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# Further investigation of endothelium-derived hyperpolarizing factor (EDHF) in rat hepatic artery: studies using 1-EBIO and ouabain

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**1** The characteristics of endothelium-dependent hyperpolarization in rat hepatic artery have been further investigated in the presence of inhibitors of cyclo-oxygenase and nitric oxide synthase.

**2** Using sharp micro-electrodes, the smooth muscle hyperpolarization induced by acetylcholine, KCl or 1-ethyl-2-benzimidazolinone (1-EBIO) in intact hepatic arteries was abolished by 30 $\mu$M barium plus 500 nM ouabain.

**3** In vessels without endothelium, the smooth muscle hyperpolarization induced by KCl was not reduced by 30 $\mu$M barium alone. However, in the presence of barium the effects of KCl were partially inhibited by 100 nM ouabain and essentially abolished by 500 nM ouabain.

**4** Using sharp micro-electrodes, the hyperpolarization of both the smooth muscle and the endothelium induced by 1-EBIO or by acetylcholine was unaffected by 100 nM iberiotoxin. However, in the presence of 100 nM charybdotoxin, the effects of 1-EBIO were abolished whereas those of acetylcholine were only partially reduced. The hyperpolarization induced by levcromakalim was unaffected by either charybdotoxin or iberiotoxin.

**5** Under whole-cell patch-clamp recording conditions, 1-EBIO induced a voltage-insensitive, charybdotoxin-sensitive $K^+$ current in cultured endothelial cells but was without effect on $K^+$ currents in smooth muscle cells isolated from hepatic arteries.

**6** It is concluded that the endothelium-dependent hyperpolarization of smooth muscle induced by either acetylcholine or by 1-EBIO in rat hepatic artery is initially associated with the opening of endothelial calcium-sensitive $K^+$-channels insensitive to iberiotoxin. The resulting accumulation of $K^+$ in the myoendothelial space activates an isoform of $Na^+/K^+$-ATPase which is sensitive to low concentrations of ouabain.

**Keywords:** EDHF; $Na^+/K^+$-ATPase; 1-EBIO; endothelium; potassium channels; hepatic artery

**Abbreviation:** EDHF, endothelium-derived hyperpolarizing factor

## Introduction

In many arteries, acetylcholine stimulates the release of the so-called 'endothelium-derived hyperpolarizing factor', EDHF (Garland *et al*., 1995; Mombouli & Vanhoutte, 1997; Edwards & Weston, 1998). Using rat mesenteric and hepatic arteries, Edwards *et al*. (1998) concluded that EDHF was $K^+$ liberated from vascular endothelial cells following agonist-induced opening of calcium-sensitive $K^+$ cells. The subsequent $K^+$-accumulation in the myoendothelial space activated both $Na^+/K^+$-ATPase and inwardly-rectifying $K^+$ channels ($K_{IR}$) in the surrounding vascular smooth muscle, resulting in the hyperpolarization attributed to 'EDHF' (Edwards *et al*., 1998).

In the present study in rat hepatic artery, attempts were made to mimic the actions of EDHF using 1-ethyl-2-benzimidazolinone (1-EBIO), a compound reported to activate calcium-sensitive $K^+$ channels in epithelial cells (Devor *et al*., 1996). If successful, such a strategy would allow activation of endothelial cell $K^+$ channels without using agonists like acetylcholine which raise intracellular calcium and thus also release mediators such as nitric oxide or prostacyclin (Garland *et al*., 1995; Mombouli & Vanhoutte, 1997; Edwards & Weston, 1998). In addition, the possible identity of the $Na^+/K^+$-ATPase subtype(s) (see Blanco & Mercer, 1998) present in the smooth muscle cells of the hepatic artery and involved in EDHF-induced hyperpolarizations (Edwards & Weston, 1998) was investigated using different concentrations of ouabain.

Using these approaches in a single blood vessel, it was hoped to clarify certain features of the EDHF response and to provide the basis for further studies in other vessels such as the guinea-pig internal carotid and porcine coronary arteries. In these, recent work has suggested that the EDHF response is fundamentally different from that in the rat hepatic artery (Quignard *et al*., 1999).

## Methods

Experiments were performed on hepatic arteries dissected from male Sprague-Dawley rats (150–200 g) previously killed by stunning and cervical dislocation.

### *Micro-electrode experiments*

Intact vessels were pinned to the Sylgard base of a heated bath (volume 10 ml) and superfused (10 ml $min^{-1}$), at 37°C, with Krebs solution containing 300 $\mu$M $N^G$-nitro-L-arginine and 10 $\mu$M indomethacin and gassed with 95% $O_2$/5% $CO_2$. Smooth muscle cells were impaled from the adventitial side using micro-electrodes filled with 3 M KCl (resistance 40–80 MΩ). In some experiments, the arteries were de-endothe-

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lialized by exposing the lumen to distilled water for 20 s and the loss of the vascular endothelium was confirmed by the lack of response to 10 $\mu$M acetylcholine. Endothelial cells were impaled from the lumenal side of the artery. This surface was accessed *via* the hole in the main vessel wall produced by removal of a side branch. Experiments were performed using a conventional high impedance amplifier (Intra 767; WPI Instruments, U.S.A.). In most experiments, any 50 Hz interference at the amplifier output was selectively removed using an active processing circuit (Humbug; Digitimer, U.K.) after which the signals were digitized and analysed using a MacLab system (AD Instruments).

### *Isolation of cells*

Hepatic arteries were dissected into a nominally calcium-free physiological salt solution (Klöckner & Isenberg, 1985) and cleaned of extraneous connective tissue under a dissecting microscope. An intact artery was placed in a collagenase solution originally described for the separation of guinea-pig bladder smooth muscle cells (Klöckner & Isenberg, 1985). The tissue was agitated for 12 min in the enzyme solution at 37°C, washed using the same solution free of enzyme and cut into four segments. These segments were then briefly triturated using a wide bore, smooth tipped pipette in $Ca^{2+}$-free PSS. After a further 5 min incubation at 37°C in the enzyme solution, the segments were washed and triturated in Kraftbruhe (KB-medium; Klöckner & Isenberg, 1985). Cells were stored at 8°C in KB-medium and used within 9 h of separation. All experiments were performed at room temperature (22–26°C). Patch pipettes were made from Pyrex glass (H15/10, Jencons, U.K.) and had a resistance of 2–4 MΩ when filled with internal solution.

### *Cultured endothelial cells*

Bovine endothelial cells (BAE1) were purchased from the European Collection of Animal Cell Cultures (Salisbury) and maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco, U.K.) supplemented with 2 mM glutamine (Gibco, U.K.) and 20% foetal bovine serum at 37°C in a humidified 5% $CO_2$ : 95% air atmosphere.

### *Single-cell electrophysiology*

The whole-cell configuration of the patch clamp technique (Hamill *et al*., 1981) was employed using an Axopatch-1C amplifier (Axon Instruments, U.S.A.). The settling time of the system was less then 500 $\mu$s.

Voltage commands and data acquisition were performed on-line with an AT-compatible computer equipped with an Axon TL-1 interface. For cell stimulation and for recording, the pClamp 5.5 programme was used (Axon Instruments, U.S.A.). Data were stored on a digital audio tape recorder (Sony, U.K.) and the evoked membrane currents were monitored continuously on a Gould Windograf recorder. The data described were not corrected by linear leak subtraction. Analysis of results was performed using Axograph v.3.5.5 (Axon Instruments, U.S.A.).

The chamber containing the isolated cells was perfused with a $Ca^{2+}$-free physiological salt solution at approximately 1 ml $min^{-1}$ using a peristaltic pump (LKB Microperpex, U.K.); a second identical pump was used to remove the solution from the recording chamber. Hyperpolarizations were induced by bolus injection of the agonists into the recording chamber to give (transiently) the final concentrations indicated. The effects of inhibitors were examined by adding appropriate quantities into the reservoir of solution perfusing the bath so that responses could be examined under equilibrium conditions.

### *Drugs and solutions*

Krebs solution comprised (mM): $Na^+$ 143, $K^+$ 4.6, $Ca^{2+}$ 2.5, $Mg^{2+}$ 1.2, $Cl^-$ 126.4, $H_2PO_4^-$ 1.2, $SO_4^{2-}$ 1.2, $HCO_3^-$ 25, glucose 11.1 and was bubbled with 95% $O_2$ and 5% $CO_2$.

The composition of the solutions used for the patch-clamp experiments was as follows (mM): pipette (internal) $Na^+$ 5, $K^+$ 122.4, $Mg^{2+}$ 1.2, $Cl^-$ 129.8, $H_2PO_4^{2-}$ 1.2, glucose 11, HEPES 10, oxalacetic acid 5, sodium pyruvate 2, sodium succinate 5, pH 7.30; bath $Na^+$ 125 (135 mM for BAE1 cells), $K^+$ 6, $Ca^{2+}$ 2.5, $Mg^{2+}$ 1.2, $H_2PO_4^{2-}$ 1.2, $Cl^-$ 137.2, glucose 11, HEPES 10, pH 7.30 aerated with $O_2$.

The enzyme solution comprised (mM): KOH 130, $CaCl_2$ 0.05 taurine 20, pyruvate 5, creatine 5, HEPES 10, collagenase (1 mg $ml^{-1}$; Sigma Type 8), pronase (0.2 mg $ml^{-1}$; Calbiochem), fatty acid-free albumin (1 mg $ml^{-1}$; Sigma). The pH was adjusted to 7.40 with methanesulphonic acid.

KB-medium was composed of (mM): KCl 85, $KH_2PO_4$ 30, $MgSO_4$ 5, $Na_2ATP$ 5, K-pyruvate 5, creatine 5, taurine 20, $\beta$-hydroxybutyrate 5, fatty acid-free albumin 1 mg $ml^{-1}$ and the pH was adjusted to 7.20 with KOH.

The following substances were used: acetylcholine chloride, barium chloride, synthetic charbydotoxin (Latoxan, France), 1-EBIO (1-ethyl-2-benzimidazolinone; Aldrich), synthetic iberiotoxin (Latoxan), indomethacin, $N^{\omega}$-nitro-L-arginine, NS1619 (1-(2′-hydroxy-5′trifluoromethylphenyl)-5-trifluoromethyl-2(3*H*)benzimidazolone; Research Biochemicals International, U.K.), ouabain, phenylephrine hydrochloride. Unless otherwise stated, all compounds were obtained from Sigma.

### *Statistics*

Student's *t*-test (paired or unpaired as appropriate) was used to assess the probability that differences between mean values had arisen by chance; $P<0.05$ was considered to be statistically significant.

## Results

### *Effects of 1-EBIO on rat intact hepatic arteries*

In the presence of an intact endothelium, 600 $\mu$M 1-EBIO induced a hyperpolarization of the vascular smooth muscle cells ($12.4\pm0.6$ mV, $n=4$) which was not modified by 100 nM iberiotoxin ($13.1\pm1.0$ mV, $n=4$) but which was abolished by subsequent exposure to 100 nM charybdotoxin ($0.8\pm0.7$ mV, $n=4$). In the same blood vessels, the hyperpolarization to acethylcholine ($22.4\pm0.7$ mV, $n=4$) was also unaffected by iberiotoxin ($21.3\pm1.2$ mV, $n=4$) but was partially inhibited by charybdotoxin ($16.8\pm1.2$ mV, $n=4$) (Figure 1a,c). In hepatic artery segments from the same animals but without endothelium, 1-EBIO was without effect (hyperpolarization: 10 $\mu$M acetylcholine $0.4\pm0.2$ mV; 600 $\mu$M 1-EBIO $0.4\pm0.2$ mV; Figure 1b). However, subsequent application of 5 mM KCl produced a hyperpolarization ($20.3\pm1.2$ mV, $n=4$; Figure 1b), indicating that the smooth muscle of the vessels had not been damaged during endothelium removal. Rat hepatic artery endothelial cells had a resting membrane potential of $-64.5\pm0.4$ mV ($n=4$). Application of 600 $\mu$M 1-EBIO produced a hyperpolarization

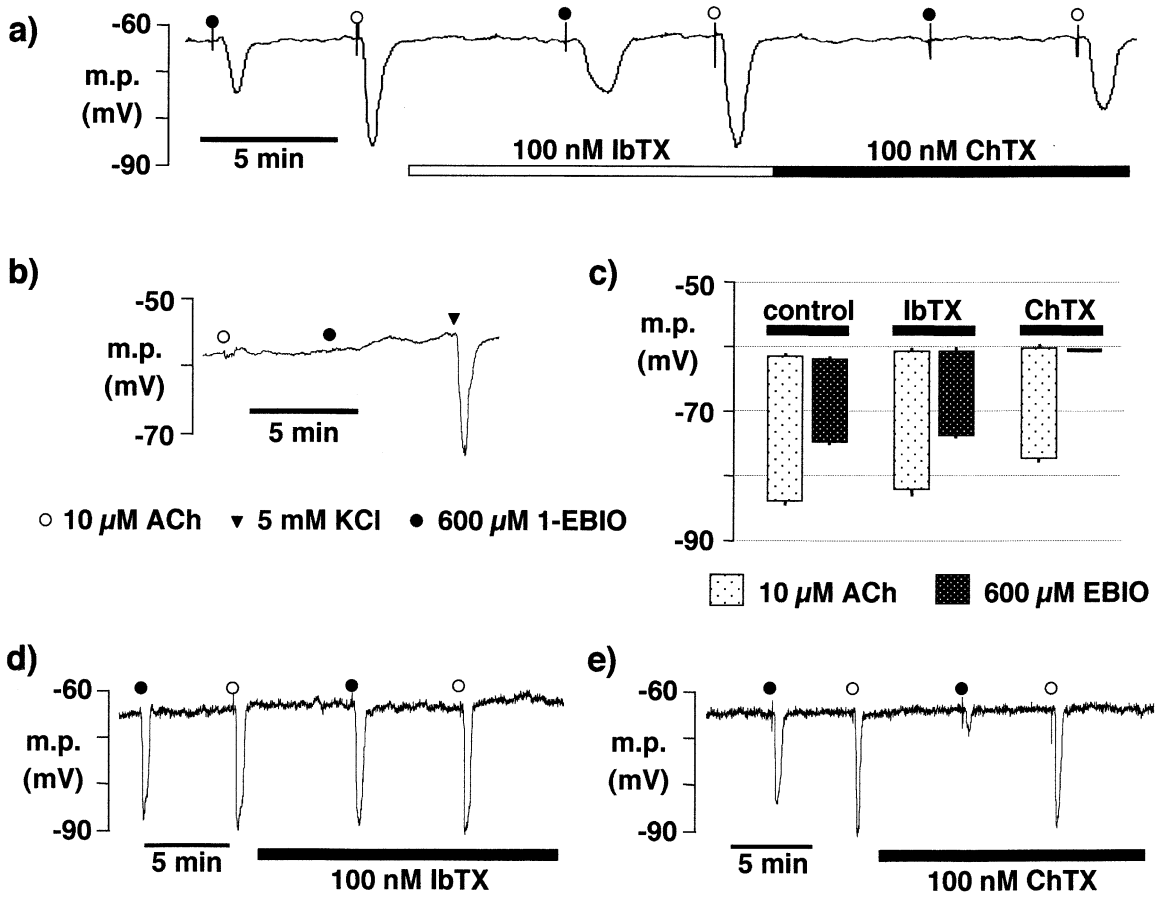


**Figure 1** Effects of iberiotoxin and charybdotoxin on hyperpolarizations induced by acetylcholine (ACh) and 1-EBIO in smooth muscle (a–c) and endothelial cells (d,e) of rat intact hepatic arteries. (a,c) In intact arteries, the smooth muscle hyperpolarization to 1-EBIO, but not that to acetylcholine (ACh) was fully inhibited by charybdotoxin (ChTX) whereas iberiotoxin (IbTX) was without effect on either response. (b) In de-endothelialized vessels neither acetylcholine nor 1-EBIO induced a hyperpolarization. (d) In endothelial cells, neither the hyperpolarization induced by 1-EBIO nor that induced by acetylcholine was sensitive to inhibition by iberiotoxin. (e) The endothelial cell hyperpolarization to 1-EBIO but not to acetylcholine was abolished by charybdotoxin alone. Data from four separate experiments are shown graphically in (c) in which the columns represent the membrane potential (m.p.)±s.e.mean before and after exposure to each hyperpolarizing agent in the absence (control) or presence of the inhibitors as indicated.

of 20.6±0.7 mV ($n=4$). In the same preparations, 10 μM acetylcholine hyperpolarized the endothelial cells by 26.1±0.6 mV (Figure 1d,e). The response to 1-EBIO, but not that to acetylcholine, was essentially abolished by 100 nM charybdotoxin (Figure 1e). Neither the endothelial cell hyperpolarization induced by 1-EBIO nor that to acetylcholine was inhibited by 100 nM iberiotoxin (Figure 1d).

In intact hepatic arteries, 33 μM NS1619 hyperpolarized the smooth muscle. Although this concentration of NS1619 produces a large increase in $I_{BK(Ca)}$ in vascular smooth muscle cells (Edwards *et al*., 1994; Holland *et al*., 1996; Mistry & Garland, 1998), the magnitude of the hyperpolarization in the hepatic artery (5.5±0.3 mV, $n=4$) was smaller than that to 600 μM 1-EBIO (15.0±0.7 mV, $n=4$) (Figure 2a,b). In the presence of 30 μM barium plus 500 nM ouabain, the response to 1-EBIO was essentially abolished (1.0±0.3 mV, $n=4$) whereas, as shown in Figure 2a,b, the hyperpolarizations to NS1619 (10.5±1.6 mV, $n=4$) and to levcromakalim were not diminished (hyperpolarization to levcromakalim: control, 24.6±1.0 mV; in presence of barium plus ouabain, 26.2±1.9 mV, $n=4$).

### *Effects of 1-EBIO on endothelial and smooth muscle cell $K^+$-currents*

The effects of 1-EBIO on cultured endothelial cells (BAE1) and on single smooth muscle cells dispersed from the hepatic artery were investigated using whole-cell patch-clamp techniques. In the presence of a quasi-physiological $K^+$ gradient, BAE1 cells were voltage-clamped at a holding potential of 0 mV and 500 ms voltage ramps from +50 to −140 mV were applied. Under these conditions a large inwardly-rectifying $K^+$ current ($I_{K(IR)}$) was observed in BAE1 cells (Figure 3a). In the presence of 600 μM 1-EBIO an additional, voltage-insensitive $K^+$ current was induced. Charybdotoxin (100 nM), which alone was without effect on $I_{K(IR)}$, abolished the 1-EBIO-induced current and revealed an inhibition of $I_{K(IR)}$ by the benzimidazolinone ($n=5$, Figure 3a). In six cells, the charybdotoxin-sensitive current induced by 1-EBIO was not modified by exposure to iberiotoxin (100 nM) (data not shown). Exposure of BAE-1 cells to 33 μM NS1619 only induced a small increase in current (current at +50 mV: control, 23.1±10.9; 33 μM NS1619, 65.3±16.2, $n=5$).

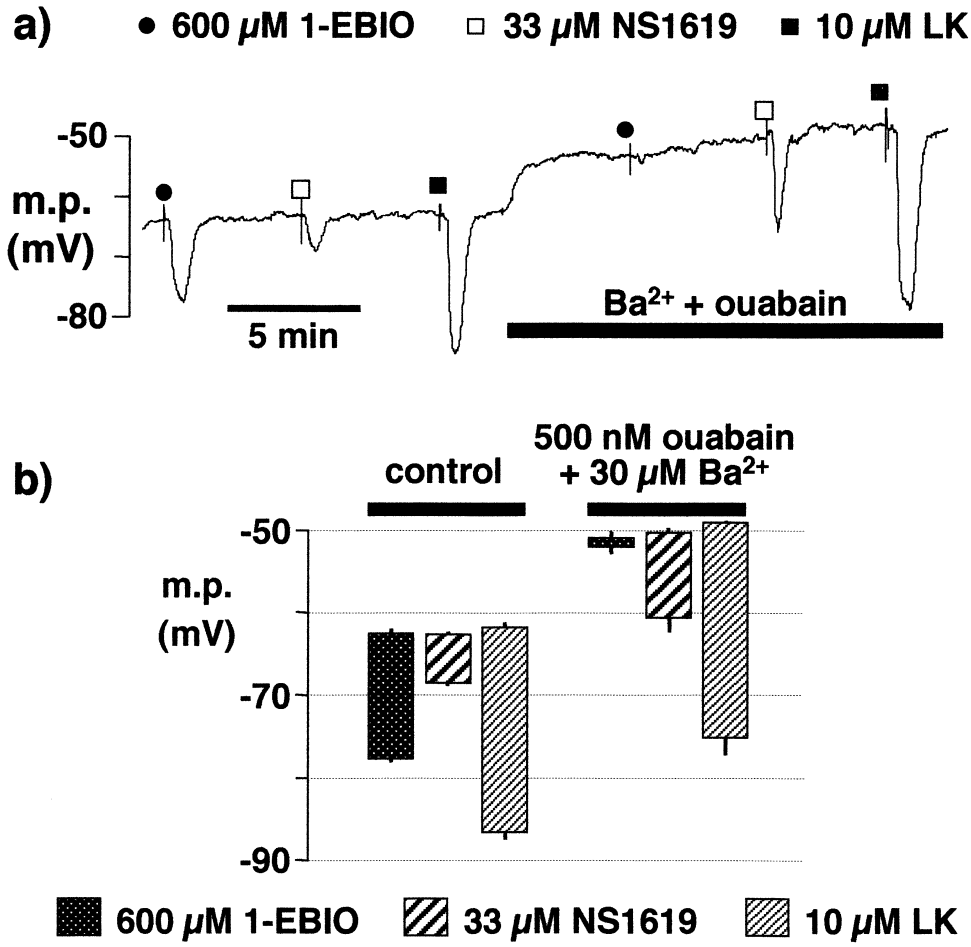


**Figure 2** Effects of barium and ouabain on hyperpolarizations induced by 1-EBIO, NS1619 and levcromakalim (LK) in rat intact hepatic arteries. As shown in the typical recording (a) and summarized graphically in (b), the membrane potential was depolarized by barium and ouabain. In the presence of these two inhibitors the response to 1-EBIO was abolished whereas the hyperpolarizations to NS1619 and levcromakalim were enhanced. In (b) the columns represent the membrane potential (m.p.)±s.e.mean ($n=4$) before and after exposure to each hyperpolarizing agent in the absence (control) or presence of the inhibitors as indicated.

Rat hepatic artery myocytes were held at 0 mV and then subjected to 500 ms voltage ramps from −140 to +50 mV. Using this voltage protocol, no current was induced by 1-EBIO in 9 cells (Figure 3b).

*Ouabain-sensitivity of hyperpolarizations to acetylcholine and $K^+$*

In the presence of the endothelium, 30 μM barium depolarized the hepatic artery smooth muscle by 3.0±0.8 mV, $n=6$. In separate cells, the combination of 30 μM barium and 500 nM ouabain depolarized smooth muscle by 5.8±0.6 mV ($n=4$) from a resting membrane potential of −61.2±0.9 mV. Under these latter conditions, the hyperpolarization to acetylcholine or to $K^+$ was almost abolished whereas the response to levcromakalim was unaffected (Figure 4a,c). In preparations without endothelium, the resting membrane potential (−60.0±0.79 mV) was essentially unaffected by exposure to 30 μM barium (−58.4±1.2 mV; depolarization 1.68±0.6 mV, $n=4$). However, in the continued presence of barium, addition of 100 nM ouabain produced a 3.9±0.5 mV depolarization (to −54.5±1.2 mV). This change in membrane potential was significantly enhanced by increasing the ouabain concentration to 500 nM (membrane potential −49.1±1.6 mV; additional depolarization 5.4±1.3 mV, $n=4$). In the absence of a functional endothelial cell layer (hyperpolarization to 10 μM acetylcholine 0.5±0.2 mV, $n=4$), the hyperpolarization induced by 5 mM KCl was inhibited by 100 and 500 nM ouabain (each plus 30 μM barium) in a concentration-dependent manner (Figure 4b,d).

## Discussion

In rat hepatic and mesenteric arteries, Edwards *et al.* (1998) showed that acetylcholine induced the opening of two calcium-sensitive $K^+$ channel types (one apamin-sensitive and one charybdotoxin-sensitive) allowing $K^+$ efflux from the endothelial cells. The resultant elevation of $K^+$ in the myoendothelial space activated the $Na^+/K^+$-ATPase and inwardly-rectifying $K^+$ channels ($K_{IR}$) on the smooth muscle cells resulting in their hyperpolarization (Edwards *et al.*, 1998). Since elevation of extracellular $K^+$ also hyperpolarized the smooth muscle in rat hepatic and mesenteric arteries, it was concluded that EDHF was $K^+$ in these vessels. If this were correct, then an agent which selectively opens endothelial cell $K^+$ channels should mimic EDHF.

The initial objective of the present study was thus to determine whether 1-EBIO would hyperpolarize vascular endothelial cells in intact hepatic arteries. This benzimidazoli-

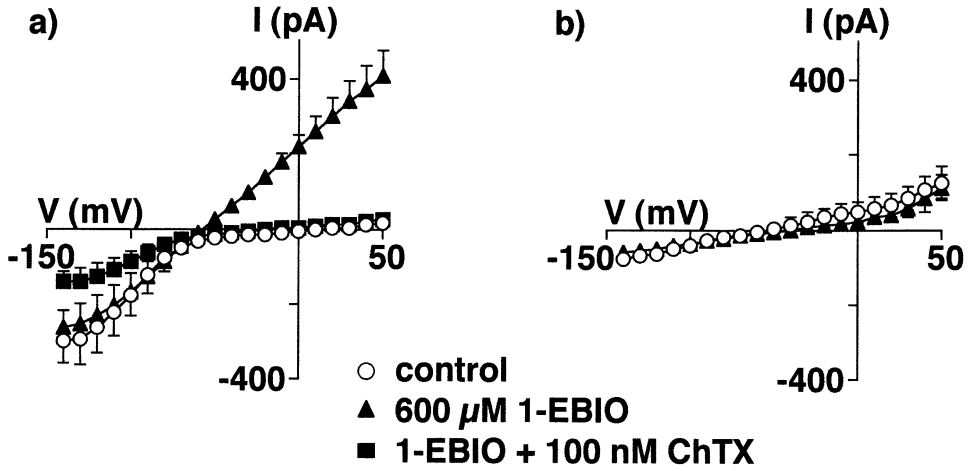


**Figure 3** Effects of 1-EBIO on whole-cell $K^+$ currents. (a) The current induced by 1-EBIO in cultured endothelial cells (BAE1) was abolished by charybdotoxin (ChTX) ($n=5$). (b) 1-EBIO was without effect on currents in freshly-isolated smooth muscle cells (rat hepatic artery myocytes) ($n=9$). Each point represents the mean ± s.e.mean.

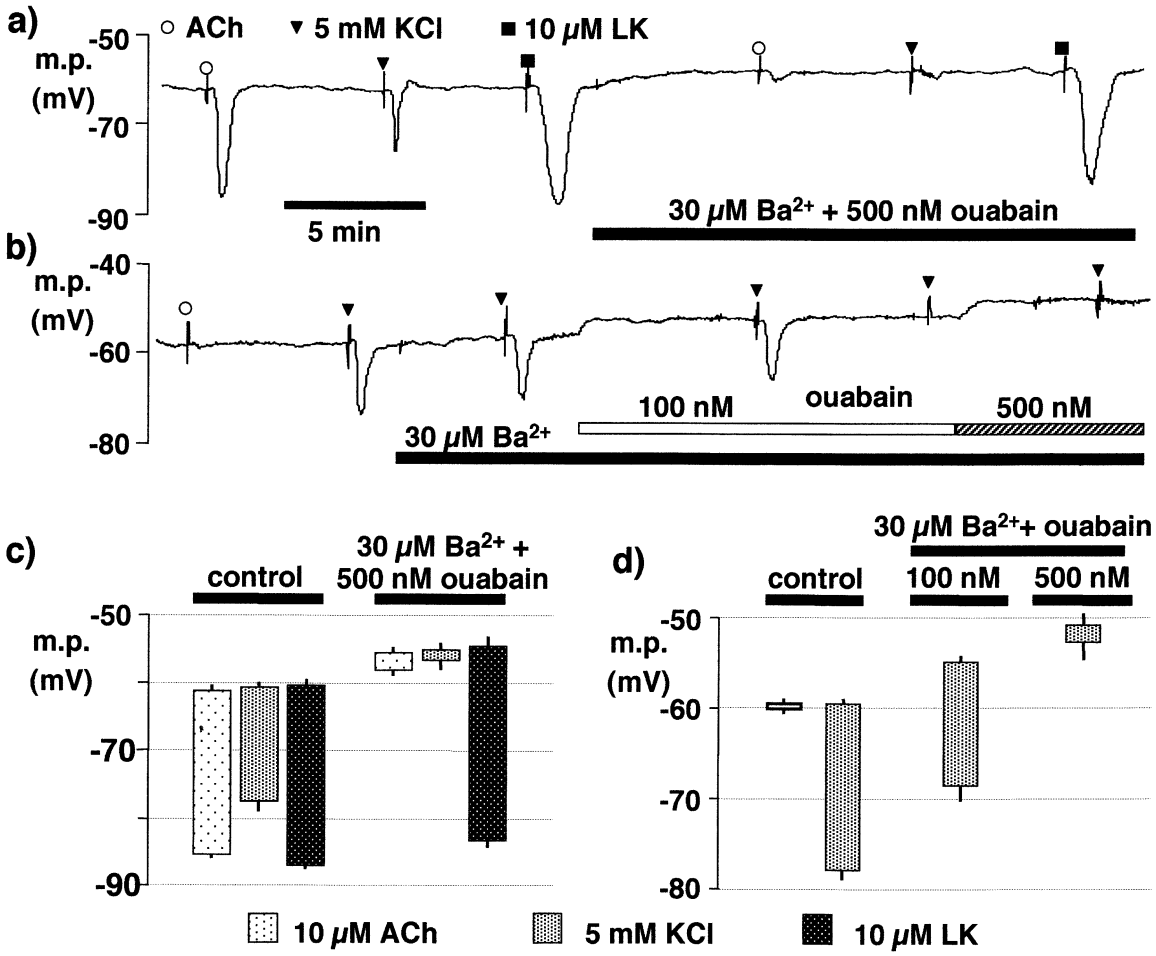


**Figure 4** Effects of barium and ouabain on hyperpolarizations induced by acetylcholine (ACh), KCl and levcromakalim (LK) in rat hepatic arteries. Typical recordings of responses in (a) intact and (b) de-endothelialized preparations are shown. Note that in (b) the preparation was first exposed to barium and then to increasing concentrations of ouabain. Data from four or six separate experiments are shown graphically in (c) and (d), respectively, in which the columns represent the membrane potential (m.p.) ± s.e.mean before and after exposure to each hyperpolarizing agent in the absence (control) or presence of the inhibitors as indicated.

none opens both epithelial cell and endothelial cell $K^+$ channels (Devor *et al*., 1996; Cai *et al*., 1998) and the cloned intermediate-conductance calcium-sensitive $K^+$-channel, $IK_{Ca}$ (Jensen *et al*., 1998). We compared its effects with those of the structurally-related benzimidazolone, NS1619, an opener of the large-conductance calcium-sensitive K-channel ($BK_{Ca}$; Olesen *et al*., 1994; Gribkoff *et al*., 1996). NS1619 induces $I_{BK(Ca)}$ and hyperpolarizes vascular smooth muscle cells (Edwards *et al*.,

1994; Holland *et al*., 1996) but does not open the endothelial cell $IK_{Ca}$, a channel which is inhibited by charybdotoxin but not by iberiotoxin (Cai *et al*., 1998). Unlike smooth muscle cells, freshly-isolated endothelial cells do not possess $BK_{Ca}$ (Kestler *et al*., 1998; Jow *et al*., 1999), a channel which is inhibited by both charybdotoxin and iberiotoxin (Garcia *et al*., 1991). However, such a channel may be induced during endothelial cell culture (Jow *et al*., 1999) and may be responsible for the small increase in current induced by NS1619 in the cultured endothelial (BAE1) cells used in the present study.

1-EBIO induced a charybdotoxin-sensitive, iberiotoxin-insensitive hyperpolarization of rat intact hepatic arteries. However, it had no effect on de-endothelialized vessels, a strong indication that it might be a selective opener of endothelial cell $K^+$ channels. Confirmation of this was obtained using patch-clamp techniques. Thus, 1-EBIO induced a charybdotoxin-sensitive, iberiotoxin-insensitive current in the endothelial (BAE1) cells but it was without effect on currents in myocytes isolated from rat hepatic arteries. Like the responses to acetylcholine or to KCl (present study; see also Edwards *et al*., 1998), the effects of 1-EBIO were inhibited by the combination of barium + ouabain. Thus, we were able to demonstrate that opening of endothelial $K^+$ channels could mimic the effects of 'EDHF'. These results are thus consistent with the evidence (Edwards *et al*., 1998) that EDHF is indeed $K^+$ in rat hepatic and mesenteric arteries.

### *Role of $Na^+/K^+$-ATPase subtypes in the EDHF response in the rat hepatic artery*

In an earlier study using rat blood vessels, Edwards *et al*. (1998) used a high concentration of ouabain (1 mM) to inhibit EDHF in the belief that rat $Na^+/K^+$-ATPase was generally resistant to the inhibitory effects of this agent (Lingrel, 1992). However, of the subtypes of the $Na^+/K^+$-ATPase $\alpha$-subunit which exist in the rat, only one isoform, the $\alpha1$, is relatively insensitive to ouabain (see Lingrel, 1992; Blanco & Mercer, 1998). Typically, the native rat $Na^+/K^+$-ATPase comprising $\alpha1$-subunits exhibits a ouabain $IC_{50}$ value of approximately 300 $\mu$M whereas the equivalent values for the ATPases containing either the $\alpha2$ or the $\alpha3$ subunits are in the nanomolar range (Lingrel, 1992). The exact identity of the $Na^+/K^+$-ATPase $\alpha$-subunit is unknown but it may be an $\alpha3$ since this, but not the $\alpha2$ subunit, has been detected in myocytes freshly-isolated from rat mesenteric arteries (Juhaszova & Blaustein, 1997).

In the present study, we surprisingly found that, in the presence of barium, the KCl response was partially inhibited by 100 nM ouabain and almost abolished by 500 nM ouabain. This strongly indicates that the smooth muscle $Na^+/K^+$-ATPase which is involved in the response to 'EDHF' and to exogenous application of KCl is not the 'type 1' (containing the $\alpha$-1 subunit). The existence of numerous forms of $Na^+/K^+$-ATPase in different tissues, and the changes in the relative expression of these forms in certain tissues during development, suggests that they each have a specific physiological role. The finding of the present study that the $Na^+/K^+$-ATPase which is involved in the EDHF response is unlikely to be the type 1 would be consistent with the apparent situation in neurones in which type 1 $Na^+/K^+$-ATPase is considered to have essentially a 'housekeeping' role in the maintenance of membrane potential (Blanco & Mercer, 1998). Thus, whereas the type 1 $Na^+/K^+$-ATPase may be maximally active under basal conditions, the activity of other types may be enhanced either by elevation of extracellular $K^+$ or by a reduction in $[Ca^{2+}]_i$ (see Blanco & Mercer, 1998). In neurones, isoforms which are less sensitive to $K^+$ than the type 1 are recruited to restore ionic gradients after nerve impulses (Lingrel, 1992; Blanco & Mercer, 1998), a situation which may have a parallel in the rat hepatic artery.

### *Models for the EDHF response*

The importance of elevation of endothelial cell intracellular calcium in the EDHF response is known (Garland *et al*., 1995; Mombouli & Vanhoutte, 1997; Edwards & Weston, 1998). The initial agonist-induced increase in intracellular calcium is thought to result from the emptying of intracellular calcium stores (Marchenko & Sage, 1994), depletion of which initiates calcium entry *via* voltage-independent cation channels (Fukao *et al*., 1997). The rise in intracellular calcium stimulates the opening of calcium-sensitive endothelial $K^+$ channels which hyperpolarizes the endothelial cells (Kukovetz *et al*., 1992; Marchenko & Sage, 1996; Muraki *et al*., 1997; Edwards *et al*., 1998). This should further enhance calcium influx *via* the voltage-insensitive cation channels and in porcine aortic endothelial cells, changes in the intracellular calcium concentration indeed mirror changes in the membrane potential (Kukovetz *et al*., 1992).

In vessels such as the rat hepatic and mesenteric arteries, the results of the present and earlier (Edwards *et al*., 1998) studies combine to provide strong evidence that the phenomenon of EDHF is explained by the loss of $K^+$ from the endothelium *via* Ca-sensitive $K^+$ channels. One of these is likely to be a subtype of $SK_{Ca}$ (Edwards *et al*., 1998; Edwards & Weston, 1998). The other can be activated by 1-EBIO and is probably $IK_{Ca}$, a channel inhibited by charybdotoxin but not by iberiotoxin.

Charybdotoxin also inhibits Kv1.2 and Kv1.3 (see Garcia *et al*., 1995 for review) but it is unlikely that these channels form the endothelial cell target for the toxin. Thus, dendrotoxin, an inhibitor of Kv1.2 (Grissmer *et al*., 1994) cannot substitute for charybdotoxin as an inhibitor of EDHF in rat hepatic or guinea-pig basilar arteries (Petersson *et al*., 1997; Zygmunt *et al*., 1997). Although 300 $\mu$M margatoxin (a very potent inhibitor of Kv1.3, $IC_{50}$ approximately 50 nM in T-lymphocytes; Garcia-Calvo *et al*., 1993) had a significant inhibitory effect on the EDHF response in the presence of apamin this effect was small (Zygmunt *et al*., 1997). Furthermore, margatoxin inhibits $IK_{Ca}$, although with a lower potency than for Kv1.3 channels ($IC_{50}$ 459 nM on cloned hIK1 channels expressed in HEK293 cells; Jensen *et al*., 1998). Additionally, the relative order of potency in inhibiting EDHF (charybdotoxin > > margatoxin; Zygmunt *et al*., 1997) is similar to that for inhibition of hIK1 or the endothelial cell $IK_{Ca}$ (Cai *et al*., 1998; Jensen *et al*., 1998) but differs from that for inhibition of Kv1.3 (margatoxin > > charybdotoxin; Garcia-Calvo *et al*., 1993).

### *Conclusions*

Clearly, the EDHF phenomenon in all vessels cannot be explained by activation of smooth muscle $Na^+/K^+$-ATPase and $K_{IR}$ since EDHF is not inhibited by ouabain plus $Ba^{2+}$ in the guinea-pig carotid artery (Quignard *et al*., 1999). However, movement of $K^+$ out of the endothelial cells must play a pivotal role in the EDHF-induced smooth muscle hyperpolarization in all vessels (including the guinea-pig carotid artery) since the response is always abolished by charybdotoxin and/or apamin (see Edwards & Weston, 1998). The possible role of gap junctions in certain vessels is currently under investigation.

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