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Characterization of the potassium channels involved in EDHF-mediated relaxation in cerebral arteries

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1 In the presence of N^G-nitro-L-arginine (L-NOARG, 0.3 mM) and indomethacin (10 μM), the relaxations induced by acetylcholine and the calcium (Ca) ionophore A23187 are considered to be mediated by endothelium-derived hyperpolarizing factor (EDHF) in the guinea-pig basilar artery.

2 Inhibitors of adenosine 5′-triphosphate (ATP)-sensitive potassium (K)-channels (K_{ATP}; glibenclamide, 10 μM), voltage-sensitive K-channels (K_V; dendrotoxin-I, 0.1 μM or 4-aminopyridine, 1 mM), small (SK_{Ca}; apamin, 0.1 μM) and large (BK_{Ca}; iberiotoxin, 0.1 μM) conductance Ca-sensitive K-channels did not affect the L-NOARG/indomethacin-resistant relaxation induced by acetylcholine.

3 Synthetic charybdotoxin (0.1 μM), an inhibitor of BK_{Ca} and K_V, caused a rightward shift of the concentration-response curve for acetylcholine and reduced the maximal relaxation in the presence of L-NOARG and indomethacin, whereas the relaxation induced by A23187 was not significantly inhibited.

4 A combination of charybdotoxin (0.1 μM) and apamin (0.1 μM) abolished the L-NOARG/indomethacin-resistant relaxations induced by acetylcholine and A23187. However, the acetylcholine-induced relaxation was not affected by a combination of iberiotoxin (0.1 μM) and apamin (0.1 μM).

5 Ciclazindol (10 μM), an inhibitor of K_V in rat portal vein smooth muscle, inhibited the L-NOARG/indomethacin-resistant relaxations induced by acetylcholine and A23187, and the relaxations were abolished when ciclazindol (10 μM) was combined with apamin (0.1 μM).

6 Human pial arteries from two out of four patients displayed an L-NOARG/indomethacin-resistant relaxation in response to substance P. This relaxation was abolished in both cases by pretreatment with the combination of charybdotoxin (0.1 μM) and apamin (0.1 μM), whereas each toxin had little effect alone.

7 The results suggest that K_V, but not K_{ATP} and BK_{Ca}, is involved in the EDHF-mediated relaxation in the guinea-pig basilar artery. The synergistic action of apamin and charybdotoxin (or ciclazindol) could indicate that both K_V and SK_{Ca} are activated by EDHF. However, a single type of K-channel, which may be structurally related to K_V and allosterically regulated by apamin, could also be the target for EDHF.

Keywords: Cerebral arteries; endothelium-derived hyperpolarizing factor; potassium channels; vascular endothelium; hyperpolarization

Introduction

As shown by Nishiye *et al.* (1989), acetylcholine elicits an endothelium-dependent hyperpolarization and relaxation, which is unaffected by the nitric oxide (NO) scavenger oxyhaemoglobin, in the guinea-pig basilar artery. This hyperpolarization and the relaxation induced by acetylcholine and the calcium (Ca) ionophore A23187 in the presence of N^G-nitro-L-arginine (L-NOARG, 0.3 mM) and indomethacin (10 μM), inhibitors of nitric oxide synthase and cyclo-oxygenase, are considered to be mediated by endothelium-derived hyperpolarizing factor (EDHF) in this preparation (Nishiye *et al.*, 1989; Petersson *et al.*, 1996a). Vasodilator responses mediated by EDHF have also been demonstrated in human cerebral arteries (Petersson *et al.*, 1995). Although EDHF is believed to open K-channels in vascular smooth muscle, thereby causing membrane hyperpolarization and vasodilatation, the identity of these K-channels is unclear (Garland *et al.*, 1995; Zygmunt & Högestätt, 1996).

In certain blood vessels, apamin which is an inhibitor of small conductance Ca-sensitive K-channels (SK_{Ca}) prevents EDHF-mediated responses (Adeagbo & Triggle, 1993; Hecker *et al.*, 1994; García-Pascual *et al.*, 1995; Murphy & Brayden, 1995; Parsons *et al.*, 1996). However, in other vascular preparations, the effects of EDHF are antagonized by charybdotoxin (Cowan *et al.*, 1993; Lischke *et al.*, 1995), an inhibitor of large conductance Ca-sensitive K-channels (BK_{Ca}) and certain voltage-sensitive K-channels (K_V). Furthermore, in the rat hepatic artery, a combination of apamin and charybdotoxin is necessary to suppresss EDHF-mediated relaxation (Zygmunt & Högestätt, 1996). Collectively, these findings could indicate that different types of K-channel are activated by EDHF in different blood vessels.

In rabbit middle cerebral arteries, it has been shown that glibenclamide inhibits acetylcholine-induced smooth muscle hyperpolarization, suggesting that EDHF activates ATP-sensitive K-channels (K_{ATP}) in cerebral arteries (Standen *et al.*, 1989; Brayden, 1990). However, in preliminary studies on guinea-pig basilar and human pial arteries, glibenclamide did not affect the endothelium-dependent, L-NOARG/indomethacin-resistant relaxation or hyperpolarization (Petersson *et al.*, 1996a,b). Glibenclamide also had no effect on EDHF-mediated responses in a variety of other blood vessels (Zygmunt *et al.*, 1994; for review see Garland *et al.*, 1995).

The objective of the present study was to characterize the K-channels activated by EDHF in cerebral arteries. Such a study is highly relevant, since these channels could serve as pharmacological targets in conditions, such as subarachnoid haemorrhage and cerebral ischaemia, which have been associated with loss of endothelium-derived relaxing factors (Nakagomi *et al.*, 1987; Mayhan *et al.*, 1988; Hatake *et al.*, 1992). It has also been suggested that drugs that cause cerebral vasodilatation by opening K-channels and subsequent hyperpolarization may be of clinical value in situations where pathological vasospasm is caused by membrane depolarization (Zhang & Cook, 1994;

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Petersson *et al*., 1996a). The experimental approach adopted in the present study was to examine the effects of different K-channel inhibitors on endothelium-dependent relaxation in the presence of inhibitors of NO synthase and cyclo-oxygenase in the guinea-pig basilar artery and human pial arteries. Some of these results have been presented to the British Pharmacological Society (Petersson *et al*., 1996a; Zygmunt *et al*., 1996).

Methods

Male guinea-pigs (300 g) were killed by CO_2 asphyxia followed by exsanguination. After removal of the brainstem, the basilar artery was dissected and flushed with physiological salt solution (PSS; composition in mM: NaCl 119, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 15, NaH_2PO_4 1.2 and (+)-glucose 6) to remove blood products. Human pial arteries from cortical tissue which was macroscopically free of tumour infiltration, were obtained from four patients who had decompressive lobectomy performed due to malignant cerebral glioma.

Wall tension was measured in organ baths filled with PSS. The temperature was maintained at 37°C. To provide oxygenation and a pH of 7.4, the PSS was continuously bubbled with carbogen (95% O_2, 5% CO_2). Ring preparations (2 mm long) of the arteries were gently threaded on two stainless steel wires (Högestätt *et al*., 1983). One of the wires was connected to a force-displacement transducer (model FT03 C, Grass Instruments, U.S.A.) for isometric tension recording. The output from the transducer was displayed on a polygraph (model 7D, Grass Instruments, U.S.A.). The vessels were subjected to a passive load of 3 mN. After an equilibration period of 1 h, a solution containing 60 mM K^+ (prepared as the PSS, but 55 mM NaCl was replaced with KCl) was added to assess the contractile capacity of the preparation. All experiments were performed in the presence of L-NOARG (0.3 mM) and indomethacin (10 μM) to suppress the synthesis of NO and prostanoids. In the guinea-pig basilar artery, prostaglandin $F_{2\alpha}$ $PGF_{2\alpha}$; (0.1 – 1 μM) was used to induce stable contractions. Acetylcholine (0.01 – 100 μM) or A23187 (0.1 – 3 μM) was then added cumulatively to elicit endothelium-dependent relaxations. Human pial arteries were pre-contracted with the thromboxane receptor agonist U46619 (1 μM), and endothelium-dependent relaxations were induced with substance P (0.1 – 10 nM; Petersson *et al*., 1995).

Calculations and statistics

The negative logarithm of the drug concentration eliciting half maximal relaxation (pEC_{50}) was calculated by linear regression by using the values immediately above and below half maximal response. E_{max} refers to the maximal relaxation achieved (100% denotes a complete reversal of agonist-induced contractions). Results are given as arithmetic mean values ± s.e.mean. Two-tailed Student's *t* test for unpaired observations was used for statistical comparison, and a *P* value less than 0.05 was considered as statistically significant.

Drugs

Acetylcholine chloride, 4-aminopyridine, A23187, glibenclamide, N^G-nitro-L-arginine, substance P and tetraethylammonium chloride were purchased from Sigma, St Louis, MO, U.S.A.; synthetic charybdotoxin from Latoxan, Rosans, France; ciclazindol from Pfizer, U.K.; U46619 (9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$) and prostaglandin $F_{2\alpha}$ (Prostin) from Upjohn, Kalamazoo, MI, U.S.A.; indomethacin (Confortid) from Dumex, Copenhagen, Denmark; iberiotoxin, apamin and dendrotoxin-I from Alomone Labs, Jerusalem, Israel.

Results

Guinea-pig basilar arteries

Effects of inhibitors of Ca-sensitive K-channels In the presence of L-NOARG (0.3 mM) and indomethacin (10 μM), acetylcholine and A23187 elicited concentration-dependent relaxations in guinea-pig basilar arteries contracted with $PGF_{2\alpha}$. The acetylcholine-induced relaxation was inhibited by charybdotoxin (0.1 μM; Figure 1a); both the sensitivity and maximal response to acetylcholine were reduced (Table 1). Raising the concentration of charybdotoxin to 0.3 μM did not cause fur-

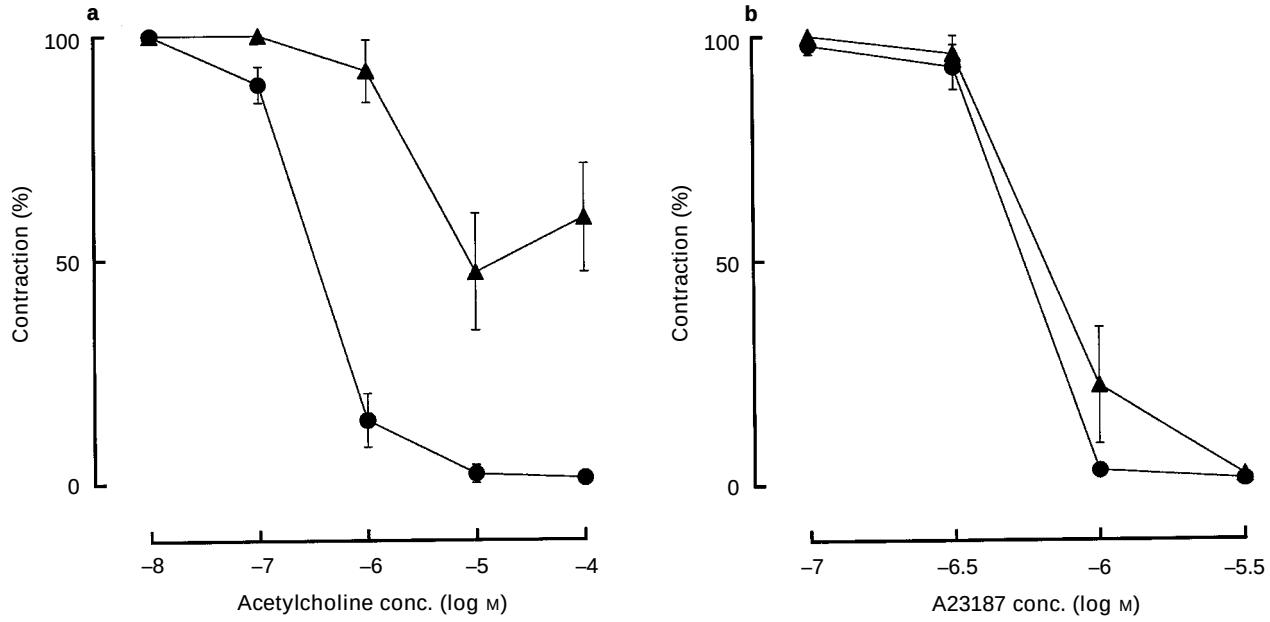

Figure 1 Relaxation induced by acetylcholine (a; $n=11$) or A23187 (b; $n=7-8$) in guinea-pig basilar arteries contracted with $PGF_{2\alpha}$ in the presence (▲) or absence (●) of charybdotoxin (0.1 μM). The level of contraction induced by $PGF_{2\alpha}$ before addition of acetylcholine or A23187 was set to 100%. L-NOARG (0.3 mM) and indomethacin (10 μM) were present throughout. Data are presented as means and vertical lines show s.e.mean.

ther inhibition ($n=4$, not shown). However, charybdotoxin (0.1 μM) did not affect the A23187-induced relaxation (Figure 1b, $n=8$); pEC_{50} for A23187 was 6.16 ± 0.07 vs 6.27 ± 0.02 in controls, and E_{max} was $99\pm1\%$ in both treated and control vessels. Iberiotoxin (0.1 μM), a selective inhibitor of BK_{Ca}, was devoid of inhibitory activity on the acetylcholine-induced relaxation (Table 1). Pretreatment with apamin (0.1 μM) had no effect on the relaxations induced by acetylcholine (Table 1) and A23187; pEC_{50} for A23187 was 6.20 ± 0.07 vs 6.27 ± 0.02 in controls, and E_{max} was $99\pm1\%$ in both treated and control vessels ($n=7$). Tetraethylammonium (TEA; 1 mM) caused a small but significant rightward shift of the concentration-response curve for acetylcholine without affecting the maximal response (Table 1).

Effects of inhibitors of ATP- and voltage-sensitive K-channels Pretreatment with the K_{ATP} inhibitor glibenclamide (10 μM) did not affect the relaxation induced by acetylcholine (Table 1). Dendrotoxin-I (0.1 μM) and 4-aminopyridine (4-AP; 1 mM), inhibitors of K_V, also had no effect on this relaxation (Table 1). However, ciclazindol (10 μM), which inhibits K_V in rat portal vein (Noack *et al*., 1992), antagonized the relaxations induced by acetylcholine (Figure 2a, Table 1) and A23187 (Figure 2b); pEC_{50} and E_{max} for A23187 were 5.77 ± 0.01 and $80\pm19\%$ in the presence of ciclazindol (10 μM) and 6.23 ± 0.02 and $99\pm1\%$ in controls, respectively ($n=5$).

Effects of combinations of K-channel inhibitors A combination of charybdotoxin (0.1 μM) and apamin (0.1 μM) completely inhibited the relaxations induced by acetylcholine and A23187 (Figure 3). When ciclazindol (10 μM) was combined with apamin (0.1 μM), the relaxations induced by acetylcholine and A23187 were also abolished (Figure 4). However, a combination of iberiotoxin (0.1 μM) and apamin (0.1 μM) had no effect on the acetylcholine-induced relaxation; pEC_{50} for acetylcholine was 6.40 ± 0.06 vs 6.49 ± 0.04 in controls and E_{max} was $99\pm0\%$ vs $99\pm1\%$ in controls ($n=5$). Apamin (0.1 μM) and 4-AP (1 mM) combined elicited a small significant rightward shift of the concentration-response curve for acetylcholine without affecting the maximal response (Figure 5); pEC_{50} for acetylcholine was 5.52 ± 0.03 in treated arteries vs 6.46 ± 0.07 in controls, and E_{max} was $98\pm1\%$ and $96\pm2\%$, respectively ($n=5$).

Human pial arteries

Substance P elicited a L-NOARG/indomethacin-resistant relaxation in pial arteries from two out of four patients. Pretreatment with a combination of charybdotoxin (0.1 μM) and apamin (0.1 μM) abolished the relaxation in both cases,

Table 1 Effects of K channel inhibitors on acetylchloine-induced relaxation in the guinea-pig basilar artery in the presence of indomethacin (10 μM) and N^G-nitro-L-arginine (0.3 mM)

		pEC_{50}		E_{max}	
Treatment	n	*Control*	*Treated*	*Control*	*Treated*
Charybdotoxin 0.1 μM	11	6.46 ± 0.09	4.92 ± 0.51*	99 ± 0	55 ± 12*
Apamin 0.1 μM	6	6.21 ± 0.20	6.20 ± 0.12	93 ± 6	95 ± 4
Iberiotoxin 0.1 μM	4	6.27 ± 0.21	6.45 ± 0.29	99 ± 1	99 ± 1
4-Aminopyridine 1 mM	5	6.43 ± 0.20	6.18 ± 0.18	99 ± 1	100 ± 0
Dendrotoxin-I 0.1 μM	4	6.28 ± 0.13	6.85 ± 0.32	95 ± 2	97 ± 2
Glibenclamide 10 μM	4	6.40 ± 0.26	6.61 ± 0.12	99 ± 1	99 ± 1
TEA 1 mM	5	6.46 ± 0.07	5.99 ± 0.14*	96 ± 2	97 ± 2
Ciclazindol 10 μM	7	6.35 ± 0.04	5.22 ± 0.19*	97 ± 1	38 ± 12*

pEC_{50} and E_{max} values for the concentration-response curves were calculated as described in the Methods section. *Indicates a significant difference compared to controls ($P<0.05$).

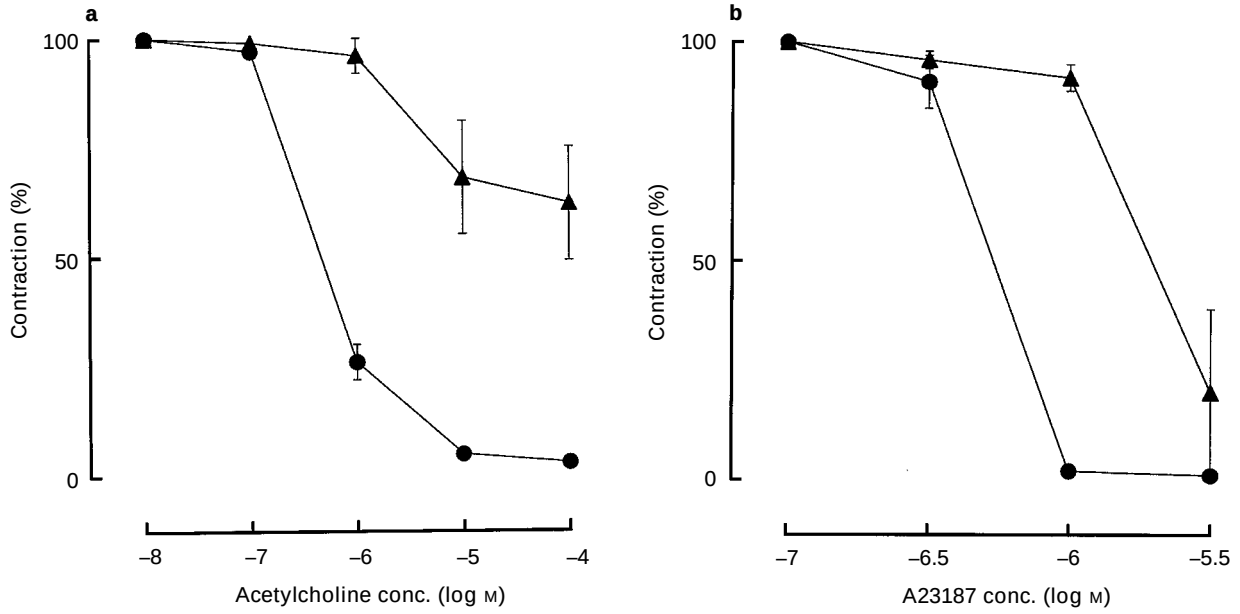

Figure 2 Relaxation induced by acetylcholine (a; $n=7$) or A23187 (b; $n=5$) in guinea-pig basilar arteries contracted with $PGF_{2\alpha}$ in the presence (▲) or absence (●) of ciclazindol (10 μM). The level of contraction induced by $PGF_{2\alpha}$ before addition of acetylcholine or A23187 was set to 100%. L-NOARG (0.3 mM) and indomethacin (10 μM) were present throughout. Data are presented as means and vertical lines show s.e.mean.

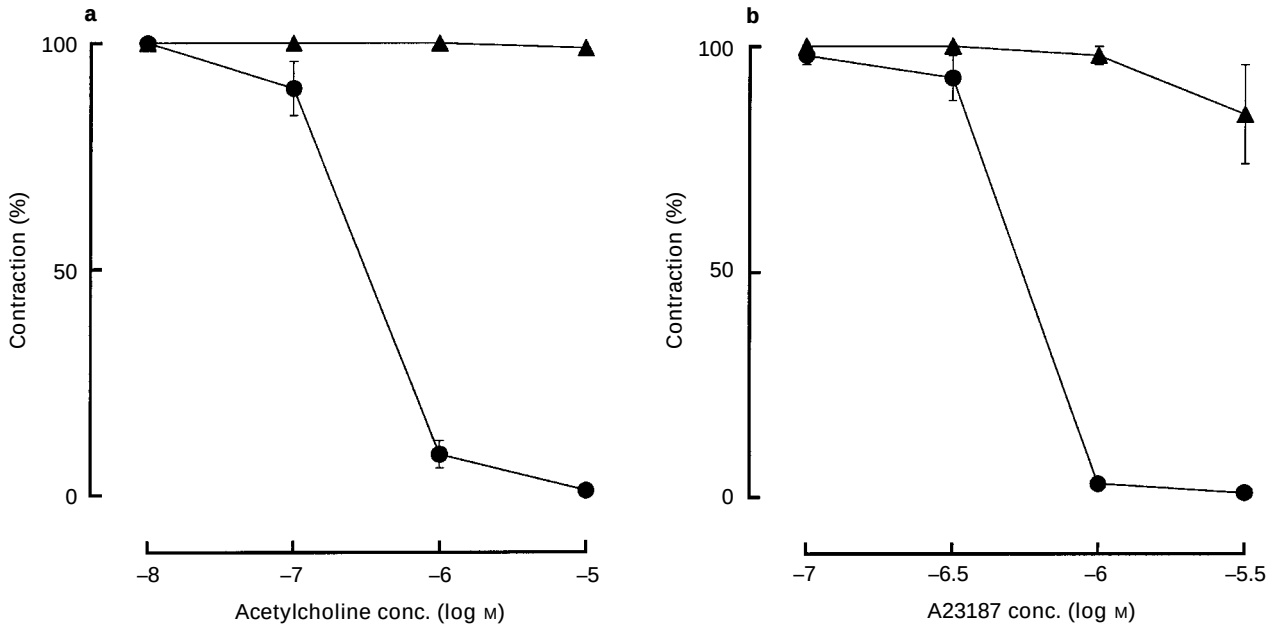

Figure 3 Relaxation induced by acetylcholine (a; $n=6$) or A23187 (b; $n=7-8$) in guinea-pig basilar arteries contracted with $PGF_{2\alpha}$ in the presence (▲) or absence (●) of a combination of charybdotoxin (0.1 μM) and apamin (0.1 μM). The level of contraction induced by $PGF_{2\alpha}$ before addition of acetylcholine or A23187 was set to 100%. L-NOARG (0.3 mM) and indomethacin (10 μM) were present throughout. Data are presented as means and vertical lines show s.e.mean.

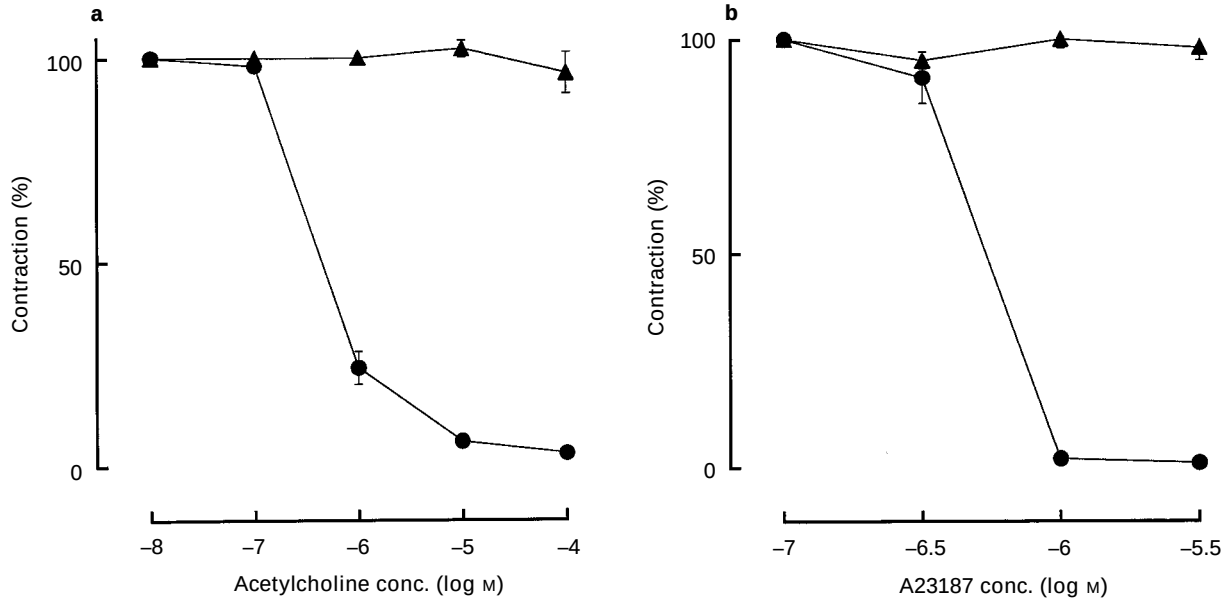

Figure 4 Relaxation induced by acetylcholine (a; $n=6$) or A23187 (b; $n=4$) in guinea-pig basilar arteries contracted with $PGF_{2\alpha}$ in the presence (▲) or absence (●) of a combination of ciclazindol (10 μM) and apamin (0.1 μM). The level of contraction induced by $PGF_{2\alpha}$ before addition of acetylcholine or A23187 was set to 100%. L-NOARG (0.3 mM) and indomethacin (10 μM) were present throughout. Data are presented as means and vertical lines show s.e.mean.

whereas charybdotoxin (0.1 μM) or apamin (0.1 μM) alone had little effect (Figure 6).

Discussion

It has previously been shown in the guinea-pig basilar artery that acetylcholine induces both hyperpolarization and relaxation in the presence of oxyhaemoglobin (Nishiye *et al.*, 1989). It was therefore concluded that EDHF is released by acetylcholine in this preparation. In the same study, A23187 failed to induce a significant hyperpolarization. However, the highest concentration of A23187 used was 1 μM, which is close to the threshold concentration for relaxation in the present study, and may thus have been too low to induce a significant hyperpolarization. The results of the present study suggest that activation of K-channels mediates the L-NOARG/indomethacin-resistant relaxation induced by acetylcholine and A23187 in the guinea-pig basilar artery. Since this relaxation was unaffected by glibenclamide, it is unlikely that K_{ATP} is the target K-channel for EDHF in this preparation. In the rabbit middle cerebral artery, glibenclamide inhibited both the hyperpolarization and relaxation induced by acetylcholine (Brayden, 1990). Although the hyperpolarization was unaffected by the soluble guanylate cyclase inhibitor methylene blue, the effect of cyclo-oxygenase inhibition on this response was not examined (Brayden, 1990). This raises the possibility that the glibenclamide-sensitive hyperpolarization and relaxation were mediated

by prostacyclin or another prostanoid (Fredricks *et al*., 1994; Murphy & Brayden, 1995; Parkington *et al*., 1995).

The inhibitory action of charybdotoxin and TEA may suggest that EDHF mediates relaxation by activation of BK_{Ca}. However, this assumption is contradicted by the lack of effect of iberiotoxin, a more selective inhibitor of BK_{Ca} than charybdotoxin and TEA (Edwards & Weston, 1991, 1994; Chandy & Gutman, 1995). Furthermore, TEA possesses anti-muscarinic properties (see Cohen & Vanhoutte, 1995), which could account for the effect of TEA in the present study. Since charybdotoxin also inhibits Kv1.2 and Kv1.3 (Chandy & Gutman, 1995), this raises the possibility that charybdotoxin is interacting with K_V in the guinea-pig basilar artery. This is supported by the inhibitory action of ciclazindol, which is known to inhibit K_V in vascular smooth muscle (Noack *et al*., 1992).

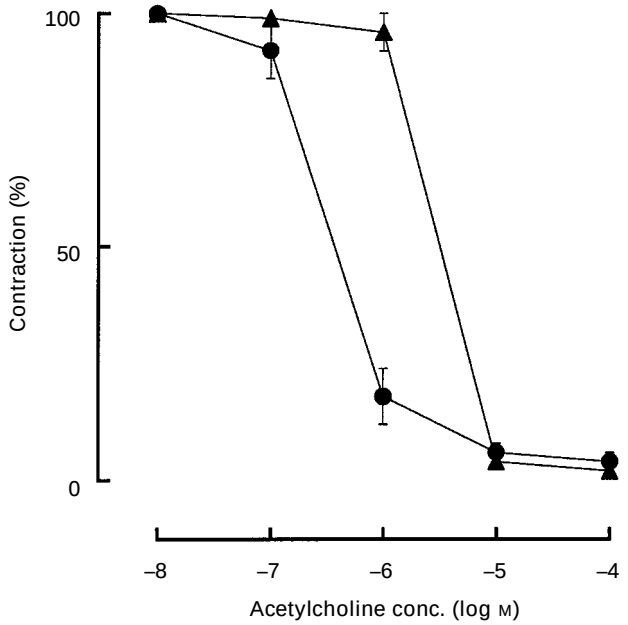

Figure 5 Relaxation induced by acetylcholine in guinea-pig basilar arteries contracted with $PGF_{2\alpha}$ in the presence (▲) or absence (●) of a combination of 4-aminopyridine (1 mM) and apamin (0.1 μM). The level of contraction induced by $PGF_{2\alpha}$ before addition of acetylcholine was set to 100%. L-NOARG (0.3 mM) and indomethacin (10 μM) were present throughout. Data are presented as means ($n=5$) and vertical lines show s.e.mean.

It has been shown previously that a combination of synthetic charybdotoxin and apamin abolishes the EDHF-mediated relaxation induced by either acetylcholine or A23187 in the rat hepatic artery, whereas each toxin had no effect when given alone (Zygmunt & Högestätt, 1996). Similarly, apamin and charybdotoxin had to be given together in order to abolish the EDHF response in the guinea-pig basilar artery. In a study on human pial arteries, we found evidence for the release of EDHF when the endothelium was stimulated with substance P (Petersson *et al*., 1995). The present study shows that the EDHF-induced relaxation in human pial arteries is also abolished by charybdotoxin plus apamin, whereas each toxin had little effect when given alone. Furthermore, the toxin combination abolished EDHF-mediated relaxation in rat isolated small mesenteric arteries (unpublished results), an observation also made by Waldron & Garland (1994).

In the guinea-pig basilar artery, the EDHF-mediated relaxation was completely inhibited when apamin was combined with ciclazindol (instead of charybdotoxin), which may indicate that ciclazindol and charybdotoxin were acting on the same K-channel in this vessel. Although the K_V inhibitors 4-AP and dendrotoxin-I (the latter has been shown to inhibit neuronal K_V; Garcia *et al*., 1991), did not antagonize the EDHF-mediated relaxation by themselves, the combination of 4-AP and apamin did cause a significant inhibition of the EDHF-induced relaxation. Thus, the effect of 4-AP when combined with apamin provides further evidence for the involvement of K_V in the EDHF-mediated relaxation. The combination of iberiotoxin and apamin was without any inhibitory effect, again indicating that BK_{Ca} is not of importance for the EDHF-mediated response in cerebral arteries.

The fact that a combination of two different K-channel inhibitors was necessary to abolish the EDHF response could indicate that EDHF mediates relaxation by activation of two

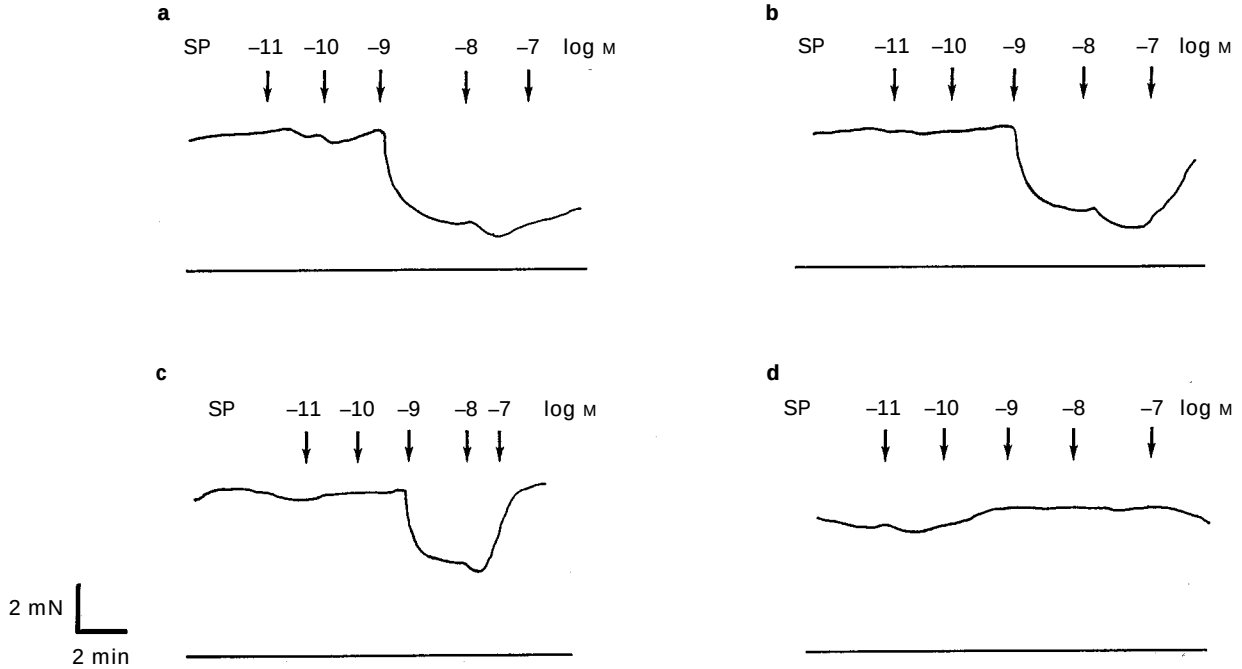

Figure 6 Traces showing effects of K-channel inhibitors on the relaxation induced by substance P (SP) in four separate ring preparations of a human pial artery contracted with U46619. The arterial segments were either untreated (a), or pretreated with charybdotoxin (0.1 μM; b), apamin (0.1 μM; c) or a combination of charybdotoxin (0.1 μM) and apamin (0.1 μM; d). L-NOARG (0.3 mM) and indomethacin (10 μM) were present throughout.

distinct types of K-channel, one sensitive to apamin and the other to charybdotoxin or ciclazindol. Provided that these K-channels are differently distributed in the vascular system, this concept could explain the apparently conflicting findings that apamin inhibits EDHF-mediated relaxation in some blood vessels, while charybdotoxin is effective in other vessels (see Introduction). A chemical interaction between apamin and charybdotoxin seems unlikely, since the EDHF-mediated relaxation was also inhibited when apamin was combined with the structurally different K-channel inhibitors ciclazindol and 4-AP.

Although attractive, the two channel hypothesis cannot account for the lack of effect of apamin and charybdotoxin on the A23187-induced relaxation when each toxin was given alone, unless these channels can fully compensate for each other. However, these findings would be consistent with the view that the target for EDHF is a single type of K-channel, which is inhibited by apamin and charybdotoxin (or ciclazindol) in a synergistic manner. The binding site for charybdotoxin has been located to the external vestibule of K_V and BK_{Ca} (Garcia *et al*., 1993; Miller, 1995). However, the molecular site of action of apamin is unclear. An apamin binding protein has been isolated and cloned from porcine vascular smooth muscles (Sokol *et al*., 1994), but the structure of this protein is distinct from known K-channel subunits, and it has not been shown whether this protein can function as a K-channel in expression systems. Apamin modulates allosterically charybdotoxin binding in erythrocytes (Brugnara *et al*., 1995), and charybdotoxin inhibits apamin binding in rat brain synaptosomes (Chicchi *et al*., 1988), indicating that these two toxins can interact with common membrane proteins. Recordings of single channel K currents in isolated membrane patches from rat renal arterioles have demonstrated the presence of a K-channel, which is sensitive to both apamin and charybdotoxin (Gebremedhin *et al*., 1996). In view of these findings, it may be speculated that EDHF activates a single type of K-channel, which is structurally related to both SK_{Ca} and K_V, in the guinea-pig basilar artery, and that apamin may facilitate binding of charybdotoxin and ciclazindol to this channel by an allosteric mechanism.

In contrast to the present study, apamin inhibited the NO-independent hyperpolarization and relaxation in rabbit mesenteric and bovine oviductal arteries, and porcine and bovine coronary arteries (Hecker *et al*., 1994; García-Pascual *et al*., 1995; Murphy & Brayden, 1995; Parsons *et al*., 1996). Charybdotoxin was inactive in two of these test systems (Hecker *et al*., 1994; García-Pascual *et al*., 1995), whereas the effect of this toxin was not examined in the other studies. It remains to be shown whether EDHF activates a K current with characteristics typical of SK_{Ca} in these preparations. Only one study has so far demonstrated an apamin-sensitive SK_{Ca} current in vascular smooth muscle, but this current was also sensitive to charybdotoxin (Gebremedhin *et al*., 1996).

Interestingly, charybdotoxin when given alone inhibited the acetylcholine-induced relaxation, whereas it had no effect on the non-receptor-coupled A23187-induced relaxation. It seems unlikely that this difference is due to an interaction between charybdotoxin and muscarinic receptors (Zygmunt & Högestätt, 1996). However, the results could indicate that EDHF is not a single factor, but perhaps a family of factors, which are released in different proportions by acetylcholine and A23187, and that one of these factors preferentially activates K-channels sensitive to charybdotoxin. However, it must also be taken into account that charybdotoxin may interact with endothelial K-channels (see below), an effect that would not be expected to have any bearing when a Ca ionophore is used to elicit endothelial stimulation.

Potassium channels sensitive to charybdotoxin or apamin have been demonstrated in the vascular endothelium (Marchenko & Sage, 1996), and charybdotoxin, but not apamin, prevents the hyperpolarization of the endothelium induced by endothelium-dependent vasodilators (Chen & Cheung, 1992; Marchenko & Sage, 1996). Depolarization of the endothelial cell reduces Ca influx as a consequence of a decreased electrical driving force, and this in turn may suppress Ca-dependent formation of endothelium-derived relaxing factors (Suzuki & Chen, 1990; Lückhoff & Busse, 1990; Groschner *et al*., 1992). In a recent study with fura-2 microfluorometry and tension recordings of intact porcine coronary arteries, evidence was provided that the formation of EDHF is indeed regulated by the intracellular Ca^{2+} concentration (Higuchi *et al*., 1996). This raises the question whether the K-channel inhibitors were acting at the level of the endothelium rather than on the smooth muscle cells in the present study. However, several pieces of evidence suggest that these compounds were indeed acting on the smooth muscle cells. First, apamin plus charybdotoxin (or ciclazindol) abolished the L-NOARG/indomethacin-resistant relaxation induced by the Ca ionophore A23187. This mode of activation of the endothelium should be little affected by the membrane potential. Furthermore, charybdotoxin plus apamin only marginally affected the acetylcholine-induced relaxation in the absence of L-NOARG, i.e. when NO was allowed to be co-released with EDHF (unpublished observation). Similarly, the toxin combination had no effect on the component of the acetylcholine-induced relaxation mediated by endothelium-derived NO in the rat hepatic artery (Zygmunt & Högestätt, 1996). This suggests that the Ca-dependent formation of endothelium-derived relaxing factors is preserved in the presence of charybdotoxin plus apamin.

The results suggest that K_V, but not K_{ATP} and BK_{Ca}, is involved in the EDHF-mediated relaxation in the guinea-pig basilar artery. The synergistic action of apamin and charybdotoxin (or ciclazindol) could indicate that both K_V and SK_{Ca} are activated by EDHF. Alternatively, a single type of K-channel, which may be structurally related to K_V and allosterically regulated by apamin, could be the target for EDHF.

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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.

BRAYDEN, J.E. (1990). Membrane hyperpolarization is a mechanism of endothelium-dependent cerebral vasodilation. *Am. J. Physiol*., **259,** H668–H673.

BRUGNARA, C., ARMSBY, C.C., DE FRANCESCHI, L., CREST, M., MARTIN EUCLAIRE, M.-F. & ALPER, S.L. (1995). Ca^{2+}-activated K^+ channels of human and rabbit erythrocytes display distinctive patterns of inhibition by venom peptide toxins. *J. Membr. Biol*., **147,** 71–82.

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. & CHEUNG, D.W. (1992). Characterization of acetylcholine-induced membrane hyperpolarization in endothelial cells. *Circ. Res*., **70,** 257–263.

CHICCHI, G.G., GIMENEZ-GALLEGO, G., BER, E., GARCIA, M.L., WINQUIST, R. & CASCIERI, M.A. (1988). Purification and characterization of a unique, potent inhibitor of apamin binding from leiurus quinquestriatus hebraeus venom. *J. Biol. Chem*., **263,** 10192–10197.

COHEN, R.A. & VANHOUTTE, P.M. (1995). Endothelium-dependent hyperpolarization: beyond NO and cGMP. *Circulation*, **92,** 3337 – 3349.

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.

EDWARDS, G. & WESTON, A.H. (1991). Potassium channel modulators. In *Receptor Data for Biological Experiments: a Guide to Drug Selectivity*. ed. Doods, H.N. & Van Meel, J.C.A. pp. 194 – 208. Chichester: Ellis Horwood Limited.

EDWARDS, G. & WESTON, A.H. (1994). Effect of potassium channel modulating drugs on isolated smooth muscle. In *Handbook of Experimental Pharmacology*. ed. Szekeres & Papp. pp. 469 – 531. Heidelberg: Springer Verlag.

FREDRICKS, K.T., LIU, Y., RUSCH, N.J. & LOMBARD, J.H. (1994). Role of endothelium and arterial K^+ channels in mediating hypoxic dilation of middle cerebral arteries. *Am. J. Physiol.*, **267,** H580 – H586.

GARCIA, M.L., GALVAREZ, A., GARCIA-CALVO, M., KING, V.F., VAZQUEZ, J. & KACZOROWSKI, G.J. (1991). Use of toxins to study potassium channels. *J. Bioenerg. Biomemb.*, **23,** 615 – 646.

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.

GEBREMEDHIN, D., KALDUNSKI, M., JACOBS, E.R., HARDER, D.R. & ROMAN, R.J. (1996). Coexistence of two types of Ca^{2+}-activated K^+ channels in rat renal arterioles. *Am. J. Physiol.*, **270,** F69 – F81.

GROSCHNER, K., GRAIER, W.F. & KUKOVETZ, W.R. (1992). Activation of a small-conductance Ca^{2+}-dependent K^+ channel contributes to bradykinin-induced stimulation of nitric oxide synthesis in pig aortic endothelial cells. *Biochim. Biophys. Acta*, **1137,** 162 – 170.

HATAKE, K., WAKABAYASHI, I., KAKISHITA, E. & HISHIDA, S. (1992). Impairment of endothelium-dependent relaxation in human basilar artery after subarachnoid hemorrhage. *Stroke*, **23,** 1111 – 1117.

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

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.

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

LÜCKHOFF, A. & BUSSE, R. (1990). Calcium influx into endothelial cells and formation of endothelium-derived relaxing factor is controlled by the membrane potential. *Pflügers Archiv.*, **416,** 305 – 311.

MARCHENKO, S.M. & SAGE, S.O. (1996). Calcium-activated potassium channels in the endothelium of intact rat aorta. *J. Physiol.*, **492,** 53 – 60.

MAYHAN, W.G., AMUNDSEN, S.M., FARACI, F.M. & HEISTAD, D.D. (1988). Responses of cerebral arteries after ischemia and reperfusion in cats. *Am. J. Physiol.*, **255,** H879 – H884.

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.

NAKAGOMI, T., KASSELL, N.F., SASAKI, T., FUJIWARA, S., LEHMANN, R.M. & TORNER, J.C. (1987). Impairment of endothelium-dependent vasodilation induced by acetylcholine and adenosine triphosphate following experimental subarachnoid hemorrhage. *Stroke*, **18,** 482 – 489.

NISHIYE, E., NAKAO, K., ITOH, T. & KURIYAMA, H. (1989). Factors inducing endothelium-dependent relaxation in the guinea-pig basilar artery as estimated from the actions of haemoglobin. *Br. J. Pharmacol.*, **96,** 645 – 655.

NOACK, T., 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.

PARKINGTON, H.C., TONTA, M.A., COLEMAN, H.A. & TARE, M. (1995). Role of membrane potential in endothelium-dependent relaxation of guinea-pig coronary arterial smooth muscle. *J. Physiol.*, **484,** 469 – 480.

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., ANDERSSON, K.-E., BRANDT., L. & HÖGESTÄTT, E.D. (1996c). Modulation by the endothelium of the inhibitory effects of pinacidil and nimodipine on endothelin-induced contraction in cerebral arteries. *Pharmacol. Toxicol.*, **79**, (in press).

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., BRANDT, L. & HÖGESTÄTT, E.D. (1996b). Role of hyperpolarization in endothelium-dependent relaxations of human cerebral arteries. In *Endothelium-Derived Hyperpolarizing Factor*. ed. Vanhoutte, P.M. pp. 287 – 292. The Netherlands: Harwood Academic Publisher.

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

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. Prot. Chem.*, **13,** 117 – 128.

STANDEN, N.B., QUAYLE, J.M., DAVIES, N.W., BRAYDEN, J.E., HUANG, Y. & NELSON, M.T. (1989). Hyperpolarizing vasodilators activate ATP-sensitive K^+ channels in arterial smooth muscle. *Science*, **245,** 177 – 180.

SUZUKI, H. & CHEN, G. (1990). Endothelium-derived hyperpolarizing factor (EDHF): an endogenous potassium channel activator. *News Physiol. Sci.*, **5,** 212 – 215.

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.

ZHANG, H. & COOK, D. (1994). Cerebral vascular smooth muscle potassium channels and their possible role in the management of vasospasm. *Pharmacol. Toxicol.*, **75,** 327 – 336.

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. (1994). The endothelium mediates a nitric oxide-independent hyperpolarization and relaxation in the rat hepatic artery. *Acta Physiol. Scand.*, **152,** 375 – 384.

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