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Biphasic neurogenic vasodilatation in the bovine intraocular long posterior ciliary artery: involvement of nitric oxide and an additional unidentified neurotransmitter

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1 We have investigated the neurogenic factors inducing relaxation in the intraocular segment of the bovine long posterior ciliary artery.

2 In precontracted vessels, electrical field stimulation (EFS, 0.5–128 Hz, 10 s trains) in the presence of guanethidine (30 μM) evoked biphasic relaxation: optimal relaxation for the first and second components occurred at 10 and 50 s, respectively. The first component, but not the second, was abolished by L-NAME (100 μM) or ODQ (3 μM).

3 Relaxation to exogenous CGRP (0.1–300 nM) was inhibited by the CGRP antagonist, $CGRP_{8-37}$ (1–5 μM), but neither component of neurogenic relaxation was affected. Preincubation with the sensory nerve excitotoxin, capsaicin (1 μM), had no effect on either the first or second components of neurogenic relaxation.

4 Substance P (0.1 nM–0.1 μM) induced relaxation, but rapid and complete desensitisation occurred within minutes. Neither desensitisation to substance P (0.1 μM) nor incubation with the NK_1 antagonist, L-733,060 (0.3 μM), had any effect on the first or second components of neurogenic relaxation.

5 VIP (0.1 nM–0.3 μM) induced relaxation and this was followed by substantial desensitisation. Neither desensitisation to VIP (0.6 μM) nor treatment with the protease, α-chymotrypsin (10 U ml^{-1}), had any effect on the first or second components of neurogenic relaxation.

6 The results indicate that nitric oxide mediates the first component of neurogenic relaxation in the bovine intraocular ciliary artery. The neurotransmitter mediating the second component remains to be determined but is unlikely to be CGRP, substance P or VIP.
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Abbreviations: ATP, adenosine triphosphate; CGRP, calcitonin gene-related peptide; EFS, electrical field stimulation; i.d., internal diameter; L-NAME, N^G-nitro-L-arginine methyl ester; NANC, nonadrenergic, noncholinergic; NK, neurokinin; ODQ, 1H[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonate; TTX, tetrodotoxin; VIP, vasoactive intestinal polypeptide

Introduction

Evidence from studies conducted both *in vivo* and *in vitro* demonstrate a major role for nitric oxide in controlling vascular tone in the eye of many species (for reviews see Koss, 1999; Schmetterer & Polak, 2001). In keeping with this, we have recently reported that nitric oxide exerts a tonic vasodilator action in the bovine ciliary circulation (McNeish *et al*., 2001; 2002). Our work further demonstrated that endothelium-derived hyperpolarising factor (EDHF) is the sole mediator of agonist-induced vasodilatation in this vascular bed. In order to improve our understanding of vasomotor control in the bovine ciliary circulation, we wished to investigate the role of autonomic nerves. Previous reports describe neurogenic vasodilatation in retinal and ciliary arteries from a range of species, including dog, pig, monkey and human (Nyborg & Nielsen, 1994; Toda *et al*., 1994; 1997b; 1998; 1999; Ayajiki *et al*., 2000). In each of these studies, inhibitors of nitric oxide synthase abolished the dilatation demonstrating that it was mediated entirely by nitrergic nerves. In the bovine eye, neurogenic vasodilatation has been reported in the intraocular long posterior ciliary artery (Wiencke *et al*., 1994). These authors reported that the dilator response was monophasic but that it decayed with either a fast or a slow time course. They concluded that nitric oxide and CGRP were responsible for the fast- and slow-decaying components of the dilatation, respectively. As a first step in investigating the role of nerves in regulating vasomotor tone in the bovine ciliary circulation, we wished to determine if, as previously reported (Wiencke *et al*., 1994), the bovine intraocular long posterior ciliary artery was innervated by both nitrergic nerves and nerves that release CGRP as neurotransmitter.

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Methods

Preparation of bovine intraocular long posterior ciliary artery rings for electrical field stimulation and tension measurement

Bovine eyes were obtained from a local abattoir within 90 min of killing. At the laboratory, the intraocular long posterior ciliary artery ($\sim$400 μm internal diameter (i.d.)) was dissected out and cut into 2 mm long ring segments and mounted in wire myographs (Multi Myograph model 610; Danish Myo Technology). The vessels were maintained at 37°C in Krebs solution (pH 7.4) containing (mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $NaHCO_3$, 25; glucose, 11.5; and gassed with O_2 containing 5% CO_2. Isometric tension was recorded and displayed using a Powerlab 4/20 and Chart v5 (both ADInstruments, Hastings, U.K.). Tension was applied to the vessels to give a transmural pressure equivalent to $\sim$100 mmHg (actual equivalents 102.7$\pm$0.2 mmHg, $n=167$). Tissues were allowed to equilibrate for 30 min before experiments were carried out. When endothelial denudation was required, this was accomplished by inserting a human hair through the lumen of the vessel and gently rubbing the walls of the vessel for 2 min, prior to mounting in the myograph. Absence of the dilator response to bradykinin (1 μM) provided evidence of successful denudation.

Electrical field stimulation

Rings were mounted on myograph jaws containing a pair of parallel platinum electrodes (Danish Myo Technology) for electrical field stimulation (EFS). EFS, consisting of square wave pulses (10–18 V, 0.3 ms pulse width, 10 s train length at each frequency ranging from 0.5 to 128 Hz), was delivered using an S88 stimulator (Grass, Quincy, U.S.A.). A minimum period of 5 min was left between trains of stimuli to permit recovery of vessel tone. Stimulation parameters were designed to evoke maximal neurogenic and minimal myogenic response. Tetrodotoxin (TTX, 0.1 μM) was used to confirm the neurogenic origin of responses obtained. All experiments were carried out in the presence of submaximal tone (60–80%) induced by the thromboxane A_2 mimetic, U46619 (0.1–1 μM), and adrenergic neurone blockade using guanethidine (30 μM). Preliminary experiments showed that noradrenaline produced a poor level of tone, which was not sustained, and KCl could not be used since it activates perivascular nerves. A previous study demonstrated that nitrergic relaxation can be elicited under tone induced by a wide variety of contractile stimuli, although the magnitude of the relaxation was observed to vary slightly (Gibson *et al*., 1994). Care was taken to ensure that the level of tone was similar in control and drug-treated vessels. A control frequency response curve (0.5–128 Hz, 10 s trains) was obtained for each vessel before a second curve was generated in the presence of the appropriate blocking agent. Preliminary experiments demonstrated the reproducibility of the vasodilator responses, such that a second control frequency–response curve did not differ from the first. L-NAME (100 μM) and ODQ (3 μM), respectively, were used to determine the role of nitric oxide synthase and soluble guanylate cyclase in the relaxant response to EFS. The $CGRP_1$ receptor antagonist, $CGRP_{8-37}$ (1 and 5 μM), and the NK_1 receptor antagonist, L-733,060 (0.3 μM), were used to explore the involvement of CGRP and substance P, respectively, in the relaxation to EFS. VIP antiserum (dilution 1 : 250, incubated for 2 h) and α-chymotrypsin (10 U ml^{-1}) were used in attempts to investigate the involvement of VIP.

Furthermore, additional protocols involving desensitisation of receptors were used to investigate involvement of VIP and substance P in the relaxation to EFS. In all, 0.3 μM VIP was introduced to promote relaxation and the tone allowed to stabilise following loss of the relaxant response. The same concentration of the peptide was added again and lack of response was taken as an indication that receptors had become desensitised. In contrast, desensitisation to substance P was established by constructing a concentration–response curve (0.1 nM–0.1 μM) to the peptide, during which complete desensitisation occurred. Full frequency–response curves to EFS were then carried out in the presence of receptors desensitised to VIP or substance P.

In experiments where concentration–response curves were produced, they were generated in cumulative fashion. At the end of all experiments, papaverine (500 μM) was added to determine the level of tone present and all relaxations were expressed as a percentage of this active tone.

Drugs and chemicals

α-Chymotrypsin, bradykinin triacetate, capsaicin (8-methyl-*N*-vanillyl-*trans*-6-nonenamide), $CGRP_{8-37}$, guanethidine sulphate, N^G-nitro-L-arginine methyl ester (L-NAME), papaverine hydrochloride, substance P, VIP, VIP antiserum (rabbit anti-vasoactive intestinal peptide) and U46619 (9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin$F_{2\alpha}$) were all obtained from Sigma (Poole, U.K.). L-733,066 ((2 s,3 s)-3-[(3,5-bis (trifluoromethyl)phenyl)methoxy]-2-phenylpiperidine hydrochloride) was obtained from Sigma (Schnelldorf, Germany). TTX was obtained from Biomol (Plymouth Meeting, U.S.A.). ODQ (1H[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) was obtained from Alexis Biochemicals (Nottingham, U.K.) and CGRP was obtained from Merck Biosciences (Nottingham, U.K.). All drugs were dissolved and diluted in 0.9% saline, with the following exceptions: U46619 (1 mM stock) in 50% ethanol; TTX (1 mM) in 0.1 M acetic acid; ODQ (1 mM) and L-733,060 (100 μM) in DMSO; capsaicin (1 mM) in ethanol; and CGRP (300 μM) and $CGRP_{8-37}$ (300 μM) in 1% acetic acid.

Statistical analysis

Results are expressed as the mean$\pm$s.e.m. of n observations, each from a separate vessel from a different eye. Statistical comparisons were made using one-way analysis of variance (ANOVA) and the Bonferroni post test, with the aid of a computer program, Prism (GraphPad, San Deigo, U.S.A.). A probability (P) less than or equal to 0.05 was considered significant.

Results

Relaxation of the bovine intraocular long posterior ciliary artery in response to EFS

In the presence of submaximal U46619 (0.1–1 μM)-induced tone and the adrenergic neurone blocker, guanethidine

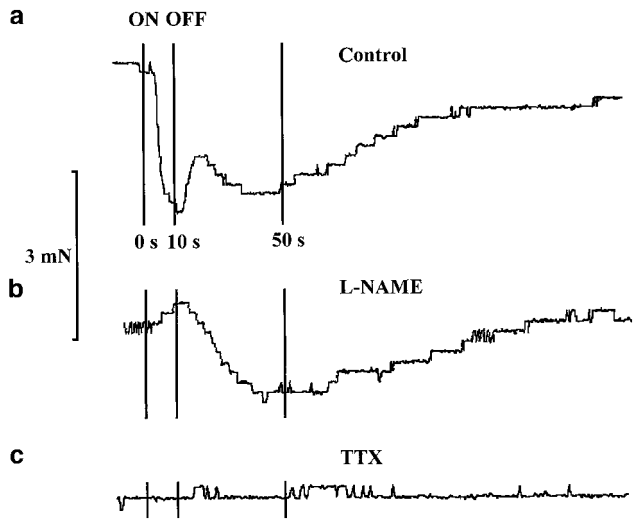

Figure 1 The biphasic relaxant response of bovine intraocular long posterior ciliary artery rings to EFS (10–18 V, 0.3 ms pulse width, 10 s train length, 16 Hz). The first solid line indicates the onset of stimulation; the second (at 10 s) represents both the end of the stimulation period and the peak of the first component of relaxation. The third line (at 50 s) indicates the peak of the second component of relaxation. (a) The control biphasic response. (b) The first component of relaxation was inhibited by L-NAME (100 μM), but the second was not. (c) Both components were abolished by TTX (0.1 μM).

(30 μM), EFS (10–18 V, 0.3 ms pulse width, 10 s train length) of bovine intraocular long posterior ciliary artery rings evoked frequency (0.5–128 Hz)-dependent relaxation that was biphasic in nature (Figures 1a and 2). The peak times for the first and second components of relaxation to occur were at 10 and 50 s, respectively. Moreover, the optimal frequency for both components of relaxation was 32 Hz (Figure 2). Removal of the endothelium had no effect on the magnitude of the first or second components of relaxation at any frequency (data not shown).

Effects of tetrodotoxin, L-NAME and ODQ

In the presence of the neurotoxin, TTX (0.1 μM), both components of relaxation of ciliary artery rings to EFS were abolished (Figure 1c), except for a small second component of relaxation that persisted at very high frequencies (64 and 128 Hz, Figure 2).

The first component of relaxation to EFS at all frequencies was abolished by the nitric oxide synthase inhibitor, L-NAME (100 μM), but the second component remained entirely unaffected (Figures 1b and 2). Likewise, the inhibitor of soluble guanylate cyclase, ODQ (3 μM), abolished the first component of relaxation at all frequencies, but had no effect on the second (data not shown).

Role of CGRP and substance P in neurogenic relaxation

The sensory nerve excitotoxin, capsaicin (1 μM), induced a transient fall in tone of ciliary artery rings (Figure 3a), which recovered to predrug levels within 15 min. A second addition of capsaicin (1 μM) failed to induce relaxation, but instead promoted a small contraction. Furthermore, pretreatment of

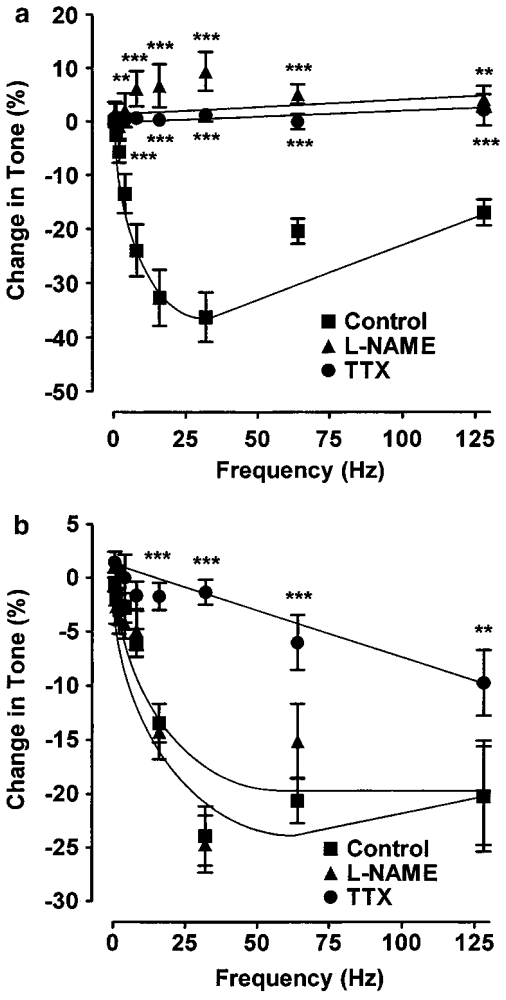

Figure 2 Frequency–response curves to EFS (0.5–128 Hz) in control bovine intraocular long posterior ciliary artery rings and in those treated with L-NAME (100 μM) or TTX (0.1 μM). (a) The first component of relaxation, measured at 10 s, was abolished by L-NAME or TTX at all frequencies. (b) The second component, measured at 50 s, was unaffected by L-NAME, but was abolished by TTX at frequencies of 32 Hz and below. At very high frequencies (64 and 128 Hz), a small degree of relaxation was seen in the presence of TTX. Data are mean$\pm$s.e.m.; $n \geqslant 8$; $^{**}P<0.01$ and $^{***}P<0.001$ indicate differences from control.

vessels that had not been previously exposed to capsaicin with the CGRP antagonist, $CGRP_{8-37}$ (1 μM), inhibited the capsaicin (1 μM)-induced relaxation. Moreover, pretreatment with capsaicin (1 μM) for 60 min prior to EFS had no effect on either the first or second components of relaxation at any frequency (Figure 4).

CGRP (0.1–300 nM) induced concentration-dependent relaxation of ciliary artery rings, with maximal relaxation of 89.5$\pm$1.8% and a pEC_{50} of 8.90$\pm$0.04 (Figure 3b). In the presence of the $CGRP_1$ antagonist, $CGRP_{8-37}$ (1 and 5 μM), the curve was shifted to the right, with an apparent pK_B of 6.65, but the maximal response was unaffected. In contrast, the first and second components of relaxation to EFS remained unaffected at all frequencies in ciliary artery rings pretreated with $CGRP_{8-37}$ (5 μM, Figure 4). These experiments were repeated in the presence of L-NAME (100 μM) to remove the first component, but again $CGRP_{8-37}$ (5 μM) had no effect on the second component of relaxation to EFS (data not shown).

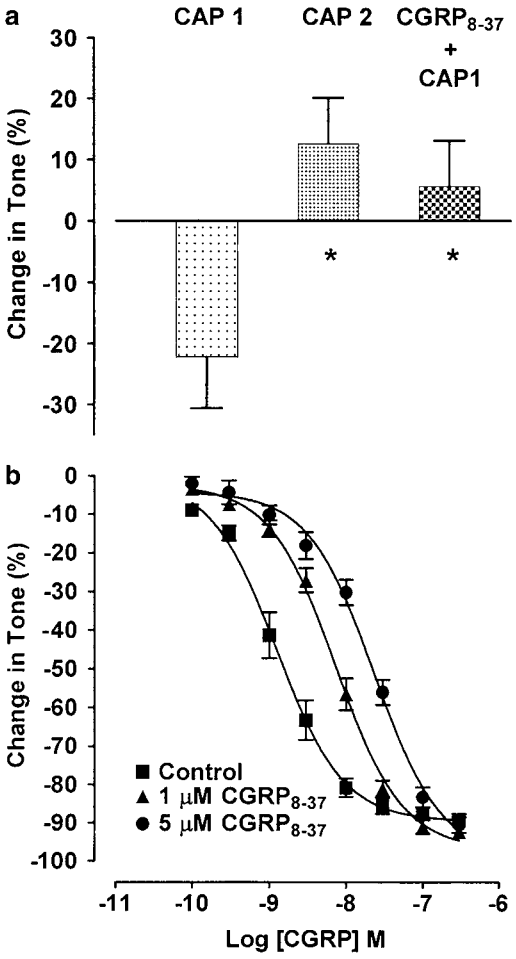

Figure 3 (a) A first addition of capsaicin (CAP 1, 1 μM) induced relaxation of bovine intraocular long posterior ciliary artery rings that was transient in nature. When the response had completely resolved, a second addition of capsaicin (CAP 2) failed to elicit relaxation, but produced a slight contraction. Furthermore, pretreatment with the $CGRP_1$ antagonist, $CGRP_{8-37}$ (1 μM), inhibited the relaxant response to a first addition of capsaicin. (b) CGRP (0.1–300 nM) induced concentration-dependent relaxation and this was inhibited following pretreatment with the $CGRP_1$ antagonist, $CGRP_{8-37}$ (1 and 5 μM). Data are mean $\pm$ s.e.m.; $n \geqslant 8$; $^*P < 0.05$ indicates difference from control.

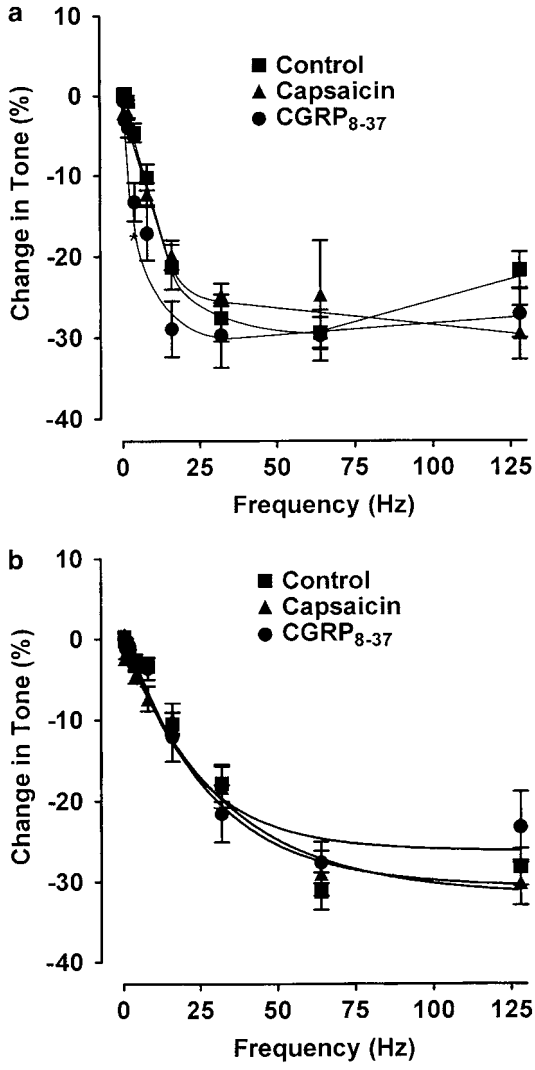

Figure 4 Frequency–response curves to EFS (0.5–128 Hz) in control bovine intraocular long posterior ciliary artery rings and in those pretreated with capsaicin (1 μM) or $CGRP_{8-37}$ (5 μM). Neither the first (a) nor the second (b) component of relaxation was affected by capsaicin or $CGRP_{8-37}$. Data are mean $\pm$ s.e.m.; $n \geqslant 4$.

Substance P (0.1 nM–0.1 μM) induced concentration-dependent relaxation of ciliary artery rings, but rapid desensitisation occurred, such that within $\sim$5 min the responses had completely disappeared (data not shown). Following the development of complete desensitisation to substance P (0.1 μM), the first and second components of relaxation to EFS remained unaffected at all frequencies (Figure 5). Moreover, in the presence of the NK_1 antagonist, L-733,060 (0.3 μM), neither the first nor second components of relaxation were inhibited (Figure 5).

Role of VIP in neurogenic relaxation

VIP (0.1 nM–0.3 μM) induced concentration-dependent relaxation, with maximal relaxation of 76.8 $\pm$ 3.4% and a pEC_{50} of 8.47 $\pm$ 0.14. A VIP desensitisation protocol was then applied to assess the role of the peptide in relaxation to EFS. Specifically, addition of VIP (0.3 μM) induced relaxation (73.0 $\pm$ 2.3%, $n = 12$) in control rings (Figure 6). Approximately 25 min later, this relaxation had declined to 30.1 $\pm$ 2.4% ($n = 12$) and a second addition of VIP (0.3 μM) now failed to promote further relaxation (Figure 6). In this desensitised state, further U46619 (0.2–0.5 μM) was added to re-establish tone to the original level, but the first and second components of relaxation to EFS remained unaffected at all frequencies (Figure 7).

The protease, α-chymotrypsin, was used in an attempt to degrade any endogenous VIP that might contribute to the EFS-induced relaxation. However, the addition of α-chymotrypsin (10 U ml^{-1}) to ciliary artery rings led to a pronounced fall in tone (43.1 $\pm$ 2.7%, $n = 7$), which did not recover spontaneously. Removal of the endothelium had no effect on this relaxation (data not shown). Additional U46619 (0.2 μM) was added in an attempt to restore tone, but it continued to fall thus preventing full frequency–response curves from being constructed. Nevertheless, in the continued presence of

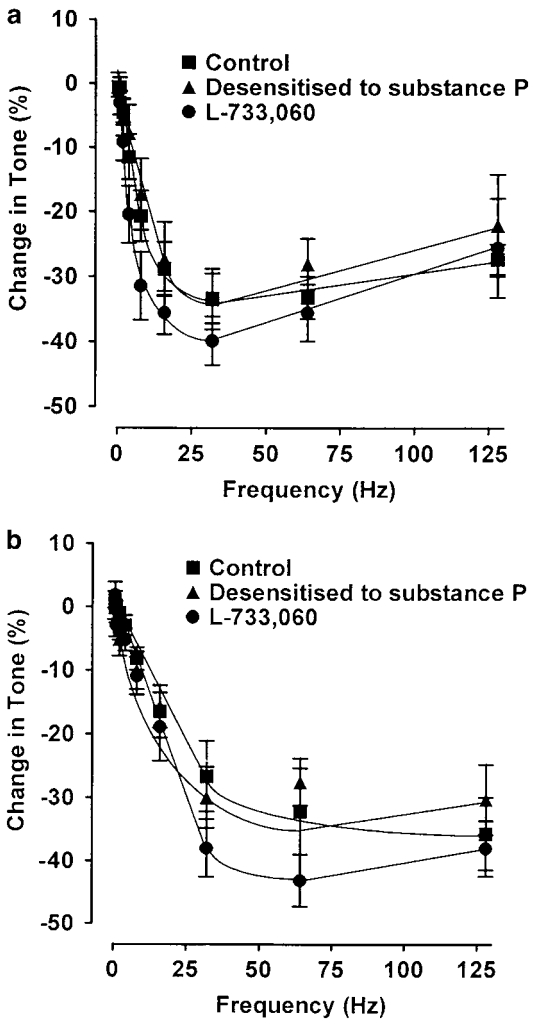

Figure 5 Frequency–response curves to EFS (0.5–128 Hz) in control bovine intraocular long posterior ciliary artery rings and in those desensitised to substance P (0.1 μM) or in the presence of NK_1 antagonist, L-733,060 (0.3 μM). Desensitisation to substance P or incubation with L-733,060 had no effect on either the first (a) or second (b) components of relaxation. Data are mean $\pm$ s.e.m.; $n \geqslant 5$.

α-chymotrypsin, the first and second components of relaxation at a single frequency (16 Hz) remained unaffected: first component 13.1 ± 2.3 and 16.0 ± 1.7%, for control and treated, respectively (both $n = 5$); and second component 9.0 ± 1.8 and 13.1 ± 3.5%, for control and treated, respectively ($n = 5$).

The addition of VIP antiserum (dilution 1 : 250) induced a sustained fall in tone of ciliary artery rings (25.0 ± 6.0%, $n = 7$, data not shown), which was difficult to restore to the original level with further U46619. The presence of the antiserum also promoted a great deal of frothing in the myograph chamber making responses difficult to discern. Furthermore, the antiserum failed to block the relaxation to exogenous VIP (data not shown). Its effects on relaxation to EFS could not, therefore, be satisfactorily assessed.

Discussion

Since their discovery in canine cerebral arteries (Toda, 1975), NANC vasodilator nerves are now known to be present in the

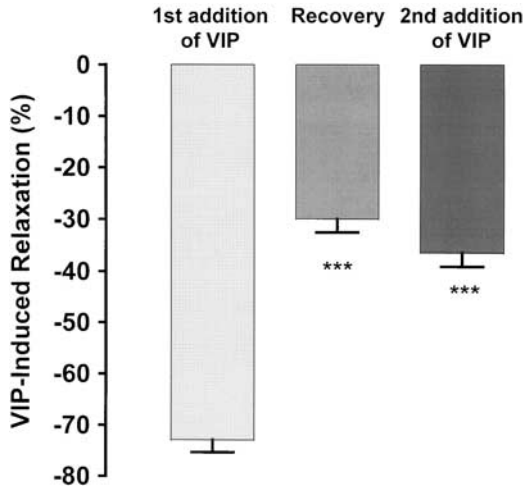

Figure 6 VIP (0.3 μM) induced powerful relaxation of bovine intraocular long posterior ciliary artery rings, which decayed to a lower but stable level within $\sim$25 min. In this state, a second addition of VIP failed to promote further relaxation. Data are mean $\pm$ s.e.m.; $n = 12$; ***$P < 0.001$ indicates difference from the maximal relaxation induced by VIP.

cerebral arteries of many species including human, as well as at other vascular sites such as in the eye and reproductive tissue (for a review, see Toda & Okamura, 2003). At some sites, for example, dog, monkey and bovine cerebral arteries the neurogenic vasodilatation appears to be exclusively nitrergic, since inhibitors of nitric oxide synthase abolish the response (Toda & Okamura, 1990; Ayajiki *et al*., 1993; Toda *et al*., 1997a). At other vascular sites, neurogenic vasodilatation appears to involve both nitric oxide and an additional transmitter. For example, in the cerebral artery of the cat the additional transmitter is CGRP (Ayajiki *et al*., 1994). In bovine mesenteric artery (Leckström *et al*., 1993) and guinea-pig uterine artery (Morris, 1993), the identity of the additional transmitter remains uncertain, but the actions of proteases suggest the involvement of a peptide, perhaps VIP. At yet other vascular sites, such as the rat mesentery (Kawasaki *et al*., 1988) and canine lingual artery (Kobayashi *et al*., 1995), neurogenic vasodilatation appears to be mediated solely by CGRP.

Having already established in the ciliary circulation of the bovine eye that endothelium-derived nitric oxide exerts a basal vasodilator tone, and that endothelium-derived hyperpolarising factor (EDHF) is solely responsible for agonist-induced vasodilatation (McNeish *et al*., 2001; 2002), we wished to determine the role of vasodilator nerves in regulating tone in this vascular bed. Previous reports describe neurogenic vasodilatation mediated solely by nitrergic nerves in retinal and ciliary arteries from a range of species, including dog, pig, monkey and human (Nyborg & Nielsen, 1994; Toda *et al*., 1994; 1997b; 1998; 1999; Ayajiki *et al*., 2000). In the bovine eye, neurogenic vasodilatation has been reported in the intraocular long posterior ciliary artery (Wiencke *et al*., 1994). These authors used an EFS train length of 20 s and described a monophasic dilator response that decayed with either a fast or a slow time course. On the basis of the actions of L-NOARG, capsaicin and $CGRP_{8-37}$, they reported that nitric oxide and CGRP were responsible for the fast- and slow-decaying components of the dilatation, respectively. By using a

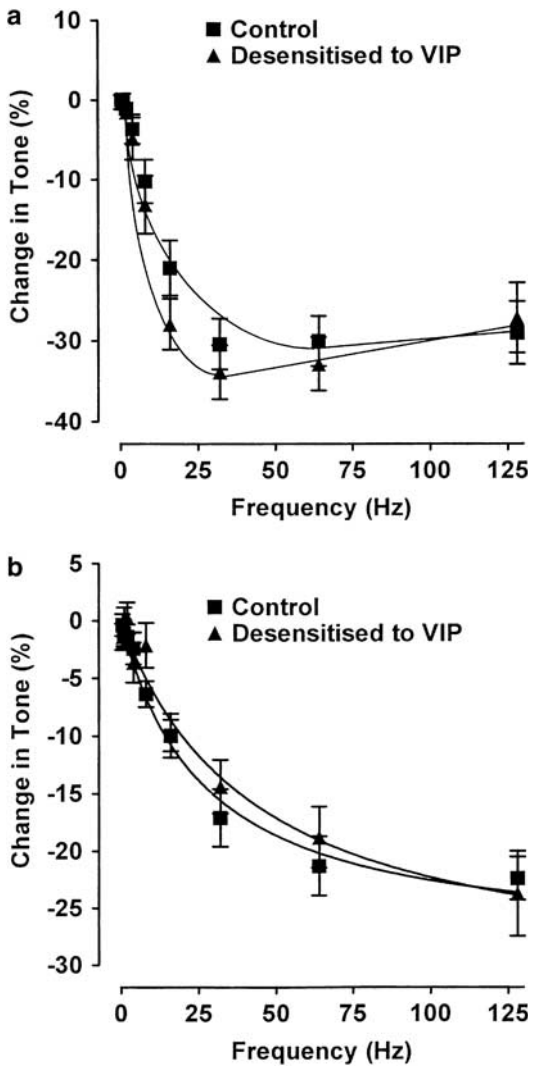

Figure 7 Frequency–response curves to EFS (0.5–128 Hz) in control bovine intraocular long posterior ciliary artery rings and in those partially desensitised to VIP (0.6 μM). Desensitisation to VIP had no effect on the first (a) or second (b) components of relaxation. Data are mean $\pm$ s.e.m.; $n \geqslant 9$.

shorter EFS train length of 10 s, we obtained a neurogenic (TTX-sensitive) vasodilator response in this artery that was clearly resolved into two separate components. This was possible because the first component of the biphasic response decayed rapidly upon termination of EFS and the latency of the second was $\sim$12 s. The clear temporal separation of the two components, peaking at 10 and 50 s, provided better conditions for pharmacological analysis that when the two components were fused together in a single response using a longer train length (Wiencke *et al*., 1994). We found that the NOS inhibitor, L-NAME (Hobbs & Gibson, 1990) and the inhibitor of soluble guanylate cyclase, ODQ (Garthwaite *et al*., 1995), each abolished the first component at all frequencies tested, but had no effect on the second. Thus, the first component of neurogenic vasodilatation appears to be exclusively nitrergic, supporting the earlier reported involvement of nitric oxide (Wiencke *et al*., 1994).

Our next major objective was to establish the identity of the transmitter responsible for the second component of neurogenic vasodilatation. In contrast to the earlier report of Wiencke *et al*. (1994), we obtained no evidence to support a role for CGRP. We found that the $CGRP_1$ receptor antagonist, $CGRP_{8-37}$, inhibited the dilator actions of exogenous CGRP with the expected pK_B of $\sim$6.7 (Brain *et al*., 2002), but it had no effect on the second (or first) component of neurogenic dilatation at any frequency. It is likely, however, that CGRP-containing sensory nerves are present in the ciliary artery, since we found that capsaicin, which activates vanilloid VR1 receptors on C and Aδ fibres (Joo *et al*., 1969; Akerman *et al*., 2003), induced a small transient dilatation that was inhibited by pretreatment with $CGRP_{8-37}$. Why these nerves were not activated by EFS is unclear, but previous findings on dural vasculature show that neurogenic vasodilatation involves only Aδ fibres, whereas neurogenic inflammation, which is associated with prolonged, high intensity stimulation, additionally involves C fibres (Williamson *et al*., 1997a, b; Akerman *et al*., 2003). It is therefore possible in the bovine ciliary artery that CGRP is present exclusively in C fibres that can be activated by capsaicin but not by the EFS parameters used either by us or by Wiencke *et al*. (1994). Indeed, further evidence against a role for CGRP in neurogenic relaxation was obtained when complete desensitisation to capsaicin had been obtained through prior exposure to the excitotoxin. Under these conditions, where prolonged inhibition of C fibre transmitter release occurs (Jancso *et al*., 1967; Lynn, 1990), we found that both the first and second components of neurogenic vasodilatation remained entirely unaffected at all frequencies. Thus, although CGRP-containing sensory fibres sensitive to capsaicin appear to be present, our enhanced ability to separate the two components of neurogenic vasodilatation in bovine ciliary artery provides no evidence of a role for CGRP in contrast to the report by Wiencke *et al*. (1994).

The inability of capsaicin desensitisation to affect neurogenic vasodilatation in the bovine ciliary artery also rules against a role for substance P, since it is also present in sensory fibres (Markowitz *et al*., 1987). Nevertheless, we further investigated the involvement of this neurokinin since it is known to participate in neurogenic responses in other blood vessels, including rat mesenteric veins (Claing *et al*., 1992; Ahluwalia & Vallance, 1997). Although substance P was able to promote dilatation of the ciliary artery, complete desensitisation occurred within minutes, thus making it an unlikely transmitter candidate for the neurogenic vasodilator response. Moreover, when complete desensitisation to substance P had been obtained or when the NK_1 receptor antagonist, L-733,060 (Seabrook *et al*., 1996), was present, neither the first nor the second components of neurogenic vasodilatation was affected. Substance P is therefore unlikely to contribute to neurogenic vasodilatation in the bovine ciliary artery.

We next considered the possibility that VIP might be a transmitter candidate for the second component of the neurogenic response. The presence of VIP-containing perivascular nerves has been demonstrated histochemically in ciliary arteries from a wide range of species, including pig, rat and human (Flugel *et al*., 1994; Toda *et al*., 1997b; Bergua *et al*., 2003). Nevertheless, all studies to-date agree that VIP has no functional role in the ciliary artery of these species and that neurogenic vasodilatation is mediated solely by nitrergic nerves. We found that VIP powerfully relaxed the bovine ciliary artery and that high concentrations led to partial

desensitisation to the peptide. In this desensitised state, which has been used previously to rule out involvement of VIP in the neurogenic vasodilatation in the dog and monkey cerebral arteries (Toda, 1982), we found that the first and second components of neurogenic vasodilatation were completely unaffected. Moreover, treatment with a protease, α-chymotrypsin, a strategy previously employed to establish successfully the role of a peptide in the neurogenic vasodilatation in guinea-pig uterine and bovine mesenteric arteries (Leckström *et al*., 1993; Morris, 1993), failed to inhibit either the first or second components of the neurogenic response in the bovine ciliary artery. A role for VIP in neurogenic vasodilatation was previously established in the sheep cerebral artery with the use of VIP antiserum (Matthew *et al*., 1997). In the bovine ciliary artery, however, VIP antiserum failed to convincingly block dilatation to exogenous VIP and caused such a profound fall in tone and frothing in the myograph baths that its effects on neurogenic vasodilatation could not be tested. Thus, none of our experimental approaches provided any evidence that VIP contributes to neurogenic vasodilatation in the bovine ciliary artery.

The final transmitter candidate we considered was ATP, since this nucleotide is believed to mediate neurogenic vasodilatation together with nitric oxide in the rabbit portal vein and mesenteric artery (Brizzolara *et al*., 1993; Kakuyama *et al*., 1998). We found that ATP did relax the bovine ciliary artery, but this was completely unaffected by the P2Y receptor antagonists, suramin (Simonsen *et al*., 1997), PPADS (Lambrecht *et al*., 1992) or reactive blue 2 (Burnstock & Warland, 1987), at concentrations that are effective in other tissues, or desensitising with the P2X receptor agonist, α,β-methylene ATP (Simonsen *et al*., 1997) (unpublished observations). Thus, lack of a suitable blocking agent prevented us from investigating the possible role of ATP in neurogenic vasodilatation of the bovine ciliary artery.

Although we have been unable to establish the identity of the agent responsible for the second component of neurogenic vasodilatation, its slow time course argues against involvement of highly diffusable neurotransmitter gases such as carbon monoxide (Ny *et al*., 1996) and hydrogen sulphide (Teague *et al*., 2002). Indeed, the slow onset and long duration of the response is more characteristic of the action of a peptide neurotransmitter (Kawasaki *et al*., 1988; Morris, 1993). It remains to be determined if neuropeptide Y, dynorphin or somatostatin, which have been localised in pelvic perivascular nerves (Morris *et al*., 1985; Morris & Gibbins, 1987), play a role in the neurogenic vasodilatation of the bovine ciliary artery.

In conclusion, we have found that neurogenic vasodilatation in response to EFS in the bovine intraocular long posterior ciliary artery is biphasic and is mediated by two distinct neurotransmitter systems. Nitrergic nerves mediate the first, rapid component in agreement with a previous report suggesting involvement of nitric oxide (Wiencke *et al*., 1994). The transmitter mediating the second component is unlikely to be CGRP, in contrast to the previous suggestion by Wiencke *et al*. (1994). It is also unlikely to be substance P or VIP. The identity of this second neurotransmitter therefore remains obscure.

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