British Journal of Pharmacology (2000) 129, 1490–1496

www.nature.com/bjp

Effects of inhibitors of small- and intermediate-conductance calcium-activated potassium channels, inwardly-rectifying potassium channels and Na^+/K^+ ATPase on EDHF relaxations in the rat hepatic artery

[1]David A. Andersson, *,[1]Peter M. Zygmunt, [1]Pouya Movahed, [1]Tomas L.G. Andersson & [1]Edward D. Högestätt

[1]Department of Clinical Pharmacology, Institute of Laboratory Medicine, Lund University, SE-221 85 Lund, Sweden

1 In the rat hepatic artery, the SK_{Ca} inhibitors UCL 1684 (300 nM) completely blocked, and scyllatoxin (1 μM) and d-tubocurarine (100 μM) partially inhibited EDHF relaxations when each of them was combined with charybdotoxin (300 nM).

2 The IK_{Ca} inhibitors clotrimazole (3 μM) and 2-chlorophenyl-bisphenyl-methanol (3 μM) strongly depressed EDHF relaxations when each of them was combined with apamin (300 nM). The cytochrome P450 mono-oxygenase inhibitor ketoconazole (10 μM) had no effect in the presence of apamin.

3 Ciclazindol (10 μM), which abolishes EDHF relaxations in the presence of apamin, almost completely prevented the calcium ionophore (A23187) stimulated $^{86}Rb^+$ influx *via* the Gardos channel (IK_{Ca}) in human erythrocytes.

4 The Na^+/K^+ ATPase inhibitor ouabain (500 μM) and the K_{IR} blocker Ba^{2+} (30 μM) neither alone nor in combination inhibited EDHF relaxations. Ba^{2+} was also without effect in the presence of either apamin or charybdotoxin.

5 In contrast to EDHF, an increase in extracellular $[K^+]$ from 4.6 mM to 9.6, 14.6 and 19.6 mM inconsistently relaxed arteries. In K^+-free physiological salt solution, re-admission of K^+ always caused complete and sustained relaxations which were abolished by ouabain but unaffected by Ba^{2+}.

6 The present study provides pharmacological evidence for the involvement of SK_{Ca} and IK_{Ca} in the action of EDHF in the rat hepatic artery. Our results are not consistent with the idea that EDHF is K^+ activating Na^+/K^+ ATPase and K_{IR} in this blood vessel.

British Journal of Pharmacology (2000) **129,** 1490–1496

Keywords: Blood vessels; endothelium-derived relaxing factor; endothelium vascular; potassium channels

Abbreviations: BK_{Ca}, large-conductance calcium-activated potassium channel; EDHF, endothelium-derived hyperpolarizing factor; IK_{Ca}, intermediate-conductance calcium-activated potassium channel; K^+, potassium; K_{IR}, inwardly-rectifying potassium channel; K_V, voltage-gated potassium channel; SK_{Ca}, small-conductance calcium-activated potassium channel

Introduction

A combination of the potassium (K^+) channel inhibitors apamin and charybdotoxin abolishes EDHF responses in many blood vessels (see Högestätt *et al*., 1999). This finding was first described independently by us in the rat hepatic artery (Zygmunt, 1995; Zygmunt & Högestätt, 1996) and by Waldron & Garland (1994) using the rat mesenteric artery. The toxin combination also completely prevents the EDHF hyperpolarization in the guinea-pig carotid and rat hepatic and mesenteric arteries (Chataigneau *et al*., 1998b; Corriu *et al*., 1996; Zygmunt *et al*., 1998), indicating that membrane hyperpolarization *via* opening of K^+ channels is crucial for EDHF relaxations in these arteries.

Apamin inhibits some but not all small-conductance calcium-activated K^+ channels (SK_{Ca}) (Köhler *et al*., 1996; Ishii *et al*., 1997a). Although apamin is considered as a specific inhibitor of SK_{Ca}, it has been shown to interact with charybdotoxin binding to voltage-gated K^+ channels (K_V) (Zygmunt *et al*., 1997a). Therefore, it would be of interest to know whether other inhibitors of SK_{Ca} can mimic the inhibitory effect of apamin on EDHF. Charybdotoxin is acting on various K^+ channels such as intermediate- and large-conductance calcium-activated K^+ channels (IK_{Ca} and BK_{Ca}, respectively) and K_V (Chandy & Gutman, 1995; Garcia *et al*., 1995; Ishii *et al*., 1997b). However, our previous findings do not support an involvement of BK_{Ca} and K_V in EDHF responses (Zygmunt *et al*., 1997a; Zygmunt & Högestätt, 1996).

Despite intense research the identity of EDHF has not been unequivocally identified (see Edwards & Weston, 1998). In the rat hepatic artery, carbon monoxide, cytochrome P450 mono-oxygenase metabolites (such as epoxyeicosatrienoic acids) and the endocannabinoid anandamide have been considered as EDHF (Zygmunt *et al*., 1994a; 1996; 1997b). The possibility that myoendothelial gap junctions mediate EDHF responses in this artery has also been examined (Zygmunt & Högestätt, 1996). The most recent proposal regarding the identity of EDHF was

*Author for correspondence; E-mail: Peter.Zygmunt@klinfarm.lu.se

provided by Edwards *et al.* (1998), who suggested that EDHF may simply be K^+ in the rat hepatic and mesenteric artery. According to their hypothesis, endothelial cell activation leads to calcium accumulation and subsequent opening of calcium-activated K^+ channels sensitive to apamin and charybdotoxin, allowing K^+ to efflux from the endothelium into the myoendothelial space. K^+ in turn causes hyperpolarization and relaxation of the smooth muscle cells *via* activation of Na^+/K^+ ATPase and inwardly-rectifying K^+ channels (K_{IR}).

The present study aimed to identify SK_{Ca} and IK_{Ca} as the K^+ channels involved in EDHF relaxation in the rat hepatic artery by using structurally different inhibitors of such K^+ channels. The actions of EDHF and K^+ with regard to Na^+/K^+ ATPase and K_{IR} were also compared. Some of these results have been presented to the British Pharmacological Society (Andersson *et al.*, 1998).

Methods

Tension studies

Female Sprague-Dawley rats (250–300 g) were killed by CO_2 asphyxia followed by exsanguination. The hepatic artery was removed and divided into ring segments, 1–2 mm long, which were suspended between two metal pins in organ baths (2.5 ml), containing warmed (37°C) physiological salt solution 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 physiological salt solution was continuously bubbled with a mixture of 95% O_2 and 5% CO_2, 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 by a force-displacement transducer (Grass Instruments FT03C, U.S.A.), connected to a polygraph (see Högestätt *et al.*, 1983). In order to assess the contractile capacity, each segment was contracted by an isosmolar 60 mM KCl solution (prepared as the physiological salt solution except for an equimolar substitution of NaCl with KCl).

Relaxations induced by acetylcholine and KCl were studied in vessels contracted by phenylephrine, the concentration of which was titrated for each vascular segment to give a contraction amounting to 70–90% of the initial response to 60 mM KCl (Zygmunt *et al.*, 1994b). When stable contractions were obtained, acetylcholine or KCl was added cumulatively to determine concentration-response relationships. Unless otherwise stated all experiments were carried out after incubation with indomethacin (10 μM) and N^{ω}-nitro-L-arginine (300 μM) for at least 40 min. The incubation time with ouabain, $BaCl_2$, ketoconazole, clotrimazole, 2-chlorophenyl-bisphenyl-methanol, charybdotoxin, apamin, UCL 1684, scyllatoxin and d-tubocurarine was 30 min. Each vessel segment was exposed to only one treatment. The EDHF relaxation induced by acetylcholine in the rat hepatic artery consists of two components–one is sensitive to apamin and the other to charybdotoxin (Zygmunt & Högestätt, 1996). Therefore, to study the effect of SK_{Ca} inhibitors on the apamin-sensitive component of the EDHF relaxation experiments were performed in the presence of charybdotoxin. Likewise, the effect of IK_{Ca} inhibitors on the charybdotoxin-sensitive component of the EDHF relaxation was studied in the presence of apamin.

$^{86}Rb^+$ influx

The method for measurement of $^{86}Rb^+$ influx in human erythrocytes as described by Brugnara *et al.* (1993) was slightly modified and used in the present study. Blood was collected in heparinized vacutainer tubes from healthy human donors and centrifuged in a 4K-1 Sigma® centrifuge at room temperature for 10 min at 150 *g*. The platelet rich plasma was removed and the blood was centrifuged once again for 10 min at $3000 \times g$, after which the platelet poor plasma and the buffy coat was removed. Erythrocytes were then washed by adding a solution containing (in mM): NaCl 140, KCl 2 and Tris-HCl (pH 7.4) 10 and centrifuged for 10 min at $3000 \times g$. The supernatant was removed and the washing procedure was repeated four times.

Erythrocytes (10^7 cells ml^{-1}) were incubated at room temperature for 90 min in a medium containing (in mM): NaCl 140, KCl 2 and Tris-HCl (pH 8.0) 10. The medium also contained the test drugs as well as ouabain (100 μM) and bumetanide (10 μM) to prevent $^{86}Rb^+$ influx *via* Na^+/K^+ ATPase and the $Na^+/K^+/Cl^-$ co-transporter, respectively (Brugnara *et al.*, 1993). The final volume in each tube was 1 ml. At the end of the incubation, the calcium ionophore A23187 (10 μM), $CaCl_2$ (50 μM) and 37 Kbq ml^{-1} $^{86}Rb^+$ were added for 5 min. Thereafter, 1 ml of 5 mM EGTA in ice cold saline (NaCl 0.9%) was added and the cell suspension was spun at $3000 \times g$ for 10 min. The supernatant was removed and 2 ml of the EGTA solution was added and the samples were re-centrifuged at $3000 \times g$ for 10 min to reduce background activity. The $^{86}Rb^+$ content in the erythrocytes was measured in a 1277 GammaMaster (Wallac®).

Calculations and statistics

Responses to acetylcholine and KCl are expressed as percentage reversal of the phenylephrine-induced contraction. The maximal relaxation induced by each concentration of acetylcholine was recorded and used in subsequent calculations. The negative logarithm ($-\log$) of the concentration eliciting half maximal relaxation (pEC_{50}) was determined by linear regression analysis, using the values immediately above and below half maximal response. E_{max} refers to the maximal relaxation achieved (100% denotes a complete reversal of the phenylephrine-induced contraction). Influx of $^{86}Rb^+$ was expressed as percentage of saline controls. Values are presented as mean ± s.e.mean and *n* indicates the number of vascular segments (animals) or individuals examined. Statistical analysis was performed by using Student's *t*-test (two-tailed) or analysis of variance (ANOVA) followed by Bonferroni Dunn's *post hoc* test (Statview 4.12). Statistical significance was accepted when $P < 0.05$.

Drugs

Acetylcholine chloride (Aldrich, Germany); apamin (Alomone Labs, Israel); $BaCl_2$, clotrimazole, N^{ω}-nitro-L-arginine hydrochloride, ouabain hydrochloride, L-phenylephrine hydrochloride, d-tubocurarine (Sigma, U.S.A.); ketoconazole (RBI, U.S.A.); synthetic charybdotoxin, scyllatoxin (Latoxan, France); indomethacin (Confortid®, Dumex, Denmark); ciclazindol (Pfizer, U.K.); UCL 1684 (Prof Robert Ganellin; Department of Chemistry, University College London, U.K.); 2-chlorophenyl-bisphenyl-methanol (Dr Carlo Brugnara; brugnara@a1.tch.harvard.edu). Clotrimazole, 2-chlorophenyl-bisphenyl-methanol, ketoconazole and ciclazindol were each dissolved in absolute ethanol. Apamin, charybdotoxin

and scyllatoxin were each dissolved in saline. All other drugs were dissolved in distilled water. Stock solutions of the substances were stored at −70°C.

Results

Effects of UCL 1684, scyllatoxin and d-tubocurarine on EDHF relaxations

The SK_{Ca} inhibitors UCL 1684 (300 nM), scyllatoxin (1 μM) and d-tubocurarine (100 μM) could each inhibit EDHF relaxations induced by acetylcholine when combined with 300 nM charybdotoxin (Figure 1). UCL 1684 at a three times higher concentration (1 μM) and d-tubocurarine (100 μM) had no effect on EDHF relaxations when tested in the absence of charybdotoxin (control: $pEC_{50}=7.5\pm0.1$, $E_{max}=93\pm2\%$; UCL 1684: $pEC_{50}=7.3\pm0.2$, $E_{max}=94\pm3\%$; d-tubocurarine: $pEC_{50}=7.4\pm0.1$, $E_{max}=97\pm1\%$; $n=6$).

The acetylcholine-induced relaxation recorded in the absence of N^{ω}-nitro-L-arginine was unaffected by a combination of 300 nM charybdotoxin and either 1 μM UCL 1684 (control: $pEC_{50}=8.2\pm0.2$, $E_{max}=100\pm1\%$; UCL 1684 plus charybdotoxin: $pEC_{50}=7.8\pm0.1$, $E_{max}=92\pm2\%$, $n=6$) or 100 μM d-tubocurarine (control: $pEC_{50}=7.7\pm0.1$, $E_{max}=100\pm1\%$ d-tubocurarine plus charybdotoxin: $pEC_{50}=7.4\pm0.1$, $E_{max}=100\pm1\%$; $n=5$).

Effects of clotrimazole, 2-chlorophenyl-bisphenyl-methanol and ketoconazole on EDHF relaxations

Clotrimazole (1 and 3 μM) and its metabolite 2-chlorophenyl-bisphenyl-methanol (3 μM), both inhibitors of IK_{Ca}, were each able to inhibit EDHF relaxations induced by acetylcholine in the presence of 300 nM apamin (Figure 2). The cytochrome P450 mono-oxygenase inhibitor ketoconazole (10 μM) was without effect in the presence of 300 nM apamin (control: $pEC_{50}=7.3\pm0.1$, $E_{max}=95\pm2\%$; ketoconazole plus apamin: $pEC_{50}=7.1\pm0.2$, $E_{max}=94\pm3\%$; $n=6$). EDHF relaxations were unaffected by clotrimazole (1 μM) in the absence of apamin (control: $pEC_{50}=7.6\pm0.2$, $E_{max}=93\pm2\%$; clotrimazole: $pEC_{50}=7.3\pm0.1$, $E_{max}=91\pm3\%$; $n=5-6$). Clotrimazole alone at a three times higher concentration causes some inhibition of such relaxations (Zygmunt *et al*., 1996).

The acetylcholine-induced relaxation in the absence of N^{ω}-nitro-L-arginine was unaffected by 3 μM clotrimazole plus 300 nM apamin. (control: $pEC_{50}=7.7\pm0.1$, $E_{max}=100\pm1\%$; clotrimazole plus apamin: $pEC_{50}=7.4\pm0.1$, $E_{max}=100\pm1\%$; $n=5$).

$^{86}Rb^{+}$ influx in human erythrocytes

The K^{+} channel inhibitor ciclazindol (10 μM) abolished EDHF relaxations when combined with apamin in the rat hepatic artery (Zygmunt *et al*., 1997a). The possibility that this effect of ciclazindol is due to inhibition of IK_{Ca} was tested using $^{86}Rb^{+}$ influx through the Gardos channel as an indicator of IK_{Ca} activity (Ishii *et al*., 1997b; Dunn, 1998). Ciclazindol (10 μM) was equally effective to prevent $^{86}Rb^{+}$ influx as known inhibitors of IK_{Ca} (charybdotoxin, clotrimazole and 2-

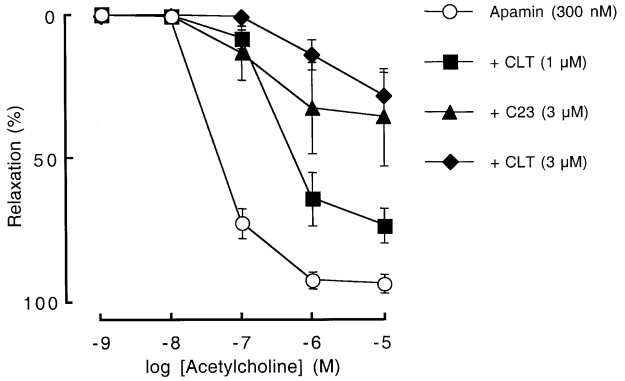

Figure 2 Effect of IK_{Ca} inhibitors on EDHF relaxations induced by acetylcholine in the presence of N^{ω}-nitro-L-arginine (300 μM) and indomethacin (10 μM) in arteries contracted by phenylephrine. The IK_{Ca} blockers clotrimazole (CLT) and 2-chlorophenyl-bisphenyl-methanol (C23) were each combined with apamin and the effect of these combinations on EDHF was compared to that of apamin alone. At this concentration (300 nM), apamin is without any effect on EDHF relaxations. Data are presented as means±s.e.mean of six experiments.

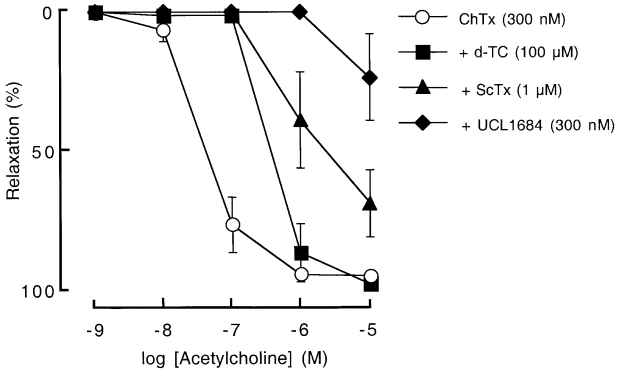

Figure 1 Effects of SK_{Ca} inhibitors on EDHF relaxations induced by acetylcholine in the presence of N^{ω}-nitro-L-arginine (300 μM) and indomethacin (10 μM) in arteries contracted by phenylephrine. The SK_{Ca} blockers UCL 1684 ($n=5$), scyllatoxin (ScTx, $n=6$) and d-tubocurarine (d-TC, $n=6$) were each combined with charybdotoxin (ChTx) and the effect of these combinations on EDHF was compared to that of charybdotoxin alone ($n=10$). At this concentration (300 nM), charybdotoxin is without any effect on EDHF relaxations. Data are presented as means±s.e.mean.

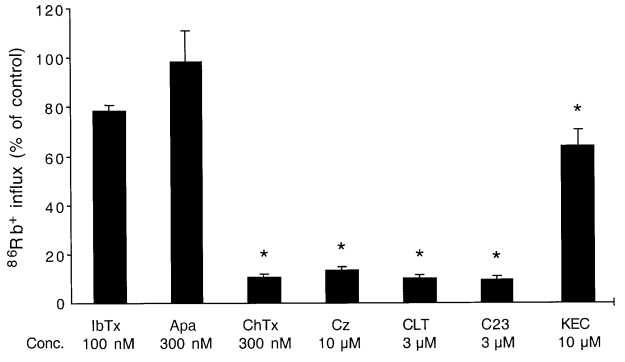

Figure 3 Effects of K^{+} channel inhibitors on calcium ionophore stimulated $^{86}Rb^{+}$ influx in human erythrocytes. The K^{+} channel inhibitors iberiotoxin (IbTx), apamin (Apa), charybdotoxin (ChTx), ciclazindol (Cz), clotrimazole (CLT) and 2-chlorophenyl-bisphenyl-methanol (C23) and the cytochrome P450 mono-oxygenase inhibitor ketoconazole (KEC) were present 85 min before erythrocytes were stimulated for 5 min by A23187 and Ca^{2+}. Data are expressed as percentage of $^{86}Rb^{+}$ influx in the absence of test drugs (control) and are presented as means±s.e.mean of six experiments (all from different individuals). Asterisks denote a statistically significant difference from control values ($P<0.05$).

chlorophenyl-bisphenyl-methanol), whereas inhibitors of BK_{Ca} (iberiotoxin) and SK_{Ca} (apamin) did not reduce $^{86}Rb^+$ influx (Figure 3). Ketoconazole (10 μM) produced a small but significant reduction (36±7%, $n=6$) of $^{86}Rb^+$ influx (Figure 3).

Effects of ouabain and Ba^{2+} on EDHF relaxations

Neither the Na^+/K^+ ATPase inhibitor ouabain (500 μM) nor the K_{IR} inhibitor Ba^{2+} (30 μM) inhibited EDHF relaxations induced by acetylcholine (Figure 4). The combination of these two inhibitors also did not significantly inhibit such relaxations (control: $pEC_{50}=7.5\pm0.1$, $E_{max}=91\pm4\%$; ouabain plus Ba^{2+}: $pEC_{50}=7.2\pm0.2$, $E_{max}=85\pm7\%$; $n=7-9$; Figure 4). In the presence of either apamin (300 nM) or charybdotoxin (300 nM), Ba^{2+} (30 μM) was also without effect on EDHF relaxations (control: $pEC_{50}=7.4\pm0.1$, $E_{max}=97\pm1\%$; apamin plus Ba^{2+}: $pEC_{50}=7.4\pm0.1$, $E_{max}=95\pm2\%$ and charybdotoxin plus Ba^{2+}: $pEC_{50}=7.4\pm0.1$, $E_{max}=100\pm1\%$; $n=6$).

Effects of ouabain and Ba^{2+} on relaxations induced by K^+

EDHF and K^+ relaxations were compared in 29 arterial segments ($n=7$). Typical EDHF relaxations induced by acetylcholine were obtained in all cases (Figure 5). In contrast, relaxations in response to cumulative additions of 5 mM K^+ (giving K^+ concentrations of 9.6, 14.6 and 19.6 mM) were transient and only observed in 14 of these preparations (Figure 5). However, K^+ could always relax preparations exposed to K^+-free physiological salt solution. These K^+ relaxations were complete and sustained in contrast to those seen in normal physiological salt solution. Treatment with ouabain (500 μM) abolished relaxations induced by re-admission of K^+ whereas Ba^{2+} (30 μM) was without effect (Figure 6).

Discussion

A combination of the K^+ channel inhibitors apamin and charybdotoxin completely prevents the hyperpolarizing and vasodilator action of EDHF in the rat hepatic artery, whereas each toxin alone is without any effect at all (Zygmunt, 1995; Zygmunt & Högestätt, 1996; Zygmunt *et al.*, 1998). The present study provides evidence to suggest that calcium-activated K^+ channels displaying pharmacological characteristics of SK_{Ca} and IK_{Ca} are involved in the EDHF relaxation.

EDHF and SK_{Ca}

Our findings that various inhibitors of SK_{Ca} like UCL 1684, scyllatoxin and d-tubocurarine inhibit EDHF relaxations when combined with charybdotoxin supports the idea that SK_{Ca} is one of the K^+ channels involved in EDHF responses. A concentration of 300 nM apamin is necessary to achieve a complete inhibition of the EDHF relaxation in the presence of charybdotoxin (Zygmunt & Högestätt, 1996). At the same concentration, UCL 1684 was as effective as apamin to inhibit such relaxations. Like apamin, UCL 1684 alone at a higher

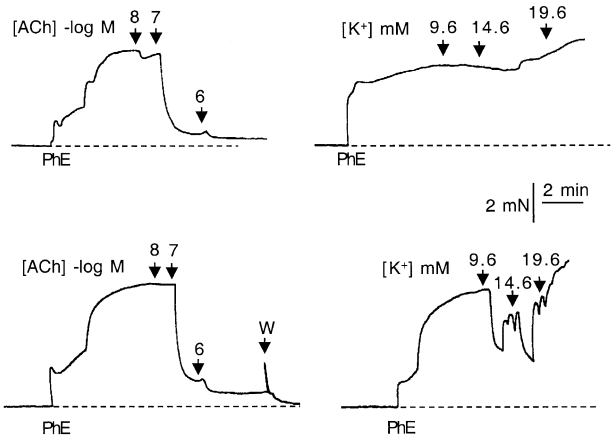

Figure 5 Traces showing differences between K^+ and EDHF relaxations in hepatic arteries contracted by phenylephrine (PhE) in the presence of N^{ω}-nitro-L-arginine (300 μM) and indomethacin (10 μM). Arterial segments were first exposed to acetylcholine (ACh) to demonstrate EDHF relaxations in a physiological salt solution with a normal K^+ content (4.6 mM KCl). After a washout period of 30 min, the relaxant effect of increased extracellular K^+ concentrations (from 4.6 mM to 9.6, 14.6 and 19.6 mM) was recorded to see whether K^+ could mimic EDHF in these arterial segments. Dashed line indicates the basal tension level before addition of phenylephrine. *Upper panel* K^+ failed to relax an arterial segment in which EDHF caused relaxation. *Lower panel* Although K^+ could relax another arterial segment the relaxation was transient and partial in contrast to the EDHF relaxation.

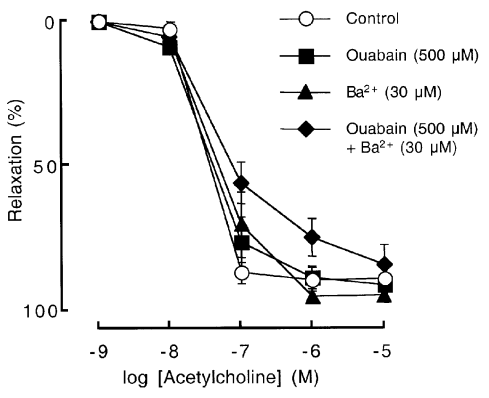

Figure 4 Effects of ouabain and Ba^{2+} on EDHF relaxations induced by acetylcholine in the presence of N^{ω}-nitro-L-arginine (300 μM) and indomethacin (10 μM) in arteries contracted by phenylephrine. The effect of ouabain and Ba^{2+} either alone or combined was examined. Control denotes EDHF relaxations in the absence of these drugs. Data are presented as means±s.e.mean of 7–9 experiments.

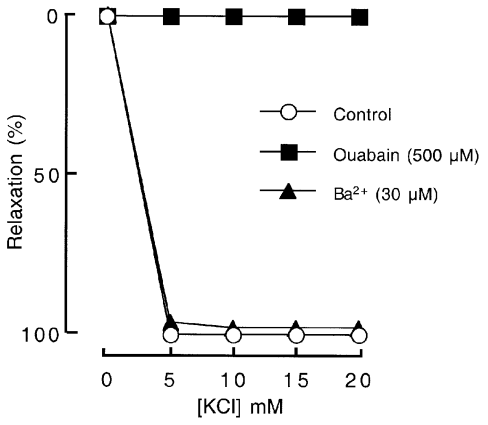

Figure 6 Effects of ouabain and Ba^{2+} on relaxation evoked by K^+ in the presence of N^{ω}-nitro-L-arginine (300 μM) and indomethacin (10 μM) in arteries contracted by phenylephrine. Preparations were incubated with either ouabain or Ba^{2+} or vehicle (control) for 30 min in K^+-free physiological salt solution before re-admission of K^+. Data are presented as means±s.e.mean of 6–9 experiments.

concentration (1 μM) had no effect on EDHF relaxations and when combined with charybdotoxin did not affect the acetylcholine-induced relaxation in the absence of N^{ω}-nitro-L-arginine. This indicates that UCL 1684 does not inhibit the charybdotoxin-sensitive component of the EDHF relaxation or possess anti-muscarinic properties. Furthermore, UCL 1684 did not change the ability of phenylephrine to contract hepatic arteries (unpublished observations). This fits very well with UCL 1684 being a potent and selective non-peptide inhibitor of SK_{Ca} (Campos Rosa *et al*., 1998; Dunn, 1999).

Scyllatoxin is a peptide which inhibits SK_{Ca} activity and interacts with apamin binding to SK_{Ca} (Auguste *et al*., 1990; Chicchi *et al*., 1988; Goh *et al*., 1992). In the present study, scyllatoxin partially inhibited the EDHF relaxation in the presence of charybdotoxin. Scyllatoxin is 5 to 10 times less potent than apamin in inhibiting either the binding of radioactively labelled apamin and scyllatoxin or the apamin-sensitive relaxation induced by epinephrine in guinea-pig taenia coli (Auguste *et al*., 1990; Chicchi *et al*., 1988). Thus, it is perhaps not surprising that scyllatoxin is less effective than apamin in the present study. Scyllatoxin has also been shown to inhibit the EDHF hyperpolarization in the guinea-pig carotid artery when combined with charybdotoxin (Corriu *et al*., 1996).

Apamin-sensitive SK_{Ca} is susceptible to high concentrations of d-tubocurarine (Ishii *et al*., 1997a; Köhler *et al*., 1996; Wadsworth *et al*., 1994). This compound can also inhibit EDHF relaxations when combined with charybdotoxin in the rat hepatic artery. An inhibitory effect of this combination on muscarinic receptors seems unlikely since relaxations induced by acetylcholine in the absence of N^{ω}-nitro-L-arginine were unaffected. d-Tubocurarine alone had no effect on EDHF relaxations suggesting that it acts selectively on the apamin-sensitive component of the relaxation.

EDHF and IK_{Ca}

To examine the possibility that the inhibitory effect of charybdotoxin on EDHF relaxations is due to a blockade of IK_{Ca}, we tested whether clotrimazole and its metabolite 2-chlorophenyl-bisphenyl-methanol could inhibit such relaxations when combined with apamin. These compounds, in contrast to apamin and iberiotoxin, all inhibit native IK_{Ca} (the Gardos channel) in human erythrocytes (Brugnara *et al*., 1995a,b; Dunn, 1998) as confirmed by us in the present study. These inhibitors also block the cloned IK_{Ca} (Ishii *et al*., 1997b; Logsdon *et al*., 1997; Vandorpe *et al*., 1998). Relaxations mediated by EDHF were almost abolished by clotrimazole and 2-chlorophenyl-bisphenyl-methanol in the presence of apamin, implying that IK_{Ca} in addition to SK_{Ca} is involved in the action of EDHF in the rat hepatic artery.

The ability of clotrimazole to also inhibit K_V and K_{ATP} (Edwards *et al*., 1996; Zygmunt *et al*., 1996) does not contribute to its action in the present study because these channels are not involved in the EDHF relaxation in the rat hepatic artery (Zygmunt *et al*., 1994b; 1997a; Zygmunt & Högestätt, 1996). An anti-muscarinic action of clotrimazole cannot explain its inhibitory effect on EDHF (Zygmunt *et al*., 1996). An inhibitory effect of the combination of clotrimazole and apamin on muscarinic receptors also seems unlikely since relaxations induced by acetylcholine in the absence of N^{ω}-nitro-L-arginine are unaffected by this combination of drugs (present study).

In the present study, we show that 2-chlorophenyl-bisphenyl-methanol, which lacks cytochrome P450 mono-oxygenase antagonistic properties and displaces ^{125}I-charybdotoxin binding in human erythrocytes (Brugnara *et al*., 1995b), was as effective as clotrimazole to inhibit the EDHF relaxation. Furthermore, the clotrimazole analogue ketoconazole, which also inhibits cytochrome P450 mono-oxygenase but only slightly affects the cloned IK_{Ca} (Ishii *et al*., 1997b) and the Gardos channel (present study), did not inhibit EDHF relaxations. There is evidence in the literature that clotrimazole binds to IK_{Ca} in a similar way as charybdotoxin, i.e. at the outer face of the channel (Benton *et al*., 1999; Rittenhouse *et al*., 1997b), although additional binding sites for clotrimazole might exist on IK_{Ca} (Dunn, 1998). Together, this suggests that clotrimazole acts directly on IK_{Ca} to inhibit EDHF responses and not *via* inhibition of cytochrome P450 mono-oxygenase. The data also support our previous conclusion that EDHF is not a cytochrome P450 mono-oxygenase metabolite in the rat hepatic artery and that some inhibitors of this enzyme may interfere directly with the K^+ channels involved in the action of EDHF (Zygmunt *et al*., 1996).

It has been reported that EDHF relaxations in the presence of apamin are abolished by ciclazindol in rat hepatic, mesenteric and guinea-pig basilar arteries (Petersson *et al*., 1997; White & Hiley, 1997; Zygmunt *et al*., 1997a). The ability of ciclazindol to inhibit K_V cannot explain this effect (Zygmunt *et al*., 1997a). In the present study, we examined the possibility that ciclazindol is an IK_{Ca} inhibitor by measuring its effect on $^{86}Rb^+$ influx through the Gardos channel in erythrocytes. This experimental model can be used to study drug effects on IK_{Ca} since the Gardos channel in human erythrocytes and the cloned IK_{Ca} display identical electrophysiological and pharmacological characteristics (Brugnara *et al*., 1993; Ishii *et al*., 1997b; Dunn 1998). By using this approach we have identified ciclazindol as a novel inhibitor of IK_{Ca}. This finding adds further evidence that IK_{Ca} is involved in EDHF relaxations in these arteries.

EDHF and BK_{Ca}

We and other groups have excluded that BK_{Ca} is involved in EDHF responses because iberiotoxin has no effect alone or when combined with apamin (Chataigneau *et al*., 1998a; Mieyal *et al*., 1998; Petersson *et al*., 1997; White & Hiley, 1997; Zygmunt & Högestätt, 1996). Inhibitors of IK_{Ca} such as charybdotoxin, clotrimazole, 2-chlorophenyl-bisphenyl-methanol and possibly ciclazindol also inhibit BK_{Ca} (Rittenhouse *et al*., 1997a). Therefore, at present it cannot be excluded that BK_{Ca}, in addition to SK_{Ca} and IK_{Ca}, is involved in EDHF responses.

Localization and function of K^+ channels involved in EDHF responses

It has been assumed that EDHF is a chemical factor activating K^+ channels on the smooth muscle cells (see Edwards & Weston, 1998). In favour of this view are the observations that apamin and charybdotoxin prevent EDHF responses by an action on the smooth muscle cells (Bolz *et al*., 1999; Yamanaka *et al*., 1998). Another option is that apamin and charybdotoxin inhibit EDHF by blocking K^+ channels located on the endothelium thereby interfering with the generation/release of EDHF (Doughty *et al*., 1999; Edwards *et al*., 1998). A reduced K^+ efflux from the endothelium by apamin and charybdotoxin could influence the Ca^{2+}-dependent generation of EDHF (Higuchi *et al*., 1996), e.g., by reducing the electrical driving force for Ca^{2+} influx into these cells (Lückhoff & Busse, 1990). However, apamin and charybdotoxin did not reduce the intracellular free calcium concentration in endothelial cells,

but abolished the associated EDHF relaxations in hamster skeletal resistance arteries and guinea-pig coronary arteries (Bolz *et al*., 1999; Yamanaka *et al*., 1998). Furthermore, in the rat hepatic artery, both nitric oxide and cyclo-oxygenase metabolites, the production of which is also Ca^{2+}-dependent, can be released from the endothelium by acetylcholine and the calcium ionophore A23187 in the presence of apamin and charybdotoxin (Zygmunt *et al*., 1998). It has been suggested that EDHF is a hyperpolarization generated in the endothelium and transmitted to smooth muscle cells *via* gap junctions (see Edwards & Weston, 1998). In the rat hepatic artery, the gap junction inhibitor heptanol does not prevent the EDHF relaxation (Zygmunt & Högestätt, 1996). Preliminary experiments also show that 18α-glycyrrhetinic acid (100 μM), an inhibitor of gap junctions (Taylor *et al*., 1998), does not affect EDHF relaxations in this artery. Therefore, it seems unlikely that the inhibitory action of apamin and charybdotoxin is by preventing propagation of current or chemical mediators from the endothelium to the smooth muscle cells *via* gap junctions in the rat hepatic artery.

Clearly, the distribution of SK_{Ca} and IK_{Ca} in the vascular wall and their precise role in EDHF responses remain to be established. Regardless of the location and function of these K^+ channels, they are crucial for the occurrence of EDHF responses in many blood vessels.

Is K^+ EDHF?

The findings of the present study do not favour the proposal by Edwards *et al*. (1998) that K^+, acting on Na^+/K^+ ATPase and K_{IR} on smooth muscle cells, is EDHF in the rat hepatic artery. Firstly, K^+ either fails or only evokes transient and partial relaxations in vascular preparations where EDHF relaxations are complete and sustained. Secondly, relaxations induced by K^+ re-admission are abolished by ouabain in contrast to EDHF responses, which are unaffected, confirming previous findings in the rat hepatic artery (Zygmunt & Högestätt, 1996). Thirdly, neither K^+ nor EDHF relaxations are sensitive to Ba^{2+} at a concentration which completely blocks K_{IR} in this preparation (Edwards *et al*., 1998). The possibility that K^+ is co-released with another EDHF, which activates K_{IR}, also seems unlikely since ouabain plus Ba^{2+} does not inhibit EDHF relaxations. The present study also shows that Ba^{2+} in combination with either apamin or charybdotoxin is without any effect on EDHF relaxations indicating that the K^+ channels on which apamin and charybdotoxin act are distinct from K_{IR}.

Two recent studies have concluded that K^+ is not EDHF in guinea-pig carotid, porcine coronary and rat mesenteric arteries, all of which exhibit typical EDHF responses (Quignard *et al*., 1999; Vanheel & Van de Voorde, 1999). Notably, the present study and that by Vanheel & Van de Voorde (1999) used the rat hepatic and mesenteric artery, respectively, preparations in which K^+ has been suggested to be EDHF (Edwards *et al*., 1998).

Conclusion

It is concluded that SK_{Ca} and IK_{Ca} are involved in EDHF responses. The distribution of these channels (endothelial and/or smooth muscle cells) and their function in EDHF responses still have to be established. We find no evidence to support the hypothesis that EDHF is K^+ acting on Na^+/K^+ ATPase and K_{IR} in the rat hepatic artery. Thus, there is no consensus regarding the identity of EDHF in this artery.

This work was supported by grants from the Swedish Medical Research Council (no. 11582) and the Medical Faculty of Lund. P.M. Zygmunt was supported by Swedish Medical Research Council (no. 13024 and no. 13275). We are grateful for the gifts of UCL 1684 from Prof Robin Ganellin and Dr Phil Dunn and 2-chlorophenyl-bisphenyl-methanol from Dr Carlo Brugnara.

References

ANDERSSON, D.A., ZYGMUNT, P.M. & HÖGESTÄTT, E.D. (1998). Pharmacological evidence for the involvement of IK_{Ca} and SK_{Ca} in EDHF-mediated relaxation of the rat hepatic artery. *Br. J. Pharmacol.,* **125,** 9P.

AUGUSTE, P., HUGUES, M., GRAVÉ, B., GESQUIÈRE, J.-C., MAES, P., TARTAR, A., ROMEY, G., SCHWEITZ, H. & LAZDUNSKI, M. (1990). Leiurotoxin I (Scyllatoxin), a peptide ligand for Ca^{2+}-activated K^+ channels. *J. Biol. Chem.,* **265,** 4753–4759.

BENTON, D.C.H., ROXBURGH, C.J., GANELLIN, C.R., SHINER, M.A.R. & JENKINSON, D.H. (1999). Differences in the actions of some blockers of the calcium-activated potassium permeability in mammalian red cells. *Br. J. Pharmacol.,* **126,** 169–178.

BOLZ, S.-S., DE WIT, C. & POHL, U. (1999). Endothelium-derived hyperpolarizing factor but not NO reduces smooth muscle Ca^{2+} during acetylcholine-induced dilation of microvessels. *Br. J. Pharmacol.,* **128,** 124–134.

BRUGNARA, C., ARMSBY, C.C., DE FRANCESCHI, L., CREST, M., EUCLAIRE, M.-F. & ALPER, S.P. (1995a). 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.

BRUGNARA, C., ARMSBY, C.C., SAKAMOTO, M., RIFAI, N., ALPER, S.L. & PLATT, O. (1995b). Oral administration of clotramizole and blockade of human erythrocyte $Ca^{(++)}$-activated K^+ channel: the imidazole ring is not required for inhibitory activity. *J. Pharmacol. Exp. Ther.,* **273,** 266–272.

BRUGNARA, C., DE FRANCESCHI, L. & ALPER, S.L. (1993). Ca^{2+}-activated K^+ transport in erythrocytes. Comparison of binding and transport inhibition by scorpion toxins. *J. Biol. Chem.,* **268,** 8760–8768.

CAMPOS ROSA, J., GALANAKIS, D., GANELLIN, C.R., DUNN, P.M. & JENKINSON, D.H. (1998). Bis-quinolinium cyclophanes: 6, 10-diaza-3 (1,3),8 (1,4)-dibenzena-1,5 (1,4)-diquinolinacyclodecaphane (UCL 1684), the first nanomolar, non-peptide blocker of the apamin-sensitive (Ca(2+)-activated K+ channel. *J. Med. Chem.,* **41,** 2–5.

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

CHATAIGNEAU, T., FÉLÉTOU, M., DUHAULT, J. & VANHOUTTE, P.M. (1998a). Epoxyeicosatrienoic acids, potassium channel blockers and endothelium-dependent hyperpolarization in the guinea-pig carotid artery. *Br. J. Pharmacol.,* **123,** 574–580.

CHATAIGNEAU, T., FÉLÉTOU, M., THOLLON, C., VILLENEUVE, N., VILAINE, J.-P., DUHAULT, J. & VANHOUTTE, P.M. (1998b). Cannabinoid CB1 receptor and endothelium-dependent hyperpolarization in guinea-pig carotid, rat mesenteric and porcine coronary arteries. *Br. J. Pharmacol.,* **123,** 968–974.

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.

CORRIU, C., FÉLÉTOU, 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.

DOUGHTY, J.M., PLANE, F. & LANGTON, P.D. (1999). Charybdotoxin and apamin block EDHF in rat mesenteric artery if selectively applied to the endothelium. *Am. J. Physiol.,* **276,** H1107–H1112.

DUNN, P.M. (1998). The action of blocking agents applied to the inner face of Ca^{2+}-activated K^+ channels from human erythrocytes. *J. Membr. Biol.,* **165,** 133–143.

DUNN, P.M. (1999). UCL 1684: a potent blocker of Ca^{2+}-activated K^+ channels in rat adrenal chromaffin cells in culture. *Eur. J. Pharmacol.,* **368,** 119–123.

EDWARDS, G., DORA, K.A., GARDENER, M.J., GARLAND, C.J. & WESTON, A.H. (1998). K^+ is an endothelium-derived hyperpolarizing factor in rat arteries. *Nature,* **396,** 269–272.

EDWARDS, G. & WESTON, A.H. (1998). Endothelium-derived hyperpolarizing factor–a critical appraisal. *Prog. Drug. Res.,* **50,** 107–133.

EDWARDS, G., ZYGMUNT, P.M., HÖGESTÄTT, E.D. & WESTON, A.H. (1996). Effects of cytochrome P450 inhibitors on potassium currents and mechanical activity in rat portal vein. *Br. J. Pharmacol.,* **119,** 691–701.

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.

GOH, J.W., KELLY, M.E., PENNEFATHER, P.S., CHICCHI, G.G., CASCIERI, M.A., GARCIA, M.L. & KACZOROWSKI, G.J. (1992). Effect of charybdotoxin and leiurotoxin I on potassium currents in bullfrog sympathetic ganglion and hippocampal neurons. *Brain Res.,* **591,** 165–170.

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.

HÖGESTÄTT, E.D., PETERSSON, J., ANDERSSON, D.A. & ZYGMUNT, P.M. (1999). Pharmacological characterization of the potassium channels targetted by endothelium-derived hyperpolarizing factor. In: Vanhoutte, P.M. (ed.). *Endothelium-Dependent Hyperpolarizations*. Harwood Academic Publishers: Amsterdam pp. 217–227.

ISHII, T.M., MAYLIE, J. & ADELMAN, J.P. (1997a). Determinants of apamin and d-tubocurarine block in SK potassium channels. *J. Biol. Chem.,* **272,** 23195–23200.

ISHII, T.M., SILVIA, C., HIRSCHBERG, B., BOND, C.T., ADELMAN, J.P. & MAYLIE, J. (1997b). A human intermediate calcium-activated potassium channel. *Proc. Natl. Acad. Sci. U.S.A.,* **94,** 11651–11656.

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.

LOGSDON, N.J., KANG, J., TOGO, J.A., CHRISTIAN, E.P. & AIYAR, J. (1997). A Novel Gene, *hKCa4*, Encodes the Calcium-activated Potassium Channel in Human T Lymphocytes. *J. Biol. Chem.,* **272,** 32723–32726.

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

MIEYAL, P., FULTON, D., MCGIFF, J.C. & QUILLEY, J. (1998). NO-dependent vasodilation to acetylcholine in the rat isolated kidney utilizes a charybdotoxin-sensitive, intermediate-conductance $Ca^{(++)}$-activated K^+ channel. *J. Pharmacol. Exp. Ther.,* **285,** 659–664.

PETERSSON, J., ZYGMUNT, P.M. & HÖGESTÄTT, E.D. (1997). Characterization of the potassium channels involved in EDHF-mediated relaxation in cerebral arteries. *Br. J. Pharmacol.,* **120,** 1344–1350.

QUIGNARD, J.-F., FÉLÉTOU, M., THOLLON, C., VILAINE, J.-P., DUHAULT, J. & VANHOUTTE, P.M. (1999). Potassium ions and endothelium-derived hyperpolarizing factor in guinea-pig carotid and porcine coronary arteries. *Br. J. Pharmacol.,* **127,** 27–34.

RITTENHOUSE, A.R., PARKER, C., BRUGNARA, C., MORGAN, K.G. & ALPER, S.L. (1997a). Inhibition of maxi-K currents in ferret portal vein smooth muscle cells by the antifungal clotrimazole. *Am. J. Physiol.,* **273,** C45–C56.

RITTENHOUSE, A.R., VANDORPE, D.H., BRUGNARA, C. & ALPER, S.L. (1997b). The antifungal imidazole clotrimazole and its major in vivo metabolite are potent blockers of the calcium-activated potassium channel in murine erythroleukemia cells. *J. Membr. Biol.,* **157,** 177–191.

TAYLOR, H.J., CHAYTOR, A.T., EVANS, W.H. & GRIFFITH, T.M. (1998). Inhibition of the gap junctional component of endothelium-dependent relaxations in rabbit iliac artery by 18-α glycyrrhetinic acid. *Br. J. Pharmacol.,* **125,** 1–3.

VANDORPE, D.H., SHMUKLER, B.E., JIANG, L., LIM, B., MAYLIE, J., ADELMAN, J.P., DE FRANCESCHI, L., CAPPELINI, M.D., BRUGNARA, C. & ALPER, S.L. (1998). cDNA cloning and functional characterization of the mouse Ca^{2+}-gated K^+ channel, mIK1. *J. Biol. Chem.,* **273,** 21542–21553.

VANHEEL, B. & VAN DE VOORDE, J. (1999). Barium decreases endothelium-dependent smooth muscle responses to transient but not to more prolonged acetylcholine applications. *Pflügers Arch.,* **439,** 123–129

WADSWORTH, J.D.F., DOORTY, K.B. & STRONG, P.N. (1994). Comparable 30-kDa apamin binding polypeptides may fulfill equivalent roles within putative subtypes of small conductance Ca^{2+}-activated K^+ channels. *J. Biol. Chem.,* **269,** 18053–18061.

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,** 115.

WHITE, R. & HILEY, C.R. (1997). A comparison of EDHF-mediated and anandamide-induced relaxations in the rat isolated mesenteric artery. *Br. J. Pharmacol.,* **122,** 1573–1584.

YAMANAKA, A., ISHIKAWA, T. & GOTO, K. (1998). Characterization of endothelium-dependent relaxation independent of NO and prostaglandins in guinea-pig coronary artery. *J. Pharmacol. Exp. Ther.,* **285,** 480–489.

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

ZYGMUNT, P.M., EDWARDS, G., WESTON, A.H., DAVIS, S.C. & HÖGESTÄTT, E.D. (1996). Effects of cytochrome P450 inhibitors on EDHF-mediated relaxation in the rat hepatic artery. *Br. J. Pharmacol.,* **118,** 1147–1152.

ZYGMUNT, P.M., EDWARDS, G., WESTON, A.H., LARSSON, B. & HÖGESTÄTT, E.D. (1997a). Involvement of voltage-dependent potassium channels in the EDHF-mediated relaxation of rat hepatic artery. *Br. J. Pharmacol.,* **121,** 141–149.

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., HÖGESTÄTT, E.D. & GRUNDEMAR, L. (1994a). Light-dependent effects of zinc protoporphyrin IX on endothelium-dependent relaxation resistant to N^{ω}-nitro-L-arginine. *Acta Physiol. Scand.,* **152,** 137–143.

ZYGMUNT, P.M., HÖGESTÄTT, E.D., WALDECK, K., EDWARDS, G., KIRKUP, A.J. & WESTON, A.H. (1997b). Studies on the effects of anandamide in rat hepatic artery. *Br. J. Pharmacol.,* **122,** 1679–1686.

ZYGMUNT, P.M., PLANE, F., PAULSSON, M., GARLAND, C.J. & HÖGESTÄTT, E.D. (1998). Interactions between endothelium-derived relaxing factors in the rat hepatic artery: focus on regulation of EDHF. *Br. J. Pharmacol.,* **124,** 992–1000.

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 September 29, 1999
Revised January 5, 2000
Accepted January 10, 2000)