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SPECIAL REPORT

Involvement of sensory nerves in vasodilator responses to acetylcholine and potassium ions in rat hepatic artery

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In the presence of ouabain (1 mM), acetylcholine and KCl (5 mM) evoked endothelium-independent relaxations in rat hepatic arteries. Treatment with capsaicin (10 μM), scopolamine (1 μM) or $CGRP_{8-37}$ (3 μM) prevented these relaxations. Acetylcholine-induced relaxations in intact arterial segments in the presence of indomethacin (10 μM) and N^G-nitro-L-arginine (0.3 mM) were only partially inhibited by ouabain plus $BaCl_2$ (30 μM). However, ouabain plus $BaCl_2$ almost abolished such relaxations in capsaicin-pre-treated preparations. In arteries without endothelium, the neurosecretagogue α-latrotoxin (1 nM) induced complete relaxations, which were abolished by $CGRP_{8-37}$ or pre-treatment with capsaicin. α-Latrotoxin also induced a smooth muscle hyperpolarization (12 ± 2 mV), which was abolished by $CGRP_{8-37}$. The ability of ouabain to disclose a CGRP-mediated neurogenic relaxation must be considered when this agent is used as a pharmacological tool. The results further suggest that CGRP is a nerve-derived hyperpolarizing factor in the rat hepatic artery.
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Abbreviations: ACh, acetylcholine; CGRP, calcitonin gene-related peptide; EDHF, endothelium-derived hyperpolarizing factor; NO, nitric oxide; PhE, phenylephrine; PS, physiological salt solution

Introduction In 1996, we reported that the nitric oxide (NO) and cyclo-oxygenase-independent relaxation induced by acetylcholine is little affected by the Na^+, K^+-ATPase inhibitor ouabain in the rat isolated hepatic artery, while the relaxation induced by re-admission of potassium (4.6 mM) to arterial segments incubated in potassium-free solution was completely abolished by this inhibitor (Zygmunt & Högestätt, 1996). These findings made us conclude that endothelium-derived hyperpolarizing factor (EDHF), which mediates the acetylcholine-induced relaxation in the presence of indomethacin and N^G-nitro-L-arginine in this artery (Zygmunt *et al*., 1994b), does not act via stimulation of the electrogenic Na^+, K^+-pump.

Edwards *et al*. (1998) recently proposed that potassium ions released from the endothelium mediate the acetylcholine-induced hyperpolarization and relaxation in the presence of indomethacin and N^G-nitro-L-arginine in rat hepatic and mesenteric arteries via activation of Na^+, K^+-ATPase and barium-sensitive inward rectifier potassium channels on the smooth muscle cells. An important argument in support of this hypothesis was the ability of exogenous potassium ions (KCl) to mimic the vascular effects of EDHF (Edwards *et al*., 1998). However, our earlier (see above) and several recent studies on a variety of blood vessels, exhibiting typical EDHF-mediated responses to acetylcholine, indicate clear differences in the actions of EDHF and potassium ions (Quignard *et al*., 1999; Vanheel & Van de Voorde, 1999; Zygmunt & Högestätt, 1996).

In preliminary experiments attempted to resolve this apparent discrepancy, we discovered that acetylcholine could evoke an endothelium-independent relaxation in the presence of ouabain and $BaCl_2$. In the present study, we have examined the mechanism behind this endothelium-independent vascular response in the rat isolated hepatic artery. The vasodilator effects of potassium ions were also compared with those of acetylcholine to further elaborate the possibility that EDHF is potassium ions in this artery.

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Methods *Experimental procedure* Female Wistar-Hannover rats (250–300 g) were killed by CO_2 asphyxia followed by exsanguination. The hepatic artery was divided into ring segments (1–2 mm long) and mounted 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 PSS was continuously bubbled with a mixture of 95% O_2 and 5% CO_2 at 37°C, resulting in a pH of 7.4 (see Zygmunt *et al*., 1994a).

Relaxations induced by acetylcholine, KCl and α-latrotoxin were studied in vessels contacted by phenylephrine (1–3 μM). The concentration of phenylephrine was titrated for each vascular segment to give a contraction equivalent to 70–90% of the maximal response (Zygmunt *et al*, 1994a). The endothelium was removed by flushing carbogen through the vessel lumen (Zygmunt *et al*., 1994a). The incubation time with indomethacin, N^G-nitro-L-arginine, $CGRP_{8-37}$, scopolamine, ouabain and $BaCl_2$ was approximately 30 min. Each vessel segment was exposed to only one treatment. Some preparations were pre-treated with capsaicin for 60 min, followed by extensive wash-out of the substance.

Electrophysiology Recording of smooth muscle membrane potential in ring segments of the rat hepatic artery was made at 37°C as described (Zygmunt *et al*., 1994b). Briefly, glass microelectrodes filled with 0.5 M KCl and of a tip resistance between 80–150 MΩ were advanced from the adventitial side of the artery at resting tension.

Calculations and statistics Relaxations are expressed as a percentage reversal of the phenylephrine-induced contraction (100% denotes a complete relaxation). The maximal relaxation (E_{max}) and the drug concentration eliciting half maximal

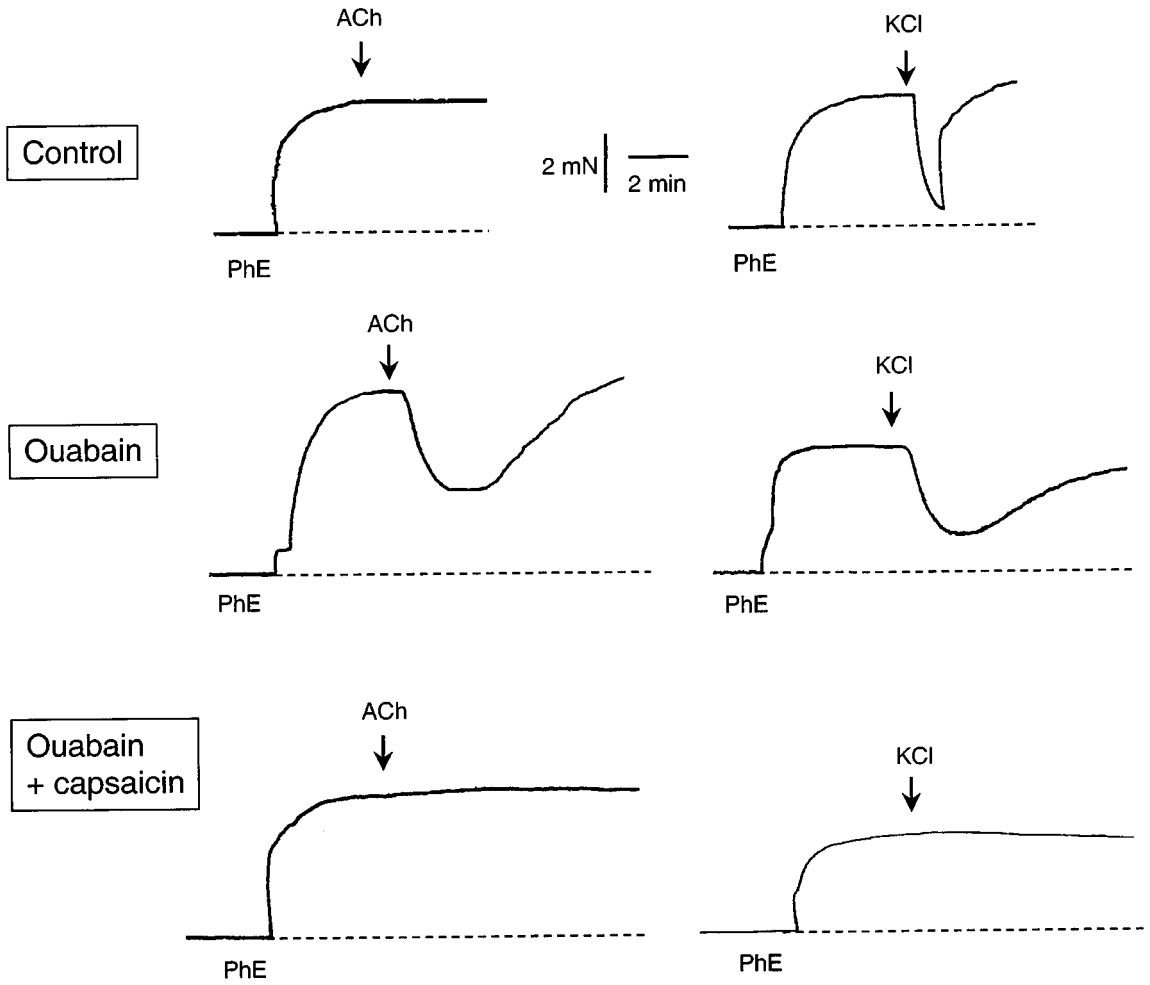

Figure 1 Vasodilator responses to acetylcholine (ACh, 10 μM) and KCl (5 mM) in arterial segments without endothelium. Traces show responses in the absence (control) and presence of ouabain (1 mM) with or without pre-treatment with capsaicin (10 μM). Indomethacin (10 μM) and N^G-nitro-L-arginine (0.3 mM) were present in all experiments. Broken lines indicate the baseline tension before addition of the vasoconstrictor phenylephrine (PhE).

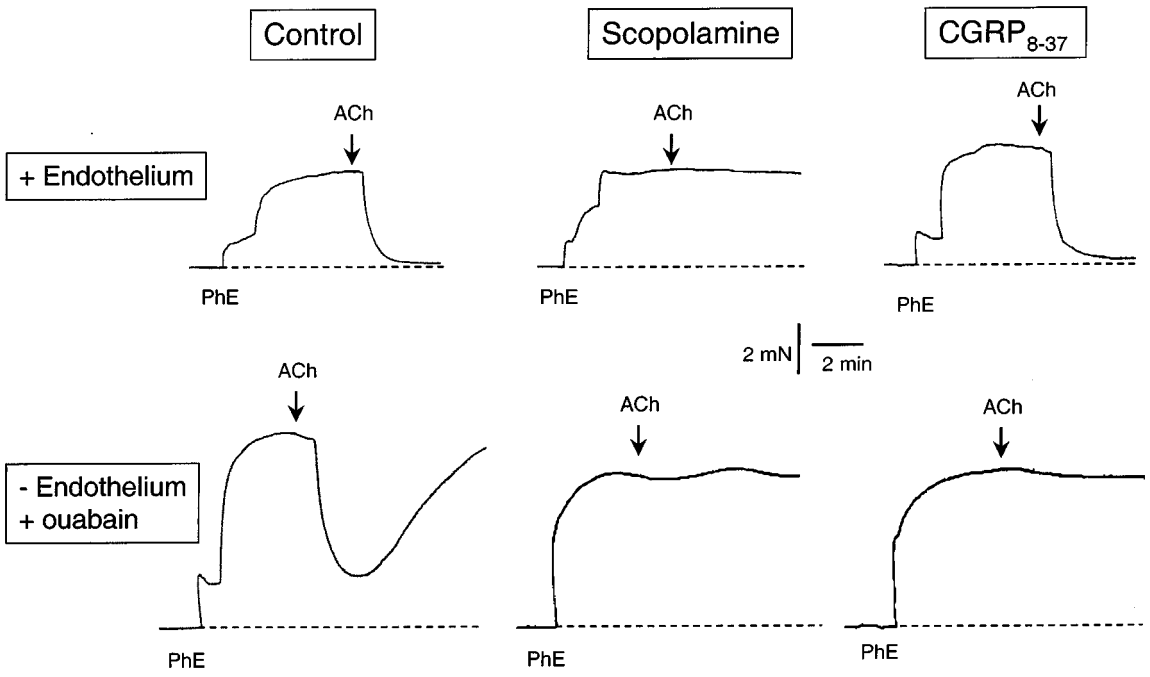

Figure 2 Effects of scopolamine (1 μM) and $CGRP_{8-37}$ (3 μM) on the vasodilator response to acetylcholine (ACh, 10 μM) in intact arterial segments (upper panel) and in arterial segments without endothelium in the presence of ouabain (1 mM; lower panel). Indomethacin (10 μM) and N^G-nitro-L-arginine (0.3 mM) were present in all experiments. Broken lines indicate the baseline tension before addition of the vasoconstrictor phenylephrine (PhE).

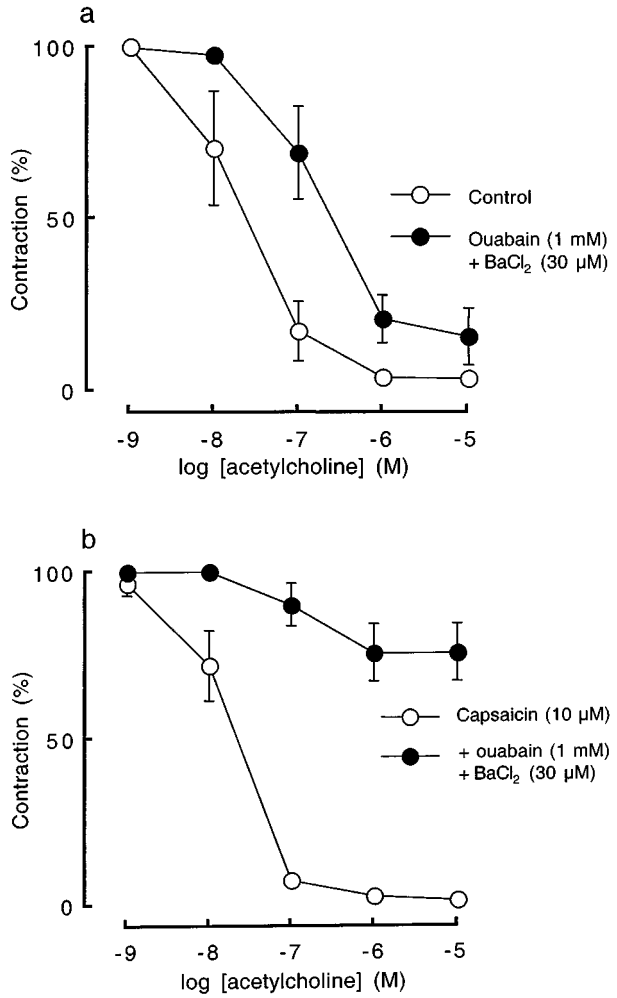

Figure 3 Effect of ouabain (1 mM) plus $BaCl_2$ (30 μM) on vasodilator responses to acetylcholine in intact arterial segments contracted with phenylephrine. (a) and (b) show preparations not pre-treated and pre-treated with capsaicin (10 μM), respectively. The level of contraction before addition of acetylcholine was set to 100%. Data are presented as mean$\pm$s.e.mean ($n=5-6$). Indomethacin (10 μM) and N^G-nitro-L-arginine (0.3 mM) were present in all experiments.

relaxation (pEC_{50}) were calculated as described (Zygmunt *et al*., 1994a). Values are presented as mean$\pm$s.e.mean, and n indicates the number of vascular segments (animals) examined. Statistical analysis of pEC_{50} and E_{max} values was performed by using Student's *t*-test (two-tailed) or multiple analysis of variance (MANOVA), followed by Bonferroni Dunn's *post hoc* test (Statview 4.12). Statistical significance was accepted when $P<0.05$.

Drugs Acetylcholine chloride (Aldrich); $BaCl_2$, $CGRP_{8-37}$, N^G-nitro-L-arginine, ouabain, L-phenylephrine hydrochloride and scopolamine (Sigma); capsaicin (Fluka); indomethacin (Confortid®, Dumex); α-latrotoxin (Alomone); levcromakalim (SmithKline Beecham). Capsaicin and levcromakalim were dissolved in ethanol. All other drugs were dissolved in distilled water. Stock solutions of the substances were stored at $-70^{\circ}C$.

Results *Vasodilator responses to acetylcholine and KCl* In the presence of indomethacin (10 μM) and N^G-nitro-L-arginine (0.3 mM), acetylcholine (10 μM) induced a complete relaxation, which lasted for more than 10 min. This relaxation is mediated entirely by EDHF (Zygmunt *et al*., 1994b). The acetylcholine-induced relaxation was abolished by scopolamine (1 μM; control: 95 ± 1%; scopolamine: -3 ± 5%; $n=5$), but unaffected by 3 μM of the selective CGRP receptor antagonist, $CGRP_{8-37}$ (control: 95 ± 1%; $CGRP_{8-37}$: 92 ± 4%; $n=6$), or pre-treatment with capsaicin (10 μM) for 1 h (control: 99 ± 1%; capsaicin: 99 ± 1%; $n=11$).

In endothelium-denuded arterial segments, which did not respond to acetylcholine, addition of KCl (5 mM) to the tissue bath (final potassium concentration of 9.6 mM) elicited a transient relaxation of greatly varying amplitude (69 ± 13%, range 11–100%, $n=6$; Figure 1). The level of tension returned to the pre-stimulation level in less than 5 min in all vascular segments (-4 ± 2%, $n=6$). This KCl-induced relaxation was not affected by 30 μM $BaCl_2$ (60 ± 7%, $n=5$).

Effect of ouabain in arteries without endothelium After a 30 min incubation period with ouabain (1 mM), acetylcholine (10 μM) and KCl (5 mM) induced a sustained (>5 min) relaxation in arterial segments without endothelium (Figure 1). The relaxation developed after a lag time of 0.5–2 min and amounted to 62 ± 5% for acetylcholine ($n=11$) and 51 ± 11% for KCl ($n=7$). Pre-treatment with capsaicin (10 μM) completely inhibited the endothelium-independent vasodilator responses to acetylcholine (0 ± 1%, $n=8$) and KCl (-2 ± 1%, $n=6$; Figure 1). The endothelium-independent, but capsaicin-sensitive, vasodilator response to acetylcholine in the presence of ouabain (1 mM) was abolished by scopolamine (1 μM; 0 ± 1%, $n=5$) and $CGRP_{8-37}$ (3 μM; 2 ± 2%, $n=5$; Figure 2).

Effect of ouabain plus $BaCl_2$ in endothelium intact arteries Ouabain (1 mM) and $BaCl_2$ (30 μM) produced a 10 fold rightward shift of the concentration-response curve for acetylcholine (control: $pEC_{50}=7.7\pm0.2$, $n=5$; ouabain + $BaCl_2$: $pEC_{50}=6.7\pm0.2$, $n=6$) in the presence of indomethacin (10 μM) and N^G-nitro-L-arginine (0.3 mM; Figure 3). The maximal response to acetylcholine was reduced by less than 20% (control: $E_{max}=97\pm2$%, $n=5$; ouabain + $BaCl_2$: $E_{max}=84\pm10$%, $n=6$). In arterial segments pre-treated with capsaicin (10 μM), which did not affect the control response to acetylcholine, ouabain (1 mM) and $BaCl_2$ (30 μM) almost abolished the acetylcholine-induced relaxation (control: $pEC_{50}=7.7\pm0.2$, $E_{max}=97\pm2$%, $n=5$; ouabain + $BaCl_2$: pEC_{50} = not determined, $E_{max}=27\pm9$%, $n=6$) in the presence of indomethacin (10 μM) and N^G-nitro-L-arginine (0.3 mM; Figure 3).

Vascular responses to α-latrotoxin In arterial segments without endothelium, α-latrotoxin (1 nM) elicited a sustained and almost complete relaxation (95 ± 4%, $n=4$) after a short lag time (1–2 min; Figure 4). This relaxation was abolished by $CGRP_{8-37}$ (3 μM; 2 ± 1%, $n=4$) or pre-treatment with capsaicin (10 μM; 0 ± 1%, $n=4$; Figure 4).

The resting membrane potential in endothelium-denuded arterial segments was -52 ± 1 mV (8 cells). α-Latrotoxin (1 nM) hyperpolarized the cell membrane by 12 ± 2 mV (basal: -50 ± 2 mV; α-latrotoxin: -62 ± 3 mV; $n=4$) in these arteries (Figure 4). This hyperpolarization was completely prevented by $CGRP_{8-37}$ (3 μM; basal: -53 ± 2 mV; α-latrotoxin: -53 ± 2 mV; $n=3$), whereas subsequent addition of levcromakalim (1 μM) hyperpolarized the cells by 22 ± 1 mV ($n=3$; Figure 4).

Discussion *Activation of sensory nerves* Many endothelium-dependent vasodilators, such as acetylcholine and bradykinin, are potential activators of primary sensory nerves, e.g., during tissue damage and ischaemia (Wood & Docherty, 1997).

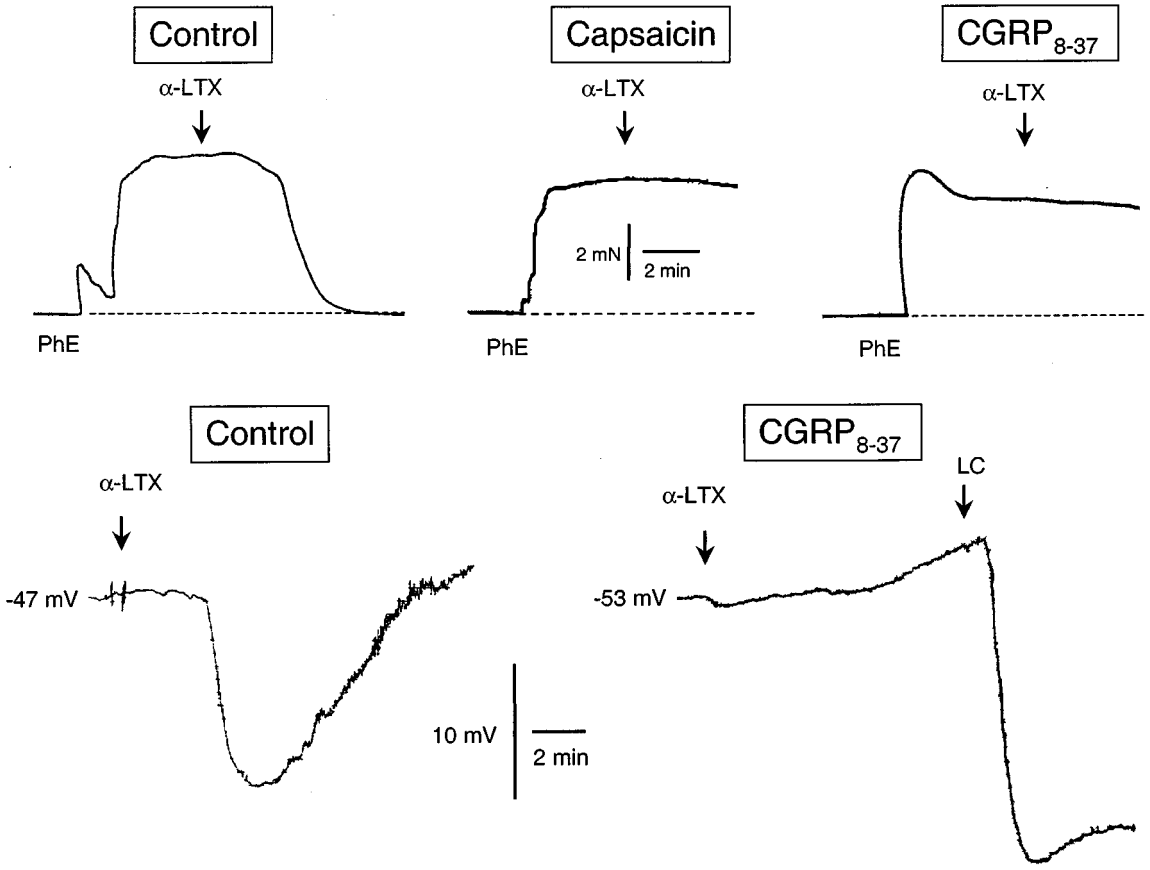

Figure 4 Effect of α-latrotoxin (α-LT, 1 nM) on vasomotor activity (upper panel) and resting membrane potential (lower panel) in arterial segments without endothelium. The relaxation and hyperpolarization induced by α-latrotoxin were abolished by capsaicin (10 μM) pre-treatment or $CGRP_{8-37}$ (3 μM), whereas levcromakalim (LC, 1 μM) always elicited hyperpolarization. Indomethacin (10 μM) and N^G-nitro-L-arginine (0.3 mM) were present in all experiments. Broken lines indicate the baseline tension before addition of the vasoconstrictor phenylephrine (PhE).

Besides transmitting sensory information to the central nervous system, capsaicin-sensitive sensory nerves have a powerful vasodilator action in many arteries and vascular beds (Holzer, 1992; Zygmunt *et al*., 1999). This study shows that both acetylcholine and potassium ions can activate such vasodilator nerves in the presence of ouabain. However, capsaicin pre-treatment of intact arterial segments did not affect the acetylcholine-induced relaxation in the absence of ouabain. This indicates that sensory nerves do not contribute to the endothelium-dependent relaxation induced by acetylcholine under control conditions. In rat skin and mesenteric vascular bed, vasodilator effects of acetylcholine are inhibited by capsaicin pre-treatment or $CGRP_{8-37}$, indicating a contribution of sensory nerves to these responses (Li & Duckles, 1992; Ralevic *et al*., 1992; Scott *et al*., 1992; Takenaga *et al*., 1995). The capsaicin-sensitive vasodilator responses to acetylcholine in the rat mesenteric vascular bed (Takenaga *et al*., 1995) and hepatic artery (present study) were endothelium-independent. Thus, besides the endothelial cells, primary sensory nerves represent another potential cellular target for acetylcholine and other endothelium-dependent vasodilators in blood vessels.

Although inhibition of Na^+, K^+-ATPase is known to cause transmitter release from many neurons, the ability of ouabain to unmask a neuroexcitatory effect of acetylcholine and potassium on perivascular sensory nerves has not been described previously. In the isolated perfused guinea-pig heart, ouabain produces a long-lasting release of CGRP from capsaicin-sensitive sensory neurons (Franco-Cereceda *et al*., 1989). This raises the possibility that ouabain may have sensitized the perivascular sensory nerves, allowing acetylcholine or potassium to act synergistically with ouabain to stimulate neurosecretion of CGRP and vasorelaxation in the present study.

Excitatory acetylcholine receptors, preferably of the nicotinic type, are present on primary sensory neurons (Wood & Docherty, 1997). However, both the neurogenic and endothelium-dependent components of the acetylcholine-induced relaxation were abolished by scopolamine, indicating that muscarinic acetylcholine receptors were mediating both these vascular responses to acetylcholine in the rat hepatic artery. Muscarinic acetylcholine receptors were also considered to mediate the capsaicin-sensitive component of the vasodilator response to acetylcholine in the rat mesenteric vascular bed, supporting the existence of such receptors on perivascular sensory nerves (Takenaga *et al*., 1995).

CGRP – a nerve-derived hyperpolarizing factor The sensory neuropeptide CGRP is a potent vasodilator and hyperpolarizing agent (Nelson *et al*., 1990). In the rat hepatic artery, CGRP increases cyclic AMP and induces an endothelium-independent relaxation (Zygmunt *et al*., 1995; 1999). As shown here, the neurogenic component of the acetylcholine-induced relaxation was abolished by $CGRP_{8-37}$, consistent with CGRP being the main neurotransmitter involved. α-Latrotoxin causes exocytosis of neurotransmitter vesicles at nerve terminals (Geppert *et al*., 1998). We have previously shown that α-latrotoxin induces a CGRP-mediated relaxation in endothelium intact vascular

segments of the rat hepatic artery (Zygmunt *et al*., 1999). This neurotoxin allowed us to study the vascular effects of sensory nerve activation without exposing the tissue to ouabain. In rat hepatic arteries without endothelium, α-latrotoxin induced a hyperpolarization and relaxation, both of which were abolished by $CGRP_{8-37}$ or capsaicin pre-treatment. Thus, apart from being a potent vasodilator, CGRP may also act as a nerve-derived hyperpolarizing factor in this artery.

Identity of EDHF Acetylcholine induces an immediate and long-lasting relaxation in the presence of indomethacin and N^G-nitro-L-arginine in the rat hepatic artery. This relaxation, which is considered to be mediated entirely by EDHF (Zygmunt *et al*., 1994b), differed markedly from the transient response to potassium ions. The failure of potassium ions to mimic the action of EDHF does not support the proposal that EDHF is potassium ions in the rat hepatic artery.

A combination of ouabain (1 mM) and $BaCl_2$ (30 μM) abolished the acetylcholine-induced hyperpolarization mediated by EDHF in rat hepatic and mesenteric arteries (Edwards *et al*., 1998). However, the corresponding vasodilator responses to acetylcholine were partially resistant to this treatment. We also found that the acetylcholine-induced relaxation in the presence of indomethacin and N^G-nitro-L-arginine was partially resistant to ouabain plus $BaCl_2$. However, the inhibition produced by this combination was substantially enhanced after capsaicin pre-treatment, indicating that ouabain plus $BaCl_2$ effectively suppress the relaxation mediated by EDHF in the rat hepatic artery. Recruitment of vasodilator nerves in the presence of ouabain can explain why the relaxations evoked by acetylcholine were partially resistant to ouabain plus $BaCl_2$. Thus, the weak correlation of electrophysiological and mechanical responses observed by Edwards *et al*. (1998) in the presence of this combination of drugs probably reflects the appearance of a neurogenic relaxation.

There are several possible explanations why Edwards *et al*. (1998) did not observe a neurogenic hyperpolarization in the presence of ouabain plus $BaCl_2$. The strength of this stimulus might have been insufficient to induce such a hyperpolarization. Since acetylcholine and KCl were injected into the flowing superfusate, the arterial segments were transiently exposed to these agents. Considering the time lag of the responses to acetylcholine and KCl, as observed in the present study, the exposure time for these agents might have been too short to evoke or detect a significant hyperpolarization. Another possibility relates to the mechanism behind the neurogenic hyperpolarization. If activation of Na^+, K^+-ATPase is involved in this hyperpolarization, no response would be observed in the presence of ouabain.

According to the original hypothesis by Edwards *et al*. (1998), potassium ions should cause hyperpolarization and relaxation of the smooth muscle cells via activation of both Na^+, K^+-ATPase and inward rectifier potassium channels. However, our findings do not support the existence of a potassium-sensitive inward rectifier in the rat hepatic artery, since potassium ions failed to evoke an ouabain-resistant relaxation in arterial segments pre-treated with capsaicin. Thus, the mechanism behind the inhibitory effect of ouabain plus $BaCl_2$ on EDHF-mediated responses is unclear and requires further investigation.

Conclusion The ability of ouabain to disclose a CGRP-mediated neurogenic vasorelaxation in response to acetylcholine and KCl must be considered when this agent is used as a pharmacological tool. Thus, in complex multicellular systems like the intact arterial wall, ouabain may interfere with Na^+, K^+-ATPase in several cell types. The results also suggest that sensory neuropeptides, including CGRP, may act as nerve-derived hyperpolarizing factors in blood vessels.

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