British Journal of Pharmacology (2001) 134, 912–920

www.nature.com/bjp

Dominant role of an endothelium-derived hyperpolarizing factor (EDHF)-like vasodilator in the ciliary vascular bed of the bovine isolated perfused eye

[1]Alister J. McNeish, [1]William S. Wilson & *,[1]William Martin

[1]Division of Neuroscience & Biomedical systems, Institute of Biomedical & Life Sciences, West Medical Building, University of Glasgow, Glasgow G12 8QQ, Scotland

1 The roles of the endothelium-derived nitric oxide, prostacyclin and endothelium-derived hyperpolarizing factor (EDHF) in mediating vasodilator responses to acetylcholine and bradykinin were assessed in the ciliary vascular bed of the bovine isolated perfused eye preparation.

2 Vasodilatation to acetylcholine or bradykinin was unaffected by the nitric oxide synthase inhibitor, L-NAME (100 μM), or the cyclo-oxygenase inhibitor, flurbiprofen (30 μM), but was virtually abolished following treatment with a high concentration of KCl (30 mM), or by damaging the endothelium with the detergent, CHAPS (0.3%, 2 min).

3 Acetylcholine-induced vasodilatation was unaffected by glibenclamide (10 μM), an inhibitor of ATP-sensitive K^+ channels (K^+_{ATP}), but was significantly attenuated by TEA (10 mM), a non-selective inhibitor of K^+ channels.

4 The small conductance calcium-sensitive K^+ channel (SK^+_{Ca}) inhibitor, apamin (100 nM), and the large conductance calcium-sensitive K^+ channel (BK^+_{Ca}) inhibitor, iberiotoxin (50 nM), had no significant effect on acetylcholine-induced vasodilatation. In contrast, the intermediate (IK^+_{Ca})/large conductance calcium-sensitive K^+ channel inhibitor, charybdotoxin (50 nM), powerfully blocked these vasodilator responses, and uncovered a vasoconstrictor response.

5 The combination of apamin (100 nM) with a sub-threshold concentration of charybdotoxin (10 nM) significantly attenuated acetylcholine-induced vasodilatation, but the combination of apamin (100 nM) with iberiotoxin (50 nM) had no effect.

6 In conclusion, blockade by a high concentration of KCl, by charybdotoxin, or by the combination of apamin with a sub-threshold concentration of charybdotoxin, strongly suggests that vasodilatation in the bovine isolated perfused eye is mediated by an EDHF.

British Journal of Pharmacology (2001) **134,** 912–920

Keywords: Bovine isolated perfused eye; endothelium; EDHF; L-NAME; nitric oxide; potassium channels; vasodilatation

Abbreviations: BK^+_{Ca}, large conductance calcium-sensitive potassium channel; COX, cyclo-oxygenase; CHAPS, 3-[(cholamidopropyl)dimethyl-ammonio]1-propanesulphonate; ChTX, charybdotoxin; EDHF, endothelium-derived hyperpolarizing factor; FBP, flurbiprofen; IbTX, iberiotoxin; IK^+_{Ca}, intermediate conductance calcium-sensitive potassium channel; K^+_{ATP}, ATP-sensitive potassium channel; K^+_{IR}, inwardly-rectifying potassium channel; L-NAME, N^G-nitro-L-arginine methyl ester; NO, nitric oxide; NOS, nitric oxide synthase; ODQ, 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one; SK^+_{Ca}, small conductance calcium-sensitive potassium channel; TEA, tetraethylammonium

Introduction

Many ocular diseases such as glaucoma (Henry *et al*., 1999) and diabetic retinopathy (Schmetterer *et al*., 1997) are associated with dysfunction of the vascular endothelium. In systemic arteries, the vascular endothelium plays a major role in the regulation of vasomotor tone through the release of the vasodilators, nitric oxide (Moncada *et al*., 1991), prostacyclin (Moncada & Vane, 1979) and the putative endothelium-derived hyperpolarizing factor (EDHF, Chen *et al*., 1988). There is now compelling evidence to support the view that endothelium-derived nitric oxide is an important mediator of vasomotor tone in the eye (for review see Koss, 1999). For example, inhibition of nitric oxide synthase *in vivo* in a wide range of species reduces basal ocular blood flow, as measured using radiolabelled microspheres (Nilsson, 1996; Hardy *et al*., 1996; Seligsohn & Anders, 2000) or laser Doppler flowmetry (Zagvazdin *et al*., 1996; Koss, 1998; Luksch *et al*., 2000). It remains to be determined whether this tonic vasodilator influence of nitric oxide on the ocular vasculature *in vivo* is derived from the endothelium as a result of basal release or release stimulated by agonists (Benedito *et al*., 1991a; Gidday & Zhu, 1995) or by flow (Rubanyi *et al*., 1986), or is derived from perivascular nitrergic nerves (Nilsson, 1996; Toda *et al*., 1998; Zagvazdin *et al*., 1996). Nevertheless, in studies using the porcine isolated perfused eye, where neural influences can be excluded (Meyer *et al*., 1993), and in isolated segments of ophthalmic, ciliary, retinal and irideal arteries from a variety of species including pig, cattle and rat (Benedito *et al*., 1991a; Haefliger *et al*., 1993; Hill & Gould, 1995; Su *et al*., 1994; Yao *et al*., 1991), where the effects of both flow and nerves

*Author for correspondence; E-mail: W.Martin@bio.gla.ac.uk

can be excluded, augmentation of vasoconstrictor tone by inhibitors of nitric oxide synthase still provides evidence of basal release of nitric oxide from the vascular endothelium. In addition to this basal release, acetylcholine, bradykinin and substance P have been shown to induce powerful, endothelium-dependent vasodilator responses sensitive to inhibition of nitric oxide synthase in many studies conducted either *in vivo* (Gidday & Zhu, 1995; Kitamura *et al*., 1993) or *in vitro* on the isolated eye (Meyer *et al*., 1993) or on isolated ocular vessels (Benedito *et al*., 1991a; Gidday & Zhu, 1995; Haefliger *et al*., 1993; Yao *et al*., 1991).

In contrast to the overwhelming evidence supporting the involvement of nitric oxide, most studies suggest a minor role for prostacyclin in the regulation of ocular vasomotor tone (Benedito *et al*., 1991b; Hardy *et al*., 1996 Meyer *et al*., 1993). Furthermore, to the best of our knowledge, the potential role of EDHF has not yet been investigated in the eye. Accordingly, the purpose of this study was to determine the respective roles of endothelium-derived nitric oxide, prostacyclin and EDHF in mediating the vasodilator actions of acetylcholine and bradykinin in the bovine isolated perfused eye preparation. A preliminary account of these findings has already been published (McNeish *et al*., 2001).

Methods

Preparation of tissues

The ciliary vascular bed of the bovine eye was perfused using the constant flow perfusion method of Wilson *et al*. (1993). In brief, bovine eyes obtained from a local abattoir within 1 h of killing were cannulated through a long posterior ciliary artery and perfused at 37°C with Krebs solution containing (mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; glucose, 11.5; and gassed with O_2 containing 5% CO_2. Flow was commenced at $\sim$0.2–0.5 ml min^{-1} and was raised in 5–10 increments to 2.5 ml min^{-1} over a 50 min period. After the final flow rate was achieved, eyes were perfused for an equilibration period of at least 30 min. Perfusion pressure was measured using Gould Statham P32 ID transducers *via* a side arm located immediately proximal to the inflow cannula. Only eyes that had a basal perfusion pressure of 20–60 mmHg after the equilibration period were used for further study. In some experiments a high K^+ (30 mM KCl)-containing Krebs solution was used; in these a proportionate reduction in the NaCl concentration was made to maintain isotonicity.

Experimental protocols

After the equilibration period, drugs were added either to the Krebs reservoir for continuous infusion, or as bolus doses immediately proximal to the cannula. The first experiments involved constructing cumulative concentration-response curves to the thromboxane A_2-mimetic, U46619 (1 nM–10 μM). In these experiments, vasoconstrictor responses to each concentration of U46619 were allowed to stabilize before a higher concentration was added. From these experiments, continuous infusion of U46619 at a concentration of 100–200 nM was chosen to achieve a submaximal perfusion pressure ($\sim$130 mmHg) suitable for conducting experiments with vasodilators. Once this perfusion pressure was established, vasodilator responses to acetylcholine and bradykinin were assessed by adding 10 μl volumes of varying doses with a Hamilton micro-syringe. In some experiments full dose-response curves to acetylcholine (1 pmol–100 nmol) or bradykinin (0.1 pmol–10 nmol) were constructed, but in others, only a single dose was employed. The endothelial dependence of vasodilator responses was tested by infusing the detergent, CHAPS (0.3%, 2 min), to selectively damage the endothelial cell layer (Randall & Hiley, 1988).

The effects of a number of blocking drugs were examined on vasodilator responses to acetylcholine and bradykinin. These drugs were: the nitric oxide synthase inhibitor, L-NAME (100 μM); the inhibitor of soluble guanylate cyclase, ODQ (10 μM); the cyclo-oxygenase inhibitor, flurbiprofen (30 μM); the non-selective K^+ channel blocker, TEA (10 mM); the ATP-sensitive K^+ channel (K^+_{ATP}) blocker, glibenclamide (10 μM); the non-selective, intermediate (IK^+_{Ca}) and large conductance (BK^+_{Ca}) calcium-sensitive K^+ channel blocker, charybdotoxin (10 and 50 nM); the selective BK^+_{Ca} channel blocker, iberiotoxin (50 nM); the selective small conductance (SK^+_{Ca}) calcium-sensitive K^+ channel blocker, apamin (100 nM); the inward rectifier (K^+_{IR}) channel blocker, Ba^{2+} (30 μM); and the Na^+/K^+ ATPase inhibitor, ouabain (10 μM). In each case the blocking drug was infused for at least 20 min before effects on vasodilator responses were tested. In some experiments the blocking drugs themselves (L-NAME, ODQ, TEA, high K^+, charybdotoxin and iberiotoxin) affected the U46619-induced perfusion pressure and these effects are described in the Results section.

Drugs and chemicals

Acetylcholine chloride, apamin (from bee venom), barium chloride, CHAPS (3-[(cholamidopropyl)dimethyl-ammonio]1-propanesulphonate), charybdotoxin (scorpion venom), L-NAME (N^G-nitro-L-arginine methyl ester), ouabain, TEA (tetraethylammonium chloride) and U46619 (9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin $F_{2\alpha}$) were obtained from Sigma (Poole, U.K.). Glibenclamide was a gift from Hoechst (Hounslow, U.K.). Iberiotoxin (synthetic) was obtained from Latoxan (Valence, France), ODQ (1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one) was obtained from Alexis (Nottingham, U.K.). Flurbiprofen was a gift from the Boots Pure Drug company (Nottingham, U.K.). All drugs were dissolved in 0.9% saline except for glibenclamide (3 mM stock), which was dissolved in ethanol, and ODQ (0.1 M stock), which was dissolved in dimethylsulphoxide.

Statistical analysis

Results are expressed as the mean $\pm$ s.e.mean of n separate observations. Vasoconstrictor responses are given in mmHg and vasodilator responses are expressed as percentage (%) reduction of U46619-induced perfusion pressure. Graphs were drawn and statistical comparisons made (Student's t-test, or one-way analysis of variance with Bonferroni's post-test, as appropriate) using the computer package Prism (GraphPad, San Diego, U.S.A.). A probability (P) less than or equal to 0.05 was considered significant.

Results

Basal and U46619-induced perfusion pressure

The basal perfusion pressure of the ciliary vascular bed of the bovine isolated perfused eye preparation at a constant flow of 2.5 ml min^{-1} was 31.8±1.5 mmHg ($n=105$). Addition of the inhibitor of nitric oxide synthase, L-NAME (100 μM), to the perfusate had no effect on this basal perfusion pressure (change of −0.6±3.3 mmHg, $n=8$). The inhibitor of soluble guanylate cyclase, ODQ (10 μM), did, however, produce a small but significant rise in perfusion pressure of 14.6±3.6 mmHg ($n=10$, $P<0.05$). The thromboxane A_2-mimetic, U46619 (1 nM–10 μM), produced a concentration-dependent rise in perfusion pressure ($pEC_{50}=5.61\pm0.03$, maximum rise of 187.6±5.6 mmHg, $n=6$). Moreover, when U46619 (~200 nM) was infused to obtain a sub-maximal rise in perfusion pressure of 86.0±4.3 mmHg, ($n=16$), the addition of L-NAME (100 μM, 20 min) produced a further rise of 37.8±5.0 mmHg ($n=16$, Figure 1b).

Endothelial dependence of vasodilator responses to acetylcholine and bradykinin and effects of L-NAME, ODQ and flurbiprofen

Following infusion of U46619 (100–200 nM), which increased the perfusion pressure to 131.8±16.3 mmHg ($n=8$), bolus injections of acetylcholine (1 pmol–100 nmol) immediately proximal to the preparation produced dose-dependent falls in the vasoconstrictor-induced perfusion pressure ($pED_{50}=9.42\pm0.16$, maximum fall of 64.1±4.3%, Figures 1a and 2a). In experiments in the presence of L-NAME (100 μM, 20 min) or ODQ (10 μM, 20 min) the concentration of U46619 was adjusted such that the final perfusion pressures obtained were not significantly different to control preparations (134.9±10.9 mmHg, $n=9$, and 116.4±17.3 mmHg, $n=5$, respectively). Under these conditions neither L-NAME (Figures 1b and 2a) nor ODQ (Figure 2a), had any effect on the vasodilator actions of acetylcholine. Further-

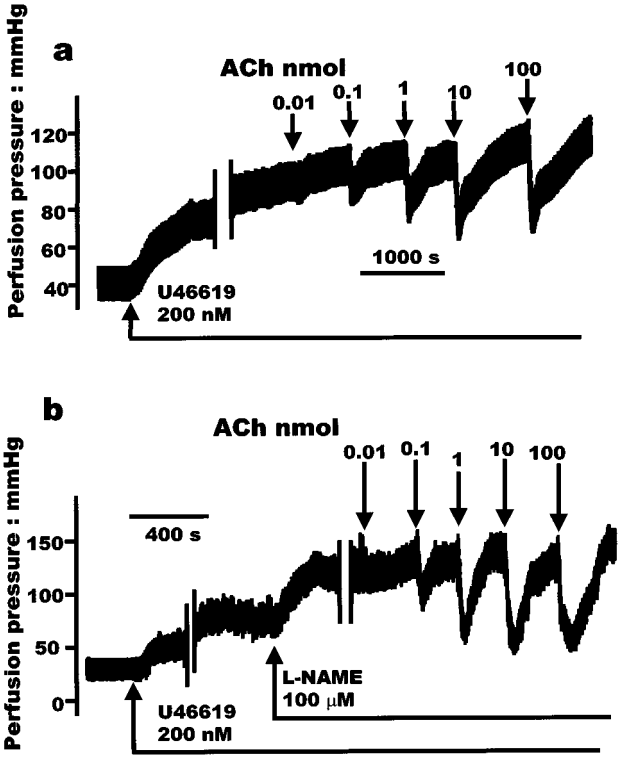


Figure 1 Original traces showing acetylcholine-induced vasodilatation in the bovine isolated perfused eye. The perfusion pressure was increased using the thromboxane A_2-mimetic, U46619 (200 nM), prior to addition of graded doses of ACh. (a) Dose-dependent vasodilatation produced by ACh (0.01–100 nmol). (b) Addition of the nitric oxide synthase inhibitor, L-NAME (100 μM), produced vasoconstriction but had no effect on ACh-induced vasodilatation.

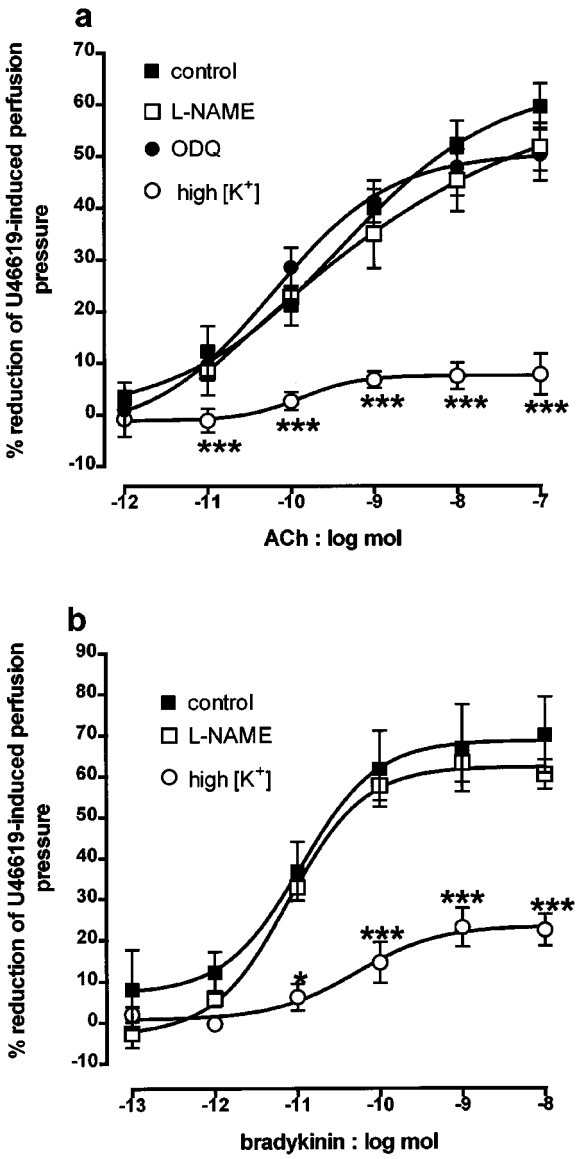


Figure 2 Control dose-response curves showing vasodilatation induced by bolus doses of (a) ACh (1 pmol–100 nmol) and (b) BK (100 fmol–10 nmol) on the bovine isolated perfused eye preparation. The effects of treatment with the nitric oxide synthase inhibitor, L-NAME (100 μM), the inhibitor of soluble guanylate cyclase, ODQ (10 μM), or a high concentration of KCl (30 mM) are also shown. Data represent mean±s.e.mean of five or more observations. *$P<0.05$ and ***$P<0.001$ indicate a difference from control.

more, the fall in perfusion pressure induced by 10 nmol acetylcholine (45.6±3.2%, $n=18$) was unaffected by the cyclo-oxygenase inhibitor flurbiprofen (30 μM, 20 min) nor by the combination of flurbiprofen and L-NAME (Figure 3a).

Bradykinin (0.1 pmol–10 nmol) also produced dose-dependent falls in U46619-induced perfusion pressure ($pED_{50}=10.95\pm0.05$, maximum fall of 68.8±1.1%, $n=6$, Figure 2b). In the presence of L-NAME (100 μM, 20 min), bradykinin-induced falls in perfusion pressure were completely unaffected (Figure 2b). In addition, the fall in perfusion pressure produced by 10 pmol bradykinin (47.0±4.4%, $n=15$) was unaffected by flurbiprofen or by the combination of flurbiprofen and L-NAME (Figure 3b).

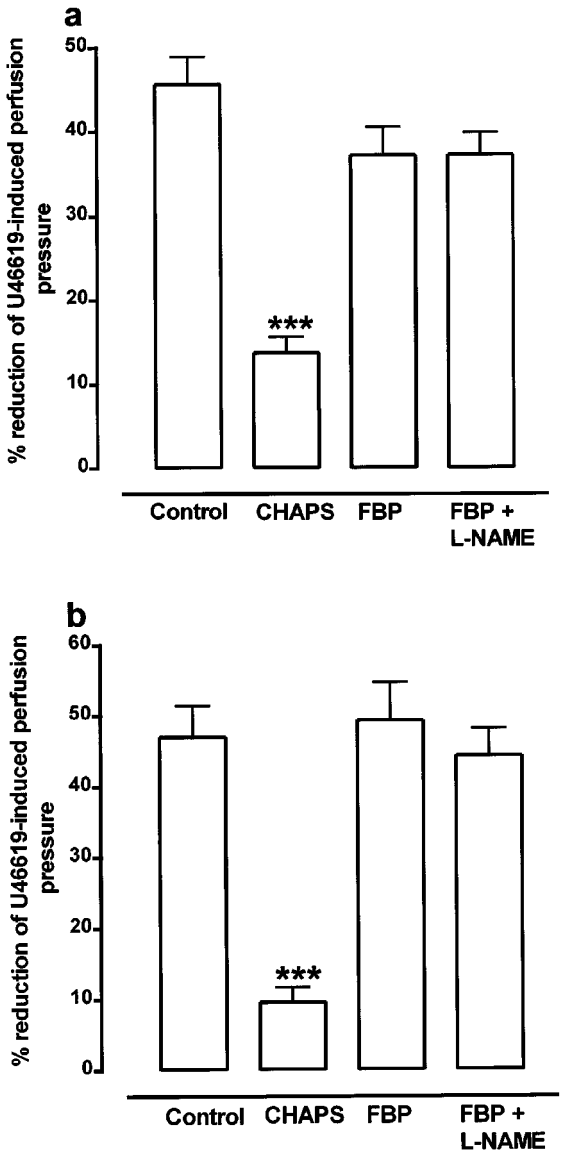


Figure 3 Histogram showing the effects of damaging the endothelium with CHAPS (0.3%, 2 min), inhibiting cyclo-oxygenase with flurbiprofen (FBP, 30 μM) and combined treatment with flurbiprofen and the inhibitor of nitric oxide synthase, L-NAME (100 μM), on vasodilatation induced by (a) ACh (10 nmol) or (b) BK (10 pmol). Data represent the mean±s.e.mean of five or more observations. ***$P<0.001$ indicates a difference from control.

Infusion of the detergent CHAPS (0.3%, 2 min), in order to damage the endothelium, had no effect on U46619-induced perfusion pressure (160.0±16.4 and 170.2±16.6 mmHg, $n=13$, before and after CHAPS, respectively), but resulted in a profound reduction in the fall in perfusion pressure induced by 10 nmol acetylcholine or 10 pmol bradykinin (Figure 3a,b).

Effect of high K^+ on vasodilator responses to acetylcholine and bradykinin

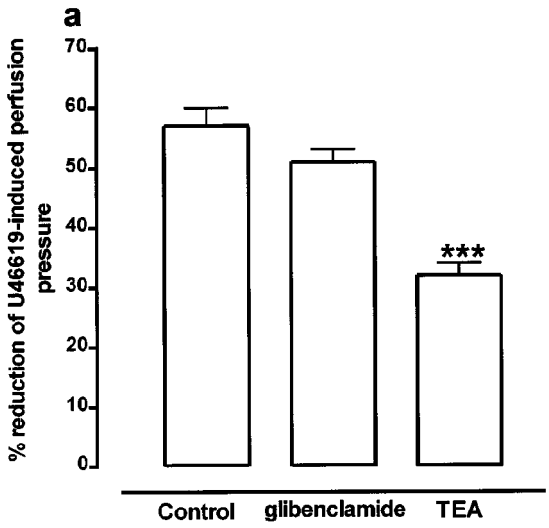
The ability of acetylcholine and bradykinin to induce vasodilator responses was examined in the presence of a high concentration of KCl (30 mM). In these experiments KCl itself produced a sustained increase in perfusion pressure to 98.7±7.1 mmHg ($n=12$), but in order to examine vasodilator responses at levels of pressure similar to control experiments the pressure was increased to 137.6±12.3 mmHg ($n=12$) with the infusion of U46619 (5–10 nM). Under these conditions, vasodilator responses to acetylcholine (1 pmol–100 nmol) and to bradykinin (0.1 pmol–10 nmol) were powerfully inhibited (Figure 2a,b).

Effect of K^+ channel blockers and ouabain on acetylcholine-induced vasodilatation

A number of agents that block specific potassium channels were infused for 20 min and their effects on acetylcholine (10 nmol)-induced vasodilator responses were assessed. The K^+_{ATP} channel blocker, glibenclamide (10 μM), had no effect on U46619-induced perfusion pressure or on acetylcholine-induced vasodilatation. In contrast, the non-selective K^+ channel blocker, TEA (10 mM), produced a large transient increase in U46619-induced perfusion pressure (291.9±57.5 mmHg, $n=4$) as well as a significant inhibition of acetylcholine-induced vasodilatation (Figure 4a).

The K^+_{IR} channel blocker, barium (30 μM), had no effect on acetylcholine-induced vasodilatation, but the Na^+/K^+ ATPase inhibitor, ouabain (10 μM), produced significant blockade (Figure 4b). The combination of barium and ouabain produced a greater blockade of acetylcholine-induced vasodilatation than was seen with ouabain alone (Figure 4b). Neither barium nor ouabain had any effect on U46619-induced perfusion pressure.

The SK^+_{Ca} channel blocker, apamin (100 nM) had no significant effect on U46619-induced perfusion pressure but the IK^+_{Ca}/BK^+_{Ca} inhibitor, charybdotoxin (50 nM; Figure 5) and the BK^+_{Ca} channel inhibitor, iberiotoxin (50 nM), each produced a large transient vasoconstriction (rise in perfusion pressure of 339.6±18.8 mmHg, $n=8$, and 298.5±38.1 mmHg, $n=7$, respectively). Neither apamin (100 nM) nor iberiotoxin (50 nM) alone had any significant effect on the acetylcholine (10 nmol)-induced vasodilatation (Figure 6b), whereas charybdotoxin (50 nM) produced a significant blockade of vasodilatation, and uncovered a vasoconstriction (Figures 5 and 6a). A lower concentration of charybdotoxin (10 nM) had no significant effect on perfusion pressure or acetylcholine-induced vasodilatation by itself, but significantly attenuated the vasodilatation when combined with apamin (100 nM, Figure 6a,b). In contrast the combination of iberiotoxin (50 nM) and apamin (100 nM) had no effect on acetylcholine-vasodilatation.



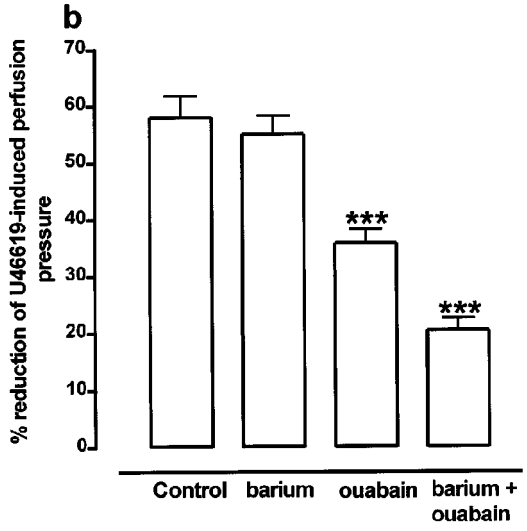


Figure 4 Histogram showing the effects of potassium channel blockers and of ouabain on vasodilatation induced by ACh (10 nmol). (a) Effects of the K^+_{ATP} channel blocker, glibenclamide (10 μM), and of the non-selective K^+_{Ca} channel blocker, tetraethylammonium (TEA, 10 mM). (b) Effects of the inward rectifier K channel (K_{IR}) blocker, barium (30 μM), and of the Na^+/K^+ ATPase inhibitor, ouabain (10 μM), each alone and in combination. Data represent mean ± s.e.mean of five or more observations. ***$P<0.001$ indicates a difference from control.

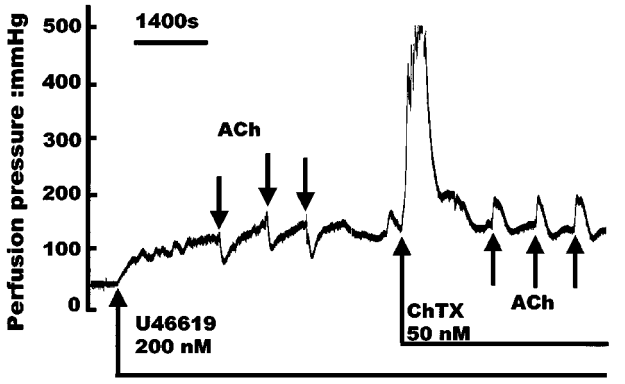


Figure 5 Original trace showing the effects of charybdotoxin (ChTX, 50 nM) on perfusion pressure and on vasodilatation to ACh (10 nmol). The addition of charybdotoxin resulted in a large transient increase in perfusion pressure followed by reversal of ACh-induced vasodilatation to vasoconstriction.

Discussion

The findings of the present study demonstrate that in the ciliary vascular bed of the bovine isolated perfused eye preparation, the vascular endothelium plays an important role in the regulation of perfusion pressure. Specifically, following preconstriction with a submaximal concentration of the thromboxane A_2-mimetic, U46619, we found that inhibition of nitric oxide synthase with L-NAME (100 μM) resulted in further increase in perfusion pressure. This suggested that a basal nitric oxide activity exerts a tonic vasodilator effect in the resistance vessels of the bovine ciliary vasculature. A similar tonic inhibitory action of nitric oxide has also been reported in the porcine isolated perfused eye preparation (Meyer *et al*., 1993).

Whether this 'basal' nitric oxide activity results from a tonic unstimulated release of nitric oxide or from release stimulated by flow-induced shear stress acting on the vascular endothelium (Rubanyi *et al*., 1986) remains to be determined. Nevertheless, studies using bovine isolated retinal rings (Benedito *et al*., 1991a) and porcine ophthalmic and ciliary arterial rings (Haefliger *et al*., 1993) in tissue baths where shear stress is absent also indicate the presence of a tonic vasodilator action of basal nitric oxide. Moreover, the ability of inhibitors of nitric oxide synthase to reduce ocular blood flow in species as diverse as rat (Koss, 1998), rabbit (Nilsson, 1996), dog (Kitamura *et al*., 1993) and human (Luksch *et al*., 2000), demonstrates an important role for nitric oxide in regulating blood flow in the eye.

We wished to investigate the role of the vascular endothelium in the vasodilator responses to acetylcholine and bradykinin in the bovine isolated perfused eye. We found that both drugs induced powerful dose-dependent vasodilator responses. Moreover, infusion of the detergent, CHAPS, which has previously been used to induce selective damage of the vascular endothelium in the rat isolated perfused mesentery preparation (Randall & Hiley, 1988), almost abolished vasodilator responses to acetylcholine and bradykinin in the bovine eye. Surprisingly, CHAPS failed to reproduce the rise in U46619-induced perfusion pressure seen when basal nitric oxide activity was abolished with L-NAME. It is possible, however, that CHAPS might have induced some slight additional damage to the smooth muscle which obscured this expected rise. It seems reasonable to conclude, however, that the vasodilator responses to acetylcholine and bradykinin are endothelium dependent in this preparation. Nevertheless, we found that vasodilator responses to acetylcholine and bradykinin were completely unaffected following the inhibition of nitric oxide synthase by L-NAME. Moreover, the inhibitor of soluble guanylate cyclase, ODQ also failed to inhibit vasodilator responses to acetylcholine. Thus, our findings suggest that nitric oxide does not contribute at all to the vasodilator responses to acetylcholine or bradykinin in the bovine eye. These findings are surprising and are in sharp contrast to those obtained in isolated rings

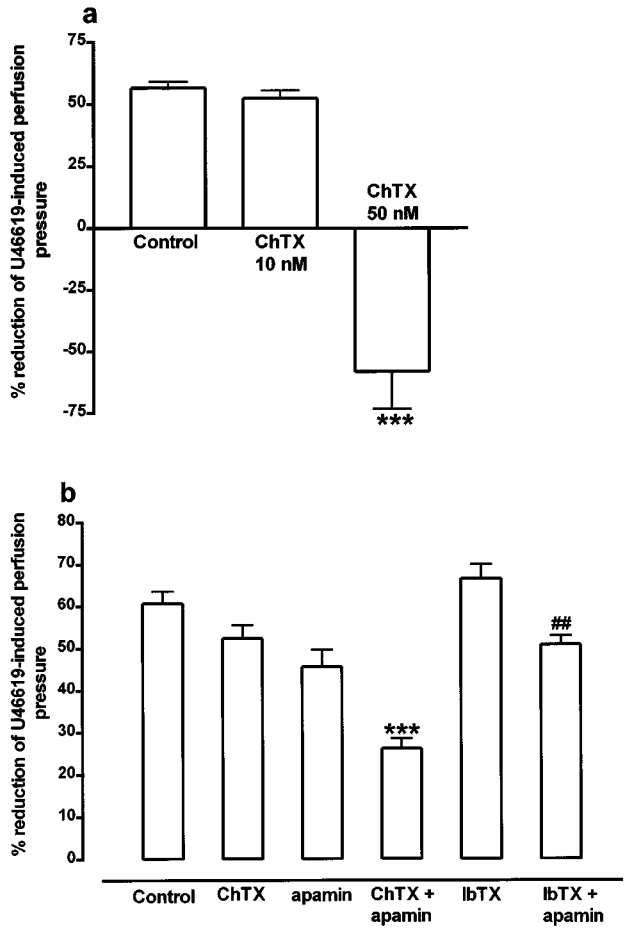


Figure 6 Histograms showing the effect of calcium-sensitive potassium channel inhibitors on vasodilatation induced by ACh (10 nmol). (a) Effects of the large/intermediate conductance (BK^+_{Ca}/IK^+_{Ca}) calcium-sensitive K^+ channel blocker, charybdotoxin (ChTX, 10 nM and 50 nM). (b) Effects of the small conductance calcium-sensitive K^+ (SK^+_{Ca}) channel blocker, apamin (100 nM), alone and in combination with a low concentration of ChTX (10 nM), and of the large conductance calcium sensitive K^+ (BK^+_{Ca}) channel blocker, iberiotoxin (IbTX, 50 nM), alone and in combination with apamin (100 nM). Data represent the mean ± s.e.mean of five or more observations. *** $P<0.001$ indicates a difference from control and ##$P<0.005$ shows that the combination of iberiotoxin and apamin was different from iberiotoxin alone.

of porcine ophthalmic artery (Yao *et al*., 1991) or ciliary artery (Haefliger *et al*., 1993; Zhu *et al*., 1997) and strips of monkey ciliary artery (Toda *et al*., 1998), where vasodilatation induced by either acetylcholine or bradykinin were sensitive to inhibition of nitric oxide synthase. It is thus possible that nitric oxide has more importance in regulating vascular tone in larger vessels than in resistance vessels in the bovine ciliary vasculature. Indeed, such a decreasing role for nitric oxide with decreasing size of blood vessel has been previously noted in the rat mesenteric vascular bed (Hwa *et al*., 1994). However, in contrast to our findings in the bovine eye, bradykinin-induced vasodilator responses in the porcine isolated perfused eye were powerfully blocked by L-NAME (Meyer *et al*., 1993), suggesting an important role for nitric oxide in such responses in this species. Furthermore, vasodilatation to acetylcholine in the retinal circulation of newborn pigs is sensitive to inhibition of nitric oxide synthase (Gidday & Zhu, 1995).

We also found that the vasodilator responses to acetylcholine and bradykinin in the bovine eye were completely unaffected following treatment with the cyclo-oxygenase inhibitor, flurbiprofen, or following combined treatment with flurbiprofen and L-NAME. It is therefore clear that neither nitric oxide nor a cyclo-oxygenase product is responsible for the powerful vasodilator responses to acetylcholine and bradykinin in this preparation. To find that acetylcholine- and bradykinin-induced vasodilatation in the bovine eye is completely independent of involvement of nitric oxide was rather surprising, but similar findings have been made in the rabbit hindlimb (Mügge *et al*., 1991) and rat renal artery (Jiang & Dusting, 2001). In blood vessels where an endothelium-derived hyperpolarizing factor (EDHF) reportedly exists, such as in the rat mesenteric vascular bed (McCulloch *et al*., 1997; Adeagbo & Triggle, 1993) or in human coronary (Kato *et al*., 1997) or forearm resistance vessels (Honing *et al*., 2000), it is more common to find it functioning together with nitric oxide.

The nature of the EDHF is still unresolved, but possible candidates include a cytochrome P_{450} metabolite (Bauersachs *et al*., 1994), perhaps an epoxyeicosatrienoic acid (EET; Fisslthaler *et al*., 1999), an endogenous cannabinoid (Randall *et al*., 1996) or potassium ions (Edwards *et al*., 1998). Moreover, evidence has been reported suggesting that the process of relaxation involves hyperpolarization of endothelial cells (Edwards *et al*., 1998; 2000) and consequent gap junctional transmission between endothelium and smooth muscle, resulting in smooth muscle hyperpolarization and relaxation (Davies *et al*., 1988; Beny, 1990; Chaytor *et al*., 1998). Regardless of the conflicting evidence for the proposed candidates, there is consensus that EDHF responses are mediated by an increase in K^+ conductance and thus are sensitive to depolarizing solutions of K^+ (Adeagbo & Triggle, 1993; Corriu *et al*., 1996a) and by inhibitors of certain types of K^+ channels. The possibility that acetylcholine- and bradykinin-induced vasodilator responses in the bovine eye were produced by an EDHF was therefore investigated further. Consistent with this possibility, we found that in the presence of 30 mM KCl, vasodilator responses to acetylcholine and bradykinin were almost abolished.

It has been suggested that K^+ released from the endothelium can act as an EDHF by stimulating the Na^+/K^+ATPase or inwardly rectifying K^+ channels (K^+_{IR}) on smooth muscle of rat hepatic arteries (Edwards *et al*., 1998). Therefore, we examined the effect of the K^+_{IR} inhibitor, barium, and the Na^+/K^+ATPase inhibitor, ouabain, on vasodilator responses to acetylcholine in the bovine perfused eye preparation. Vasodilatation was unaffected by barium, inhibited by ouabain and further inhibited by the combination of ouabain and barium. The finding that vasodilator responses to acetylcholine in the bovine eye are sensitive to inhibition of the Na^+/K^+ATPase and K^+_{IR} could be consistent with the hypothesis that K^+ ions can act as EDHF. However, the possible role of K^+ has been challenged recently by the finding that EDHF-mediated responses are unaffected by ouabain or barium in guinea-pig carotid and porcine coronary artery (Quignard *et al*., 1999) and rat hepatic artery (Andersson *et al*., 2000). Moreover, in rat mesenteric (Lacy *et al*., 2000) and renal

arteries (Jiang & Dusting, 2001), potassium-induced vasodilatation is abolished following removal of the endothelium, thus casting doubt on a role for this ion as EDHF in these vessels.

Having established that high K^+ powerfully inhibited endothelium-dependent vasodilatation in the bovine eye, we investigated the possible involvement of potassium channels. K^+_{ATP} channels are not generally considered to be involved in EDHF-mediated vasodilatation (Corriu *et al*., 1996b) and, consistent with this, we found that addition of glibenclamide, a blocker of K^+_{ATP} channels, had no effect on the vasodilatation induced by acetylcholine in the bovine eye. The vasodilatation was, however, inhibited by TEA, which is a relatively non-selective calcium-sensitive K^+ (K^+_{Ca}) channel inhibitor (Cook & Quast, 1990), although it does not block SK^+_{Ca}, even at concentrations up to 4 mM (Zima *et al*., 2000). Our ability to block acetylcholine-induced vasodilatation in the bovine eye with TEA is in keeping with its ability to inhibit EDHF-mediated vasodilatation in human forearm resistance vessels (Honing *et al*., 2000) and in rat mesenteric arteries (Chen & Cheung, 1997).

Recent evidence suggests that the action of EDHF is normally abolished by the combination of the small conductance K^+_{Ca} (SK^+_{Ca}) channel blocker, apamin, with the large and intermediate conductance K^+_{Ca} (BK^+_{Ca}/IK^+_{Ca}) blocker, charybdotoxin, but not by the combination of apamin and the large conductance K^+_{Ca} (BK^+_{Ca}) inhibitor, iberiotoxin (Zygmunt & Hogestatt, 1996; Edwards *et al*., 1998; 1999). On the basis of these results, the EDHF pathway is thought to involve the opening of SK^+_{Ca} channels, and IK^+_{Ca} channels but not BK^+_{Ca} channels on the endothelial cell (Edwards *et al*., 1998; Ohashi *et al*., 1999). We found in the bovine eye that apamin (100 nM) had no significant effect on U46619-induced perfusion pressure but charybdotoxin (50 nM) and iberiotoxin (50 nM) both caused a large transient vasoconstrictor response, similar to that seen with TEA (see above). Inhibition of BK^+_{Ca} channels is known to inhibit nitric oxide-induced vasodilatation (Demirel *et al*., 1994; Champion & Kadowitz, 1997). We think it is unlikely, however, that these powerful transient vasoconstrictions reflect inhibition of basal nitric oxide activity, since the nitric oxide synthase inhibitor, L-NAME, produced much smaller rises in perfusion pressure which were maintained for many hours. Perhaps our findings indicate that BK^+_{Ca} channels are normally open under the conditions used in our study. Moreover, apamin (100 nM) and iberiotoxin (50 nM) alone or in combination had no significant effect on the acetylcholine-induced vasodilatation. In contrast, charybdotoxin (50 nM) alone significantly attenuated acetylcholine-induced vasodilatation, and uncovered a vasoconstrictor response. In previous studies (Zygmunt & Hogestatt, 1996; Edwards *et al*., 1998; 1999) charybdotoxin alone did not attenuate acetylcholine-induced vasodilatation as powerfully as was seen in the bovine eye, and this may reflect the absence of the unusual acetylcholine-induced vasoconstriction observed in our preparation. Full characterization of this vasoconstrictor response was beyond the scope of this study, but it appears to be endothelium-dependent, since it was not observed when the endothelium was functionally impaired with CHAPS. Previous studies have reported anomalous, endothelium-dependent vasoconstrictor responses in rat aorta (Franchi-Micheli *et al*., 2000) and porcine renal interlobar artery (Derkach *et al*., 2000). A lower concentration of charybdotoxin (10 nM) had no effect on perfusion pressure or vasodilatation. Nevertheless, when this lower concentration was combined with apamin, a significant attenuation of acetylcholine-induced vasodilatation was observed. Thus, the attenuation of the acetylcholine-induced vasodilatations with the combination of apamin and charybdotoxin, and the lack of effect of the combination of apamin and iberiotoxin is consistent with the possibility that the vasodilator responses observed in the bovine perfused eye are mediated by an EDHF.

In summary, a basal level of nitric oxide regulates perfusion pressure in the cilliary vascular bed of the bovine perfused eye. Nevertheless, despite acetylcholine- and bradykinin-induced vasodilatation being endothelium-dependent, they do not involve nitric oxide or products of cyclooxygenase. The blockade of acetylcholine-induced vasodilatation by 30 mM KCl, or by K^+_{Ca} channel inhibitors, particularly charybdotoxin (50 nM), or the combination of apamin (100 nM) with a low concentration of charybdotoxin (10 nM), suggest involvement of an EDHF. To the best of our knowledge, this is the first report to suggest an important role for an EDHF in the control of vasomotor tone in the eye.

We are grateful to the British Heart Foundation and Wellcome Trust for support.

References

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

ANDERSSON, D.A., ZYGMUNT, P.M., MOVAHED, P., ANDERSSON, T.L.G. & HOGESTATT, E.D. (2000). 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. *Br. J. Pharmacol.,* **129,** 1490–1496.

BAUERSACHS, J., HECKER, M. & BUSSE, R. (1994). Display of the characteristics of endothelium-derived hyperpolarizing factor by a cytochrome P450-derived arachidonic acid metabolite in the coronary microcirculation. *Br. J. Pharmacol.,* **113,** 1548–1553.

BENEDITO, S., PRIETO, D., NIELSEN, P.J. & NYBORG, N.C.B. (1991a). Role of the endothelium in acetylcholine-induced relaxation and spontaneous tone of bovine isolated retinal small arteries. *Exp. Eye Res.,* **52,** 575–579.

BENEDITO, S., PRIETO, D., NIELSEN, P.J. & NYBORG, N.C.B. (1991b). Histamine induces endothelium-dependent relaxation of bovine retinal arteries. *Invest. Ophthalmol. Vis. Sci.,* **32,** 32–38.

BENY, J.L. (1990). Endothelial and smooth-muscle cells hyperpolarized by bradykinin are not dye coupled. *Am. J. Physiol.,* **258,** H836–H841.

CHAMPION, H.C. & KADOWITZ, P.J. (1997). Vasodilator responses to acetylcholine, bradykinin and substance P are mediated by a TEA-sensitive mechanism. *Am. J. Physiol.,* **273,** R414–R422.

CHAYTOR, A.Y., EVENS, W.H. & GRIFFITH, T.M. (1998). Central role of heterocellular gap junctional communication in endothelium-dependent relaxations of rabbit arteries. *J. Physiol.,* **508,** 561 – 573.

CHEN, G. & CHEUNG, D.W. (1997). Effect of K^+-channel blockers in acetylcholine-induced hyperpolarisation in rat mesenteric arteries. *Am. J. Physiol.,* **272,** H2306 – H2312.

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

COOK, N.S. & QUAST, U. (1990). Potassium channel pharmacology. In *Potassium channels: structure, classification, function and therapeutic potential.* pp 181 – 255. Chichester: Ellis Hardwood.

CORRIU, C., FELETOU, M., CANET, E. & VANHOUTTE, P.M. (1996a). Inhibitors of the cytochrome P450-mono-oxygenase and endothelium-dependent hyperpolarizations in the guinea pig isolated cartoid artery. *Br. J. Pharmacol.,* **117,** 607 – 610.

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

DAVIES, P.F., OLESEN, S.P., CLAPHAM, D.E., MORREL, E.M. & SCHOEN, F.J. (1988). Endothelial communication. State of the art lecture. *Hypertension,* **11,** 563 – 572.

DEMIREL, E., RUSCO, J., LASKEY, R.E., ADAMS, D.J. & VAN BREEMEN. (1994). TEA inhibits ACh-induced EDRF release: endothelial Ca^{2+}-dependent K^+ channels contribute to vascular tone. *Am. J. Physiol.,* **267,** H1135 – H1141.

DERKACH, D.N., HIRAND, K., NISHIMURA, J., TAKAHASHI, S. & KANAIDE, H. (2000). Thrombin causes endothelium-dependant biphasic regulation of vascular tone in the porcine renal interlobar artery. *Br. J. Pharmacol.,* **131,** 1635 – 1642.

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., GARDENER, M.J., FELETOU, M., BRADY, G., VANHOUTTE, P.M. & WESTON, A.H. (1999). Further investigation of endothelium-derived hyperpolarizing factor (EDHF) in rat hepatic artery: studies using 1-EBIO and ouabain. *Br. J. Pharmacol.,* **128,** 1064 – 1070.

EDWARDS, G., THOLLON, C., GARDENER, M.J., FELETOU, M., VILAINE, J.-P., VANHOUTTE, P.M. & WESTON, A.H. (2000). Role of gap junctions and EETs in endothelium-dependent hyperpolarization of porcine coronary artery. *Br. J. Pharmacol.,* **129,** 1145 – 1154.

FISSLTHALER, B., POPP, R., KISS, L., POTENTE, M., HARDER, D.R., FLEMING, I. & BUSSE, R. (1999). Cytochrome P450 2C is an EDHF synthase in coronary arteries. *Nature,* **401,** 493 – 497.

FRANCHI-MICHELI, S., FAILLI, P., MAZZETTI, L., BANI, D., CIUFFI, M. & ZILLETTI, L. (2000). Mechanical stretch reveals different components of endothelial-mediated vascular tone in rat aortic strips. *Br. J. Pharmacol.,* **131,** 1355 – 1362.

GIDDAY, J.M. & ZHU, Y. (1995). Nitric oxide does not mediate autoregulation of retinal blood flow in newborn pig. *Am. J. Physiol.,* **269,** H1065 – H1072.

HAEFLIGER, I.O., FLAMMER, J. & LÜSCHER, T.F. (1993). Heterogeneity of endothelium-dependent regulation in ophthalmic and ciliary arteries. *Invest. Ophthalmol. Vis. Sci.,* **34,** 1722 – 1730.

HARDY, P., NUYT, A.M., ABRAN, D., ST-LOUIS, J., VARMA, D.R. & CHEMTOB, S. (1996). Nitric oxide in retinal and choroidal blood flow autoregulation in newborn pigs: interactions with prostaglandins. *Pediatr. Res.,* **39,** 487 – 493.

HENRY, E., NEWBY, D.E., WEBB, D.J. & O'BRIEN, C. (1999). Peripheral endothelial dysfunction in normal pressure glaucoma. *Invest. Ophthalmol. Vis. Sci.,* **40,** 1710 – 1714.

HILL, C.E. & GOULD, D.J. (1995). Modulation of sympathetic vasoconstriction by sensory nerves and nitric oxide in rat irideal arterioles. *J. Pharmacol. Exp. Ther.,* **273,** 918 – 926.

HONING, M.L.H., SMITS, P., MORRISON, P.J. & RABELINK, T.J. (2000). Bradykinin-induced vasodilation of human forearm resistance vessels is primarily mediated by endothelium-dependent hyperpolarization. *Hypertension,* **35,** 1314 – 1318.

HWA, J.J., GHIBAUDI, L., WILLIAMS, P. & CHATTERJEE, M. (1994). Comparison of acetylcholine-induced relaxation in large and small arteries of rat mesenteric vascular bed. *Am. J. Physiol.,* **266,** H952 – H958.

JIANG, F. & DUSTING, G.J. (2001). Endothelium-dependent vasorelaxation independent of nitric oxide and K^+ release in isolated renal arteries of rats. *Br. J. Pharmacol.,* **132,** 1558 – 1564.

KATO, M., SHIODE, N., YAMAGATA, T., MATSUURA, H. & KAJIYAMA, G. (1997). Bradykinin induced dilatation of human epicardial and resistance coronary arteries *in vivo*: effect of inhibition of nitric oxide synthesis. *Heart,* **78,** 493 – 498.

KITAMURA, Y., OKAMURA, T., KANI, K. & TODA, N. (1993). Nitric oxide mediated retinal arteriolar and arterial dilatation induced by substance P. *Invest. Ophthalmol. Vis. Sci.,* **34,** 2859 – 2865.

KOSS, M.C. (1998). Role of nitric oxide in maintenance of basal anterior choroidal blood flow in rats. *Invest. Ophthalmol. Vis. Sci.,* **39,** 559 – 564.

KOSS, M.C. (1999). Functional role of nitric oxide in regulation of ocular blood flow. *Eur. J. Pharmacol.,* **374,** 161 – 174.

LACY, P.S., PILKINGTON, G., HANVESAKUL, R., FISH, H.J., BOYLE, J.P. & THURSTON, H. (2000). Evidence against potassium as an endothelium-derived hyperpolarizing factor in rat mesenteric arteries. *Br. J. Pharmacol.,* **129,** 605 – 611.

LUKSCH, A., POLAK, K., BEIER, C., POLSKA, E., WOLZT, M., DORNER, G.T., EICHLER, H.G. & SCHMETTERER, L. (2000). Effects of systemic NO synthase inhibition on choroidal and optic nerve head blood flow in healthy subjects. *Invest. Ophthalmol. Vis. Sci.,* **41,** 3080 – 3084.

MCCULLOCH, A.I., BOTTRILL, F.E., RANDALL, M.D. & HILEY, C.R. (1997). Characterization and modulation of EDHF-mediated relaxations in the rat isolated superior mesenteric arterial bed. *Br. J. Pharmacol.,* **120,** 1431 – 1438.

MCNEISH, A.J., MARTIN, W. & WILSON, W.S. (2001). Characterisation of endothelium-dependent vasodilator responses in the bovine isolated perfused eye. *Br. J. Pharmacol.,* **133,** 70P.

MEYER, P., FLAMMER, J. & LÜSCHER, T.F. (1993). Endothelium-dependent regulation of the ophthalmic microcirculation in the perfused porcine eye: role of nitric oxide and endothelins. *Invest. Ophthalmol. Vis. Sci.,* **34,** 3614 – 3621.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.,* **43,** 109 – 142.

MONCADA, S. & VANE, J.R. (1979). Pharmacology and endogenous roles of prostaglandins, endoperoxides, thromboxane A_2 and prostacyclin. *Pharmacol. Rev.,* **30,** 293 – 331.

MÜGGE, A., ANTONIO, J., LOPEZ, G., PIEGORS, D.J., BREESE, K.R. & HEISTAD, D.D. (1991). Acetylcholine-induced vasodilatation in rabbit hindlimb in vivo is not inhibited by analogues of L-arginine. *Am. J. Physiol.,* **260,** H242 – H247.

NILSSON, S.F.E. (1996). Nitric oxide as a mediator of parasympathetic vasodilation in ocular and extraocular tissues in the rabbit. *Invest. Ophthalmol. Vis. Sci.,* **37,** 2110 – 2119.

OHASHI, M., SATOH, K. & ITOH, T. (1999). Acetylcholine-induced membrane potential changes in endothelial cells of rabbit aortic valve. *Br. J. Pharmacol.,* **126,** 19 – 26.

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

RANDALL, M.D., ALEXANDER, S.P.H., BENNETT, T., BOYD, A.E., FRY, J.R., GARDINER, S.M., KEMP, P.A., MCCULLOCH, A.I. & KENDALL, D.A. (1996). An endogenous cannabinoid as an endothelium-derived vasorelaxant. *Biochem. Biophys. Res. Com.,* **229,** 114 – 120.

RANDALL, M.D. & HILEY, C.R. (1988). Detergent and methylene blue affect endothelium-dependent vasorelaxation and pressure/flow relations in rat blood perfused mesenteric arterial bed. *Br. J. Pharmacol.,* **95,** 1081 – 1088.

RUBANYI, G.M., ROMERO, J.C. & VANHOUTTE, P.M. (1986). Flow-induced release of endothelium-derived relaxing factor. *Am. J. Physiol.,* **250,** H1145 – H1149.

SCHMETTERER, L., FINDL, O., FASCHING, P., FERBER, W., STRENN, K., BRIETENEDER, H., ADAM, H., EICHLER, H.G. & WOLZT, M. (1997). Nitric oxide and ocular blood flow in patients with IDDM. *Diabetes,* **46,** 653 – 658.

SELIGSOHN, E.E. & ANDERS, B. (2000). Effects of N^G-nitro-L-arginine methyl ester on the cardiovascular system of anesthetized rabbit and on the cardiovascular response to thyrotropin-releasing hormone. *Br. J. Pharmacol.,* **109,** 1219 – 1225.

SU, E.-N., ALDER, V.A., YU, D.-Y. & CRINGLE, S.J. (1994). Adrenergic and nitrergic neurotransmitters are released by the autonomic system of the pig long posterior ciliary artery. *Curr. Eye Res.,* **13,** 907 – 917.

TODA, N., TODA, M., AYAJIKI, K. & OKAMURA, T. (1998). Cholinergic nerve function in monkey ciliary arteries innervated by nitroxidergic nerve. *Am. J. Physiol.,* **274,** H1582 – H1589.

WILSON, W.S., SHAHIDULLAH, M. & MILLAR, C. (1993). The bovine arterially perfused eye: an *in vitro* method for the study of drug mechanisms on IOP, aqueous humour formation and uveal vasculature. *Curr. Eye Res.,* **12,** 609 – 620.

YAO, K., TSCHUDI, M., FLAMMER, J. & LÜSCHER, T.F. (1991). Endothelium-dependent regulation of vascular tone of the porcine ciliary artery. *Invest. Opthalmol. Vis. Sci.,* **32,** 1791 – 1798.

ZAGVAZDIN, Y.S., FITZGERALD, M.E.C., SANCESARIO, G. & REINER, A. (1996). Neural nitric oxide mediates Edinger-Westphal nucleus evoked increase in choroidal blood flow in the pigeon. *Invest. Ophthalmol. Vis. Sci.,* **37,** 666 – 673.

ZHU, P., BÉNY, J.L., FLAMMER, J., LÜSCHER, T.F. & HAEFLIGER, I.O. (1997). Relaxation by bradykinin in porcine ciliary artery: role of nitric oxide and K^+ channels. *Invest. Ophthalmol. Vis. Sci.,* **38,** 1761 – 1767.

ZIMA, P.A.V., HARHUN, M.I. & SHUBA, M.F. (2000). Properties of the apamin-sensitive component of Ca^{2+}-dependent K^+ current in smooth muscle cells of the guinea pig taenia coli. *Neurophysiology,* **32,** 63 – 69.

ZYGMUNT, P.M. & HOGESTATT, 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.

(Received April 23, 2001
Revised July 2, 2001
Accepted July 8, 2001)