), 20 μl [^{125}I]-charybdotoxin (dissolved in 50 mM Tris-HCl buffer, containing 0.1% BSA), and 20 μl redistilled water with or without drugs. Non-specific binding was defined as the amount of binding remaining after inhibition with unlabelled charybdotoxin (0.1 μM), while specific binding was calculated as the difference between total and non-specific binding. The incubation was performed at room temperature for 10 min and was terminated by diluting the samples with 1 ml of ice-cold Tris-HCl buffer, followed by rapid filtration under reduced pressure through Whatman GF/C glass fibre filters (presoaked in 0.3% polyethyleneimine). The filters were subsequently washed twice with 5 ml portions of ice-cold Tris-HCl

buffer after which the retained radioactivity was measured with a gamma counter (LKB, Wallac, Sweden). In displacement studies, [^{125}I]-charybdotoxin (approximately 25 pM) and unlabelled competitors at various concentrations were incubated as described above.

Calculations and statistics

The negative logarithm of the drug concentration eliciting 50% of the maximal relaxation (pEC_{50}) was determined by linear regression analysis by use of the data points immediately above and below the half-maximal response. E_{max} refers to the maximal relaxation achieved (100% denotes a complete reversal of the phenylephrine-induced contraction). Values are presented as mean ± s.e.mean, and n indicates the number of vascular segments, cells or brains (from different animals) examined. Statistical analysis was performed by Student's t test (two-tailed) or multiple analysis of variance (MANOVA). Statistical significance was accepted when $P<0.05$.

Drugs

The following drugs were used: acetylcholine chloride, A23187, L-phenylephrine hydrochloride, N^{ω}-nitro-L-arginine (all from Sigma); indomethacin (Confortid, Dumex); agitoxin-2, apamin, β-dendrotoxin, kaliotoxin (Alomone); margatoxin (Alomone and Bachem); synthetic charybdotoxin (Latoxan); ciclazindol, dofetilide (Pfizer); terikalant (Rhône-Poulenc Rorer). A23187, ciclazindol, dofetilide, terikalant were each dissolved in absolute ethanol, Apamin, charybdotoxin, β-dendrotoxin, kaliotoxin were each dissolved in saline. Agitoxin-2 and margatoxin were dissolved in saline containing 0.1% BSA. All other drugs were dissolved in distilled water. Stock solutions of the substances were stored at −70°C.

Results

Tissue bath experiments

These experiments were conducted with the knowledge that the relaxant effects of EDHF in the rat hepatic artery are abolished by a mixture of apamin plus charybdotoxin, but not by apamin plus iberiotoxin, or these inhibitors individually (Zygmunt, 1995; Zygmunt & Högestätt, 1996). The initial objective of the present investigation was thus to determine which K-channel inhibitors could substitute for charybdotoxin. Unless otherwise stated, experiments were conducted in the presence of 0.3 mM N^{ω}-nitro-L-arginine (L-NOARG) and 10 μM indomethacin, under which conditions the relaxations mediated by acetylcholine in the rat hepatic artery are produced by EDHF (Zygmunt *et al*., 1994a, b).

Effects of apamin, charybdotoxin and ciclazindol Neither 1 μM apamin (Figure 1) nor 1 μM charybdotoxin, concentrations which were three times greater than those used in the previous study of Zygmunt and Högestätt (1996), had any effect on EDHF-mediated relaxations induced by acetylcholine. The pEC_{50} and E_{max} values for acetylcholine were 7.6 ± 0.1 and 96 ± 1% in the absence and 7.6 ± 0.1 and 95 ± 2% in the presence of apamin, respectively ($n=6$). The pEC_{50} and E_{max} values for acetylcholine were 7.5 ± 0.0 and 92 ± 5% in the absence and 7.6 ± 0.3 and 94 ± 3% in the presence of charybdotoxin, respectively ($n=4$). Ciclazindol (10 μM) alone produced some inhibition of the EDHF-mediated relaxation by shifting the concentration-response curve to the right (Figure 1). Thus, the pEC_{50} value for acetylcholine was significantly lower in the presence (6.6 ± 0.1) than in the absence (7.7 ± 0.2) of ciclazindol ($n=6$), whereas the E_{max} values were similar (control, 96 ± 1%; ciclazindol, 97 ± 1%; $n=6$). However, the combination of ciclazindol (10 μM) plus apamin (0.3 μM) completely abolished the EDHF-mediated relaxations (Figures 1 and 2). The combination of ciclazindol (10 μM) plus apamin (0.3 μM) did not inhibit acetylcholine-induced responses in the absence of L-NOARG, i.e., when relaxations were caused by NO (Figures 1 and 2).

Effects of margatoxin, agitoxin-2, β-dendrotoxin and kaliotoxin Margatoxin (0.3 μM) alone had no effect on EDHF-mediated relaxations induced by acetylcholine (Figure 3).

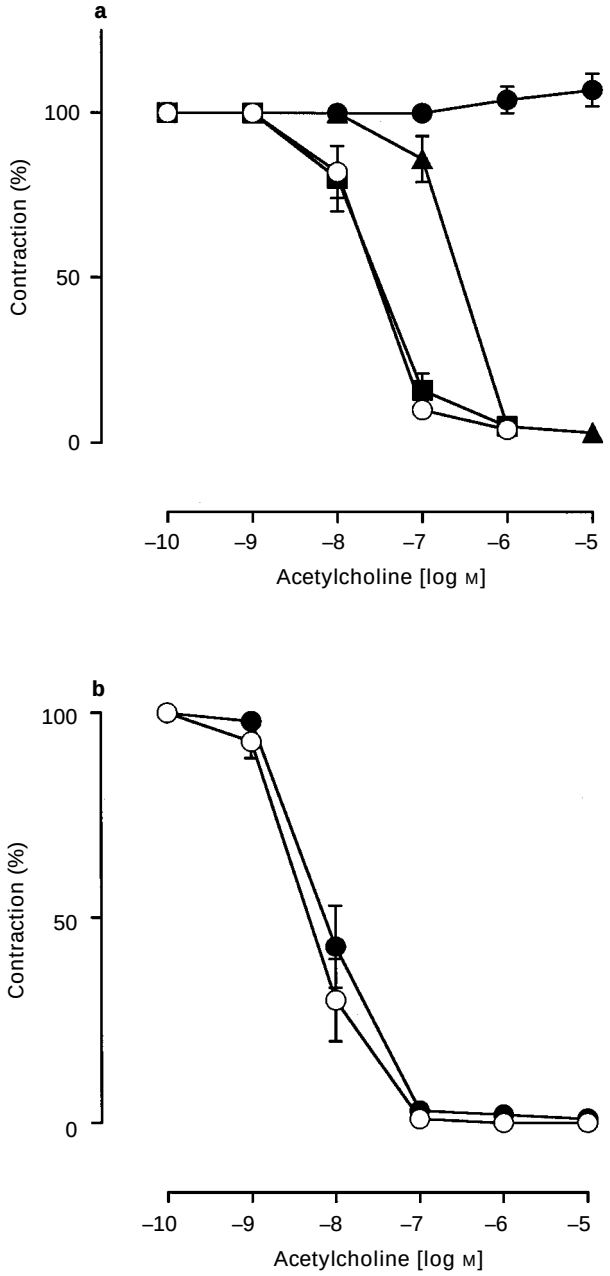


Figure 1 Effect of ciclazindol and apamin on the relaxation induced by acetylcholine in the presence (a) and absence (b) of L-NOARG (0.3 mM) in hepatic arteries contracted by phenylephrine. (a) Control (○; $n=12$), 1 μM apamin (■, $n=6$), 10 μM ciclazindol (▲, $n=6$) and 10 μM ciclazindol plus 0.3 μM apamin (●, $n=6$). (b) Control (○; $n=5$) and 10 μM ciclazindol plus 0.3 μM apamin (●, $n=5$). Indomethacin (10 μM) was present in all experiments. Responses are expressed as a percentage of the contraction before addition of acetylcholine. Data are presented as means and vertical lines show s.e.mean.

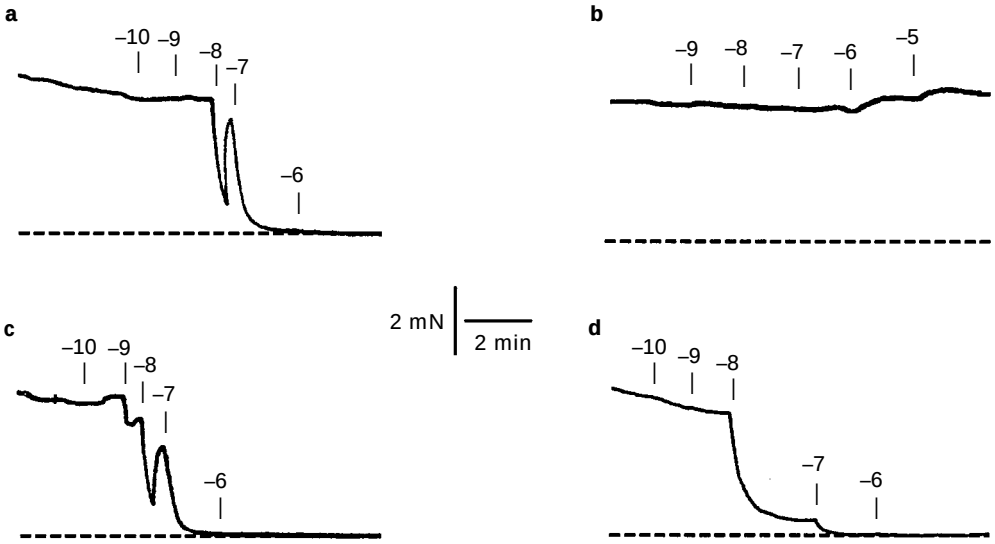


Figure 2 Traces showing endothelium-dependent relaxations elicited by log molar concentrations of acetylcholine in hepatic arteries contracted by phenylephrine in the presence (a,b) and absence (c,d) of L-NOARG (0.3 mM). Ciclazindol (10 μM) plus apamin (0.3 μM) inhibited relaxations in the presence (b) but not in the absence (d) of L-NOARG. Indomethacin (10 μM) was present throughout. Recordings (a,b and c,d) are from two different animals, respectively, and each trace was obtained from different arterial segments. Dashed line indicates the basal tension level before contraction with phenylephrine.

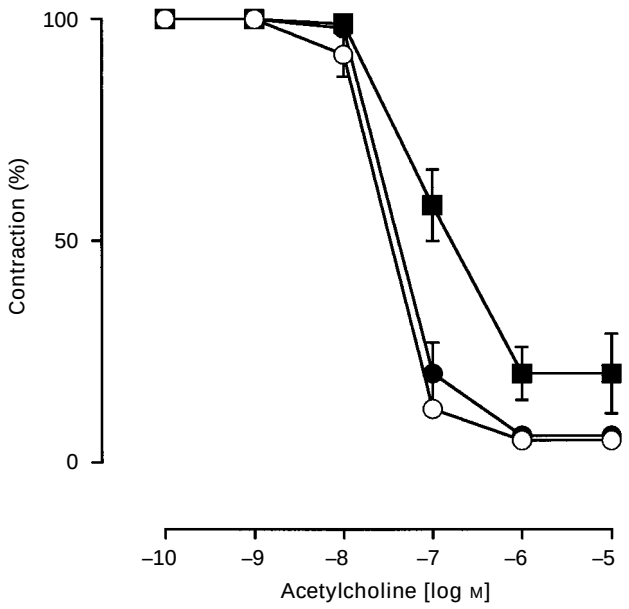


Figure 3 Relaxation induced by acetylcholine under control conditions (○) or in the presence of 0.3 μM margatoxin (●) or 0.3 μM margatoxin plus 0.3 μM apamin (■) in hepatic arteries contracted by phenylephrine. The experiments were performed in the presence of L-NOARG (0.3 mM) plus indomethacin (10 μM). Responses are expressed as a percentage of the contraction before addition of acetylcholine. Data are presented as means and vertical lines show s.e.mean of five to six experiments.

However, in six experiments, the combination of this toxin (0.3 μM) with apamin (0.3 μM) significantly inhibited the effects of EDHF with pEC_{50} and E_{max} values of 7.5 ± 0.0 and $95 \pm 1\%$ in the absence and of 7.0 ± 0.1 and $81 \pm 6\%$ in the presence of margatoxin plus apamin, respectively (Figure 3). The combination of apamin (0.3 μM) with either agitoxin-2 (0.3 μM), β-dendrotoxin (0.3 μM) or kaliotoxin (0.3 μM) had no effect on EDHF-mediated relaxations ($n = 2-3$, data not shown).

Effects of dofetilide and terikalant The K-channel inhibitors dofetilide (1 μM) and terikalant (10 μM) each combined with apamin (0.3 μM) were without effect on EDHF-mediated relaxations ($n = 3$, data not shown).

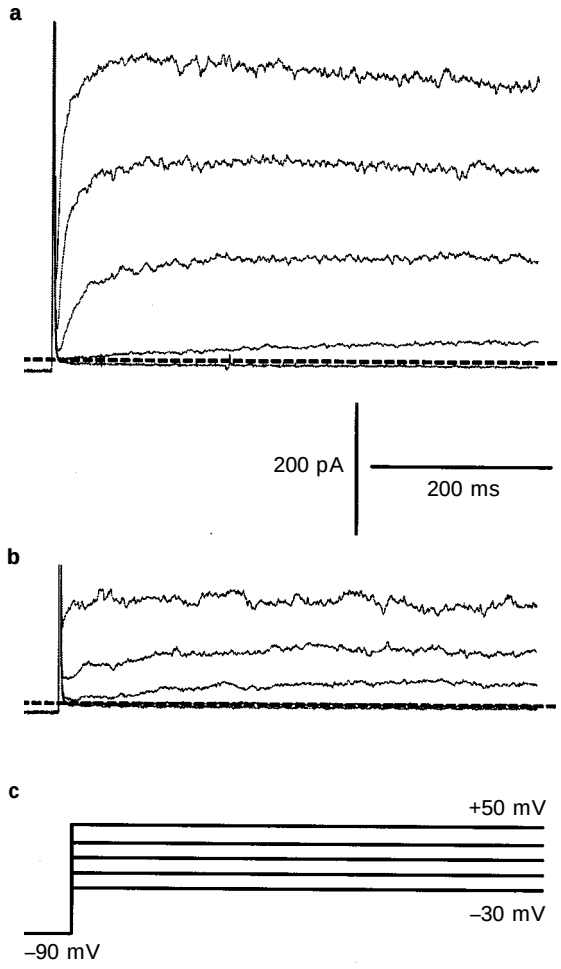
Single-cell electrophysiology

In rat hepatic arterial cells, stepping from a holding potential of -90 mV to test potentials between -80 mV and $+50$ mV in 10 mV increments generated a slowly activating and inactivating outward current (designated I_{total}) at test potentials more positive than -30 mV (Figure 4). In no cell was there evidence of a rapidly-activating and -inactivating component carried by A-like K-channels (K_A). When cells were held at -10 mV, stepping to the same series of test potentials generated a non-inactivating current $I_{(NI)}$ of much smaller amplitude than I_{total}. This finding suggests that I_{total} consists mainly of a delayed rectifier current ($I_{K(V)}$), a view confirmed by the marked and reversible inhibition of I_{total} by 4-aminopyridine (3 mM; $P < 0.05$; Figure 4).

Effects of apamin, charybdotoxin and ciclazindol Neither charybdotoxin (0.3 μM) nor ciclazindol (10 μM) in combination with apamin (0.3 μM), the concentrations which abolished EDHF-mediated relaxations in the hepatic artery, inhibited I_{total} (Figure 5). Similar results were obtained when currents were measured at the end of each 500 ms test pulse ($n = 4-5$, data not shown). Apamin (0.3 μM, $n = 3$), charybdotoxin (0.3 μM, $n = 3$) and ciclazindol (10 μM, $n = 5$) alone were also without effect on I_{total} (Figure 6). However, a ten times higher concentration (0.1 mM) of ciclazindol caused a substantial inhibition of this current, an effect which was reversed by washout of the drug. I_{total} at a test potential of $+50$ mV was significantly reduced by $45 \pm 5\%$ and by $53 \pm 4\%$ at the peak and at the end of the 500 ms test pulse, respectively ($n = 4$). No further inhibition was obtained when 0.3 μM apamin was given together with 0.1 mM ciclazindol ($n = 2$, not shown).

Radioligand-binding studies

These experiments were conducted with an homogenate of rat brain, a tissue in which [^{125}I]-charybdotoxin binds to delayed rectifier channels of the Kv1.3 type (Vasquez *et al*., 1990). Preliminary saturation studies with increasing concentrations



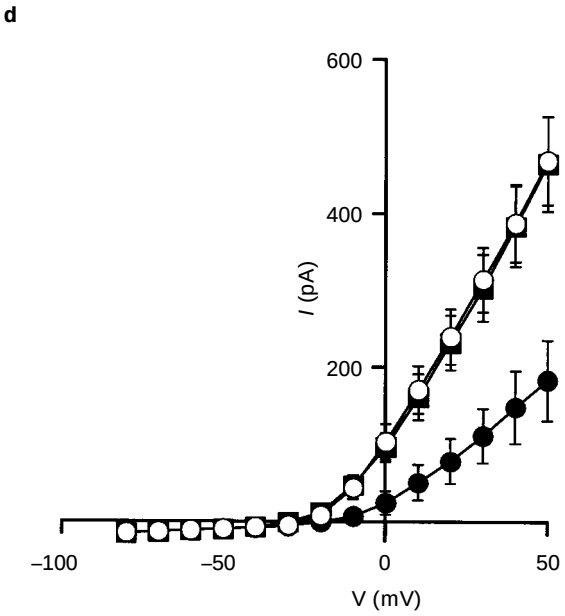
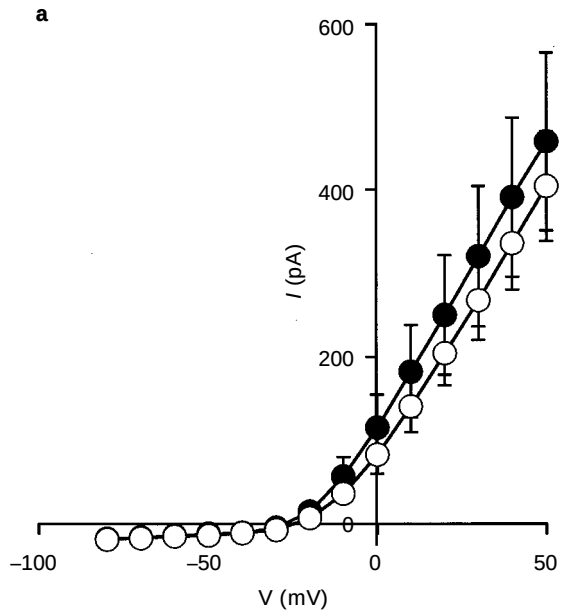


Figure 4 Whole-cell currents (I_{total}) in hepatic arterial cells under 'calcium-free' conditions before (a) and after (b) exposure to 3 mM 4-aminopyridine. (a,b) Currents (I) were generated by stepping from a holding potential of −90 mV to test potentials (V) of −30, −10, 10, 30 and 50 mV for 500 ms (c). Dashed line indicates the zero current level. (d) Full I-V relationships for peak currents in four cells before (○) and after (●) 5 min exposure to 3 mM 4-aminopyridine, and after 10 min washout (■) of 4-aminopyridine. Each point represents the mean and vertical lines show s.e.mean.

of [^{125}I]-charybdotoxin showed that [^{125}I]-charybdotoxin binding was consistent with binding to a single binding site with a K_D of approximatley 25 pM. Confirmation that the site tagged by [^{125}I]-charybdotoxin in the present study was likely to be associated with K_V (and not with BK_{Ca}) was given by the finding that iberiotoxin (0.1 μM) failed to displace [^{125}I]-charybdotoxin whereas agitoxin-2 (10 nM) inhibited the binding of this radioligand by 91 ± 3% (n = 6). In contrast to agitoxin-2, apamin alone increased [^{125}I]-charybdotoxin binding in a



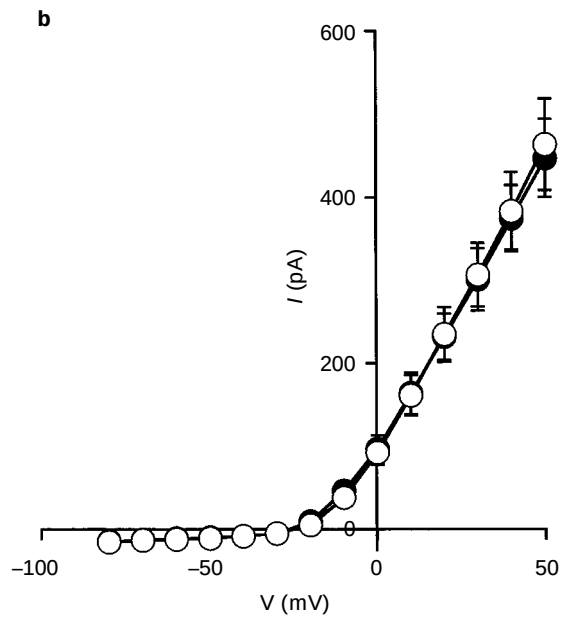


Figure 5 Effects of apamin plus charybdotoxin or apamin plus ciclazindol on I_{total} in hepatic arterial cells under 'calcium-free' conditions. Current (I)-voltage (V) relationships were determined before (○) and after (●) 10 min exposure to either (a) 0.3 μM apamin plus 0.3 μM charybdotoxin or (b) 0.3 μM apamin plus 10 μM ciclazindol. Currents at each test potential were elicited by stepping from a holding potential of −90 mV and were measured at their peak. Each point represents the mean and vertical lines show s.e.mean of four to five experiments.

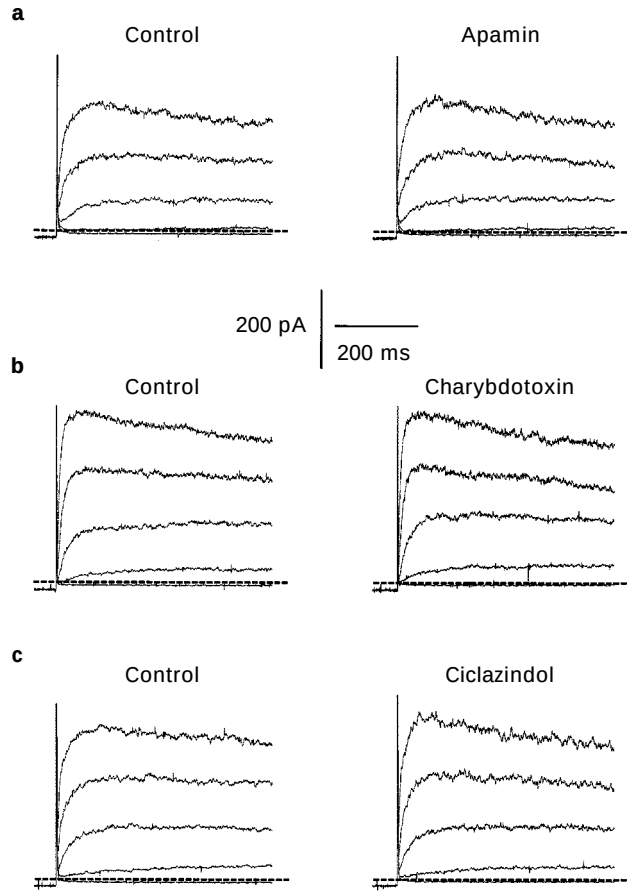


Figure 6 Whole-cell currents (I_{total}) in hepatic arterial cells under 'calcium-free' conditions before (control) and after exposure to (a) 0.3 μM apamin, (b) 0.3 μM charybdotoxin and (c) 10 μM ciclazindol for 10 min. Currents were generated by stepping from a holding potential of −90 mV to test potentials of −30, −10, 10, 30 and 50 mV. Each pair of traces was obtained from different animals; dashed lines indicate the zero current level.

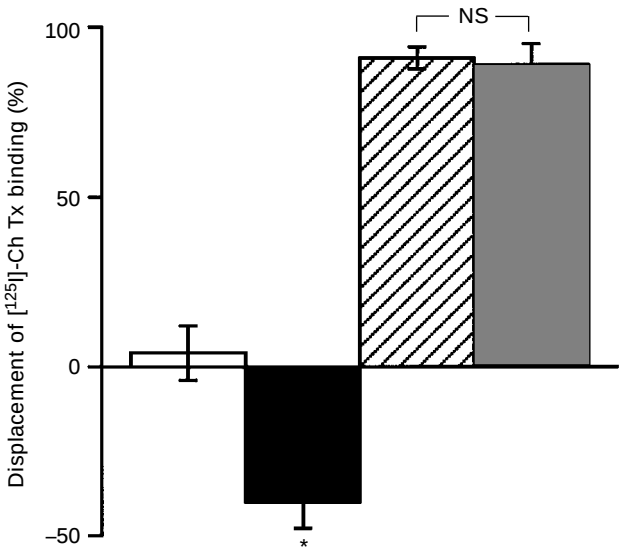


Figure 7 Displacement of [^{125}I]-charybdotoxin ([^{125}I]-ChTx) binding to rat cortical membranes by iberiotoxin (0.1 μM; open column, $n=6$), apamin (1 μM; solid column, $n=10$), agitoxin-2 (10 nM; hatched column, $n=6$) and apamin plus agitoxin-2 (stippled column, $n=6$). The results are shown as percent displacement of specific [^{125}I]-ChTx binding. Data are given as mean ± s.e.mean. *$P<0.05$ compared to [^{125}I]-ChTx binding in the absence of apamin. NS (not significant).

concentration-dependent manner (E_{max}, 43 ± 10%, $P<0.05$; pEC_{50}, 7.1 ± 0.2; $n=7-8$), but in the presence of both apamin (1 μM) and agitoxin-2 (10 nM), binding was inhibited to an extent similar to that seen in the presence of agitoxin-2 alone (Figure 7).

Discussion

Does EDHF open more than one type of K-channel?

The identity of the K-channels opened by EDHF is controversial (Garland *et al*., 1995; Zygmunt & Högestätt, 1996). In rat and rabbit mesenteric, bovine and porcine coronary and bovine oviductal arteries, EDHF-mediated responses are virtually abolished by apamin, leading to the conclusion that SK_{Ca} is the channel involved (Adeagbo & Triggle, 1993; Hecker *et al*., 1994; García-Pascual *et al*., 1995; Murphy & Brayden, 1995; Parsons *et al*., 1996). In contrast, apamin does not antagonize the effects of EDHF in rabbit carotid artery (Lischke *et al*., 1995), whereas in this tissue and in rabbit abdominal aorta, rat mesenteric and guinea-pig basilar arteries, EDHF-mediated relaxations are completely or partially inhibited by charybdotoxin, the inhibitor of BK_{Ca} (Cowan *et al*., 1993; Waldron & Garland, 1994; Petersson *et al*., 1996).

In other blood vessels, such as rat hepatic and guinea-pig carotid arteries, EDHF-mediated responses are unaffected by either apamin or charybdotoxin alone (Zygmunt, 1995; Corriu *et al*., 1996; Zygmunt & Högestätt, 1996) even when the concentration of each toxin is raised to 1 μM (present study). However, the effects of EDHF are completely inhibited when these toxins are combined (Zygmunt, 1995; Corriu *et al*., 1996; Zygmunt & Högestätt, 1996). A similar synergistic action of apamin plus charybdotoxin has also been demonstrated in rat mesenteric and guinea-pig basilar arteries (Waldron & Garland, 1994; Petersson *et al*., 1996). In these tissues, such results could indicate that EDHF simultaneously activates both SK_{Ca} and BK_{Ca}, and that combined inhibition of both channels is necessary to inhibit EDHF.

Does EDHF open BK_{Ca}?

The many workers who have used charybdotoxin have essentially employed this agent to indicate that BK_{Ca} is opened by EDHF. However, this toxin also inhibits delayed rectifier-type K-channels which are homomultimeric assemblies of the gene products Kv1.2 and Kv1.3 (Chandy & Gutman, 1995; Kaczorowski *et al*., 1996). In contrast, the selective BK_{Ca} inhibitor, iberiotoxin, which does not block K_V (Giangiacomo *et al*., 1992; Kaczorowski *et al*., 1996), has no effect on EDHF-mediated relaxations either on its own or when combined with apamin in rat hepatic and guinea-pig basilar arteries (Petersson *et al*., 1996; Zygmunt & Högestätt, 1996). Such results are a powerful indicator that BK_{Ca} is not opened by EDHF in rat hepatic or guinea-pig basilar arteries, a view supported by the lack of effect (present study) of kaliotoxin, another inhibitor of BK_{Ca} (Crest *et al*., 1992). Collectively, therefore, it is most unlikely that the inhibitory action of charybdotoxin combined with apamin indicates the involvement of BK_{Ca}.

Theoretical considerations also support the experimental evidence. Although BK_{Ca} does not inactivate (Bolton & Beech, 1992; Edwards *et al*., 1994) and could therefore be responsible for a sustained hyperpolarization, this channel only activates when the intracellular Ca^{2+} concentration is elevated and/or when the membrane is significantly depolarized (Bolton & Beech, 1992). Neither condition is required for the action of EDHF, since EDHF-mediated responses can be elicited in the absence of pre-contraction (when the intracellular Ca^{2+} concentration is low), or when the membrane potential is far be-

low the activation threshold of BK_{Ca} (Bolton & Beech, 1992; Zygmunt 1994b). This reasoning applies not only in rat hepatic and guinea-pig basilar arteries, but also to those vessels (guinea-pig and rabbit carotid and rat mesenteric arteries, rabbit abdominal aorta) in which charybdotoxin either alone or in combination with apamin inhibits EDHF.

Does EDHF open K_V?

Since charybdotoxin inhibits not only BK_{Ca}, but also Kv1.2 and Kv1.3 (Chandy & Gutman, 1995; Kaczorowski *et al*., 1996), antagonism of EDHF-mediated relaxations by this toxin (albeit in combination with apamin) could indicate that a delayed rectifier-like channel is a target for EDHF. However, in the present study, a series of potent and specific K_V inhibitors (Crest *et al*., 1992; Garcia *et al*., 1994; Miller, 1995; Kaczorowski *et al*., 1996) either had no effect (agitoxin-2, kaliotoxin) or only marginally inhibited (margatoxin) the EDHF-mediated relaxation when combined with apamin. Similarly, dofetilide (Gwilt *et al*., 1991) and terikalant (Green *et al*., 1996), inhibitors of delayed rectifier currents in cardiac and smooth muscle, respectively, were without effect in the present study. The K_V inhibitor, 4-aminopyridine, combined with apamin also failed to antagonize EDHF in the rat hepatic artery (Zygmunt & Högestätt, 1996).

In contrast to the many ineffective inhibitors of K_V, ciclazindol, an inhibitor of delayed rectifier currents in rat portal vein and pulmonary artery smooth muscle (Noack *et al*., 1992; Walker *et al*., 1996), reduced EDHF-mediated relaxations and abolished them when combined with apamin in the present study. Both EDHF and NO seem to share a common activation mechanism with regard to calcium-dependency (Busse *et al*., 1993; Nagao & Vanhoutte, 1993; Higuchi *et al*., 1996) and the type of muscarinic receptor stimulated by acetylcholine (Hammarström *et al*., 1995; Murphy & Brayden, 1995). Thus, since ciclazindol plus apamin also abolished receptor-independent EDHF relaxations elicited by the calcium-ionophore A23187 (Zygmunt *et al*., 1996) and had no effect on acetylcholine-induced relaxations in the absence of L-NOARG (when relaxations were mediated by NO), the inhibition of EDHF by ciclazindol almost certainly reflects an action on the K-channel opened by EDHF and not an effect on the release of EDHF. Ciclazindol also inhibited $I_{K(V)}$ in rat hepatic artery smooth muscle, but a ten times higher concentration was required than that necessary to antagonize EDHF-mediated relaxations. Furthermore, not only was the effect of ciclazindol on $I_{K(V)}$ not enhanced by apamin, but the combination of charybdotoxin plus apamin also failed to reduce this current in these cells.

These findings, derived from both mechanical and electrophysiological experiments, do not support the view that K_V is the target for EDHF. Indeed, since K_V does not activate until the membrane has depolarized to approximately -30 mV (present study), it is highly unlikely that such a channel could be responsible for EDHF-induced hyperpolarizations, which typically shift the membrane potential into the range -50 to -70 mV in the rat hepatic artery (Zygmunt *et al*., 1994b).

Do apamin and charybdotoxin interact with the same K-channel?

In these studies, the binding of [^{125}I]-charybdotoxin was not inhibited in the presence of the selective BK_{Ca} inhibitor, iberiotoxin, indicating that a delayed rectifier site (rather than BK_{Ca}) had been labelled (see Vazquez *et al*., 1990). Notably, the binding of [^{125}I]-charybdotoxin was increased by apamin, suggesting a possible allosteric interaction between the two toxins. If this phenomenon can be extrapolated to smooth muscle, the inhibition of EDHF by apamin plus charybdotoxin in hepatic artery could thus result from the interaction of these two toxins at a single K-channel. Interestingly, the potentiation of binding by apamin was not seen in the presence of agitoxin-2, the specific K_V inhibitor (Kaczorowski *et al*., 1996). This provides further evidence that the K-channel at which the apamin-charybdotoxin interaction was observed is not BK_{Ca}.

A unifying hypothesis

Until recently, the structure of the apamin-sensitive K-channel (SK_{Ca}) was unknown. However, the studies of Köhler *et al*. (1996) have shown that this channel, like BK_{Ca} and K_V, is a member of 'Superfamily 1', the collection of K-channels characterized by α-subunits each with six putative membrane-spanning segments (Edwards & Weston, 1997). Thus, the close structural relationship between BK_{Ca}, K_V and SK_{Ca} (Köhler *et al*., 1996; Edwards & Weston, 1997) could provide a unifying explanation not only for the antagonism of EDHF-mediated responses in the rat hepatic artery by apamin combined with ciclazindol or charybdotoxin, but also for the situation in other vessels in which either charybdotoxin (Cowan *et al*., 1993; Waldron *et al*., 1994; Lischke *et al*., 1995; Petersson *et al*., 1996) or apamin (Adeagbo & Triggle, 1993; Hecker *et al*., 1994; Garcia-Pascual *et al*., 1995; Murphy & Brayden, 1995; Parsons *et al*., 1996) is individually effective.

To account for these findings, a reasonable explanation would be that the innermost part of the K-channels opened by EDHF comprises α subunits, each containing six membrane-spanning segments. To sustain membrane hyperpolarization, it is unlikely that the channel exhibits the voltage activation threshold characteristic of K_V and BK_{Ca}. Instead, it is almost certainly voltage-insensitive, a property characteristic of SK_{Ca}, four subtypes of which were recently described by Köhler *et al*. (1996). In those tissues in which the effects of EDHF are apamin-sensitive, the channel can be regarded as a 'classical' type of SK_{Ca}. In other tissues, in which apamin alone is ineffective, the channel could be similar to one of the apamin-insensitive variants of SK_{Ca} described by Köhler *et al*. (1996). Such channels may be sensitive to charybdotoxin alone (see Van Renterghem & Lazdunski, 1992) or rendered sensitive to this agent by allosteric conformational changes induced by apamin.

Conclusions

The present investigation has provided no evidence that the voltage-sensitive K-channels, K_V or BK_{Ca}, are the targets for EDHF in the rat hepatic artery. Instead, the results are consistent with the view that the target K-channels for EDHF are structurally-related to both K_V and BK_{Ca}, and may be similar to one of the apamin-insensitive subtypes of SK_{Ca} recently described by Köhler *et al*. (1996). It also seems reasonable to speculate that the opening of closely-related channels can also account for the actions of EDHF in those tissues in which either apamin or charybdotoxin alone can antagonize EDHF-mediated responses. Further studies are in progress to identify the relevant channel in rat hepatic artery and to characterize its properties.

This work was supported by the Swedish MRC (Grant no. 11582) and the Medical Faculty of Lund. P.M.Z. was supported by a Travelling Fellowship from the Swedish MRC (Grant no. 11524 and

References

ADEAGBO, A.S.O. & TRIGGLE, C.R. (1993). Varying extracellular [K^+]: a functional approach to separating EDHF- and EDNO-related mechanisms in perfused rat mesenteric arterial bed. *J. Cardiovasc. Pharmacol.*, **21,** 423–429.

BOLTON, T.B. & BEECH, D.J. (1992). Smooth muscle potassium channels, their electrophysiology and function. In *Potassium Channel Modulators*. ed. Weston, A.H. & Hamilton, T.C. pp. 144–180. Oxford: Blackwell Scientific.

BUSSE, R., MÜLSCH, A., FLEMING, I. & HECKER, M. (1993). Mechanisms of nitric oxide release from the vascular endothelium. *Circulation*, **87,** (Suppl. V) V-18–V-25.

CHANDY, K.G. & GUTMAN, G.A. (1995). Voltage-gated potassium channel genes. In *Ligand- and Voltage-Gated Ion Channels*. ed. North, R.A. pp. 2–71. Boca Raton: CRC Press.

CHEN, G., SUZUKI, H. & WESTON, A.H. (1988). Acetylcholine releases endothelium-derived hyperpolarizing factor and EDRF from rat blood vessels. *Br. J. Pharmacol.* **95,** 1165–1174.

COWAN, C.L., PALACINO, J.J., NAJIBI, S. & COHEN, R.A. (1993). Potassium-channel-mediated relaxation to acetylcholine in rabbit arteries. *J. Pharmacol. Exp. Ther.*, **266,** 1482–1489.

CORRIU, C., FELETOU, M., CANET, E. & VANHOUTTE, P.M. (1996). Endothelium-derived factors and hyperpolarization of the carotid artery of the guinea-pig. *Br. J. Pharmacol.*, **119,** 959–964.

CREST, M., JACQUET, G., GOLAS, M., ZERROUK, H., BENSLIMANE, A., ROCHAT, H., MANSUELLE, P. & MARTIN-EAUCLAIRE, M.-F. (1992). Kaliotoxin, a novel peptidyl inhibitor of neuronal BK-type Ca^{2+}-activated K^+ channels characterized from *Androctonus mauretanicus mauretanicus* venom. *J. Biol. Chem.*, **267,** 1640–1647.

EDWARDS, G., NIEDERSTE-HOLLENBERG, A., SCHNEIDER, J., NOACK, Th. & WESTON, A.H. (1994). Ion channel modulation by NS1619, the putative BK_{Ca} channel opener, in rat portal vein. *Br. J. Pharmacol.*, **113,** 1538–1547.

EDWARDS, G. & WESTON, A.H. (1997). Effect of potassium channel modulating drugs on isolated smooth muscle. In *Molecular Biology of the Neuron*. ed. Davies, R.W. & Morris, B. pp. 145–175. Oxford: βios Scientific (in press).

FÉLÉTOU, M. & VANHOUTTE, P.M. (1988). Endothelium-dependent hyperpolarization of canine coronary smooth muscle. *Br. J. Pharmacol.*, **93,** 515–524.

GARCIA, M.L., GARCIA-CALVO, M., HIDALGO, P., LEE, A. & MCKINNON, R. (1994). Purification and characterization of three inhibitors of voltage-dependent K^+ channels from *Leiurus quinquestriatus* var. *hebraeus* venom. *Biochemistry*, **33,** 6834–6839.

GARCIA, M.L., KNAUS, H.-G., MUNUJOS, P., SLAUGHTER, R.S. & KACZOROWSKI, G.J. (1995). Charybdotoxin and its effects on potassium channels. *Am. J. Physiol.*, **269,** C1–C10.

GARCÍA-PASCUAL, A., LABADIA, A., JIMENEZ, E. & COSTA, G. (1995). Endothelium-dependent relaxation to acetylcholine in bovine oviductal arteries: mediation by nitric oxide and changes in apamin-sensitive K^+ conductance. *Br. J. Pharmacol.*, **115,** 1221–1230.

GARLAND, C.J., PLANE, F., KEMP, B.K. & COCKS, T.M. (1995). Endothelium-dependent hyperpolarization: a role in the control of vascular tone. *Trends Pharmacol. Sci.*, **16,** 23–30.

GIANGIACOMO, K.M., GARCIA, M.L. & MCMANUS, O.B. (1992). Mechanism of iberiotoxin block of the large-conductance calcium-activated potassium channel from bovine aortic smooth muscle. *Biochemistry*, **31,** 6719–6727.

GREEN, M.E., EDWARDS, G., KIRKUP, A.J., MILLER, M. & WESTON, A.H. (1996). Pharmacological characterization of the inwardly-rectifying current in the smooth muscle cells of the rat bladder. *Br. J. Pharmacol.*, **119,** 1509–1518.

GWILT, M., ARROWSMITH, J.E., BLACKBURN, K.J., BURGES, R.A., CROSS, P.E., DALRYMPLE, H.W. & HIGGINS, A.J. (1991). UK-68,798: A novel, potent and highly selective class III antiarrhythmic agent which blocks potassium channels in cardiac cells. *J. Pharmacol. Exp. Ther.*, **256,** 318–324.

HAMMARSTRÖM, A.K.M., PARKINGTON, H.C. & COLEMAN, H.A. (1995). Release of endothelium-derived hyperpolarizing factor (EDHF) by M_3 receptor stimulation in guinea-pig coronary artery. *Br. J. Pharmacol.*, **115,** 717–722.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv.*, **391,** 85–100.

HECKER, M., BARA, A.T., BAUERSACHS, J. & BUSSE, R. (1994). Characterization of endothelium-derived hyperpolarizing factor as a cytochrome P450-derived arachidonic acid metabolite in mammals. *J. Physiol.*, **481,** 407–414.

HIGUCHI, T., NISHIMURA, J., KOBAYASHI, S. & KANAIDE, H. (1996). CPA induces a sustained release in $[Ca^{2+}]_i$ of endothelial cells in situ and relaxes porcine coronary artery. *Am. J. Physiol.*, **270,** H2038–H2049.

HORN, R. & MARTY, A. (1988). Muscarinic activation of ionic currents measured by a new whole-cell recording method. *J. Gen. Physiol.*, **92,** 145–159.

HÖGESTÄTT, E.D., ANDERSSON, K.-E. & EDVINSSON, L. (1983). Mechanical properties of rat cerebral arteries as studied by a sensitive device for recording of mechanical activity in isolated small blood vessels. *Acta Physiol. Scand.*, **117,** 49–61.

KACZOROWSKI, G.J., KNAUS, H.G., LEONARD, R.J., MCMANUS, O.B. & GARCIA, M.L. (1996). High-conductance calcium-activated potassium channels; structure, pharmacology, and function. *J. Bioenerg. Biomembr.* **28,** 255–267.

KLÖCKNER, U. & ISENBERG, G. (1985). Action potential and net membrane currents of isolated smooth muscle cells (urinary bladder of the guinea-pig). *Pflügers Archiv.*, **405,** 329–339.

KÖHLER, M., HIRSCHBERG, B., BOND, C.T., KINZIE, J.M., MARRION, N.V., MAYLIE, J. & ADELMAN, J.P. (1996). Small-conductance, calcium-activated potassium channels from mammalian brain. *Science*, **273,** 1709–1714.

LISCHKE, V., BUSSE, R. & HECKER, M. (1995). Selective inhibition by barbiturates of the synthesis of endothelium-derived hyperpolarizing factor in the rabbit carotid artery. *Br. J. Pharmacol.*, **115,** 969–974.

MILLER, C. (1995). The charybdotoxin family of K^+ channel blocking peptides. *Neuron*, **15,** 5–10.

MURPHY, M.E. & BRAYDEN, J.E. (1995). Apamin-sensitive K^+ channels mediated an endothelium-dependent hyperpolarization in rabbit mesenteric arteries. *J. Physiol.*, **489,** 723–734.

NAGAO, T. & VANHOUTTE, P.M. (1993). Endothelium-derived hyperpolarizing factor and endothelium-dependent relaxations. *Am. J. Respir. Cell Mol. Biol.*, **8,** 1–6.

NOACK, Th., EDWARDS, G., DEITMER, P., GREENGRASS, P., MORITA, T., ANDERSSON, P.-O., CRIDDLE, D., WYLLIE, M.G. & WESTON, A.H. (1992). The involvement of potassium channels in the action of ciclazindol in rat portal vein. *Br. J. Pharmacol.*, **106,** 17–24.

PARSONS, S.J.W., SUMNER, M. & GARLAND, C.J. (1996). Apamin-sensitive responses to acetylcholine in rabbit isolated mesenteric arteries. *Br. J. Pharmacol. Proc. Suppl.*, **117,** 278p.

PETERSSON, J., ZYGMUNT, P.M., BRANDT, L. & HÖGESTÄTT, E.D. (1995). Substance P-induced relaxation and hyperpolarization in human cerebral arteries. *Br. J. Pharmacol.*, **115,** 889–894.

PETERSSON, J., ZYGMUNT, P.M. & HÖGESTÄTT, E.D. (1996). Charybdotoxin and apamin inhibit relaxations mediated by EDHF in the guinea-pig basilar artery. *Br. J. Pharmacol. Proc. Suppl.* **117,** 119P.

RAE, J., COOPER, K., GATES, P. & WATSKY, M. (1991). Low access resistance perforated patch recordings using amphotericin B. *J. Neurosci. Methods*, **37,** 15–26.

SOKOL, P.T., HU, W., YI, L., TORAL, J., CHANDRA, M. & ZIAI, M.R. (1994). Cloning of an apamin binding protein of vascular smooth muscle. *J. Protein Chemistry*, **13,** 117–128.

TAYLOR, S.G. & WESTON, A.H. (1988). Endothelium-derived hyperpolarizing factor: a new endogenous inhibitor from the vascular endothelium. *Trends Pharmacol. Sci.*, **9,** 272–274.

VAZQUEZ, J., FEIGENBAUM, P., KING, V.F., KACZOROWSKI, G.J. & GARCIA, M.L. (1990). Characterization of high affinity binding sites for charybdotoxin in synaptic plasma membranes from rat brain. *J. Biol. Chem.*, **265,** 15564–15571.

VAN RENTERGHEM, C. & LAZDUNSKI, M. (1992). A small conductance charybdotoxin sensitive, apamin resistant Ca^{2+} activated K^+ channel in aortic smooth muscle cells (A7r5 line and primary cultures). *Pflügers Arch.*, **420,** 417–423.

WALDRON, G.J. & GARLAND, C.J. (1994). Effect of potassium channel blockers on L-NAME insensitive relaxations in rat small mesenteric artery. *Can. J. Physiol. Pharmacol.*, **72** (S1), 115.

WALKER, S., EDWARDS, G., GREEN, M.E. & WESTON, A.H. (1996). Characterisation of potassium currents in rat pulmonary arterial smooth muscle cells. *Br. J. Pharmacol. Proc. Suppl.*, **119,** 80P.

ZYGMUNT, P.M. (1995). Endothelium-dependent hyperpolarization and relaxation of vascular smooth muscle. *PhD Thesis*, Lund University.

ZYGMUNT, P.M., GRUNDEMAR, L. & HÖGESTÄTT, E.D. (1994a). Endothelium-dependent relaxation resistant to N^{ω}-nitro-L-arginine in the rat hepatic artery and aorta. *Acta Physiol. Scand.*, **152,** 107–114.

ZYGMUNT, P.M. & HÖGESTÄTT, E.D. (1996). Role of potassium channels in endothelium-dependent relaxation resistant to nitroarginine in the rat hepatic artery. *Br. J. Pharmacol.*, **117,** 1600–1606.

ZYGMUNT, P.M., PETERSSON, J., WESTON, A.H. & HÖGESTÄTT, E.D. (1996). Effects of ciclazindol on EDHF-mediated relaxations in the rat hepatic and guinea-pig basilar arteries. *Br. J. Pharmacol. Proc. Suppl.*, **119,** 76P.

ZYGMUNT, P.M., WALDECK, K. & HÖGESTÄTT, E.D. (1994b). The endothelium mediates a nitric oxide-independent hyperpolarization and relaxation in the rat hepatic artery. *Acta Physiol. Scand.*, **152,** 375–384.

(Received December 6, 1996
Revised January 28, 1997
Accepted February 3, 1997)