Purpose. Characterization of the nonadrenergic noncholinergic (NANC) vasodilator innervation in the anterior segment in the bovine eye.

Methods. The neurogenic tetrodotoxin-sensitive response to electrical field stimulation (EFS) of the intraocular segment of the bovine long posterior ciliary artery supplying the ciliary body was recorded using isolated ring segments of this artery mounted on an isometric myograph. After adrenergic and cholinergic receptor blockade (with phentolamine, propranolol, and atropine), the preconstricted vessels were subjected to EFS by passing constant current pulses (0.3 msec, 35 mA, 0.5 to 32 Hz) between two electrodes on either side of the vessel segments.

Results. EFS resulted in 60% relaxation of the active tone in 40 vessels. Treatment with capsaicin reduced the NANC response by 16 ± 2% (P < 0.001) and inhibition of the NO synthase with 1 • 10^-4 M L-NOARG reduced the NANC response by 83 ± 10% (P< 0.001). Desensitization of the vessels to substance P had no effect. The CGRP(8-37) fragment (1 • 10^-6 M) in the presence of 1 • 10^-4 M L-NOARG reversibly and competitively inhibited the NANC response. L-arginine partly antagonized the inhibition induced by L-NOARG. About 60% of the L-NOARG-sensitive component of the NANC response was inhibited by methylene blue. Combined incubation with capsaicin and L-NOARG nearly abolished the NANC response. The L-NOARG-sensitive/capsaicin-resistant relaxation was present in endothelium denuded vessels. The responses to EFS were blocked by TTX.

Conclusions. The neurogenic NANC vasodilator response in the intraocular part of the bovine long posterior ciliary artery supplying the ciliary body is endothelium independent and consists of two components: a capsaicin-sensitive component mediated by CGRP released from sensory nerve endings and a larger L-NOARG-sensitive component mediated by a direct "nitroxidergic" neurotransmission. The size of the nitroxidergic NANC response indicates that it has a physiological relevance in vivo. Invest Ophthalmol Vis Sci. 1994;35:3268-3277.

Nonadrenergic noncholinergic (NANC) innervation was first reported by Burnstock in 1963 in isolated taenia coli smooth muscle. NANC nerves have since been demonstrated in many organs, including the eye. Evidence of the role of a given putative neurotransmitter in the NANC response is based on pharmacological, immunohistochemical, and physiological studies. The nature of the NANC neurotransmitters involved in vasodilatation seems to vary, and in many investigations it has remained elusive. Particularly CGRP, VIP (vasoactive intestinal polypeptide), and ATP have been shown to mediate the NANC response in many parts of the circulation. Among the NANC mediators, nitric oxide (NO) is a novel nonpeptide neurotransmitter originating from so-called nitroxidergic nerves and has been reported to be responsible for the NANC response in dog and monkey cerebral arteries and in monkey mesenteric arteries. The ocular circulation is innervated by different nerves containing a multitude of neurotransmitters. Earlier immunohistochemical studies showing that NADPH-diaphorase staining is present throughout
the visual system can now be taken as evidence for the presence of the NO synthase system. The particularly dense staining seen in the rat choroid could be due to staining of NO synthase present in either the endothelium (producing endothelium-derived relaxing factor now realized to be NO) or in the nerves.

Changes in the production of aqueous humor in the ciliary body may be mediated by changes in the transport of sodium and bicarbonate across the basolateral membrane and by changes in vascular resistance of the ciliary body. The neurogenic regulation of the tension in vessels to and in the ciliary body may, therefore, be of crucial importance under normal physiological conditions. It is likely that impairment of the neurotransmission or neurodegeneration may change the vascular resistance ending with degeneration of vital ocular structures. Information about the neural regulation of vascular tone in the ciliary body and anterior uvea is sparse but indicates that trigeminal CGRP and a facial nerve-derived nonmuscarinic non-VIP neurotransmitter may mediate neurogenic vasodilation of the anterior uvea.

We have in the present experiments studied the noncholinergic nonadrenergic neurotransmitter responses evoked by electrical field stimulation (EFS) in the anterior intraocular segment of the bovine long posterior ciliary artery supplying the ciliary body. We paid special attention to the possible transmitters involved in the vasodilatation of these vessels. Our results show that the isolated arteries are relaxed by nerves containing CGRP and nerves liberating NO during EFS.

MATERIALS AND METHODS

Preparation of Vessels

Eyes from cows were obtained from the local abattoir and were transported to the laboratory in ice-cold physiological saline solution of the following composition in mM: NaCl, 119; NaHCO3, 25; KCl, 4.7; CaCl2, 1.5; MgSO4, 1.18; KH2PO4, 1.17; EDTA, 0.026, and glucose 11. All studies were conducted in accordance with the ARVO Resolution on the Use of Animals in Research. The posterior segment of the eye was removed together with the vitreous. The choroid and the sclera were separated, and the intracocular branch of a long, posterior ciliary artery was located where it penetrates the ciliary body. One to two millimeter long segments of the artery before it enters the ciliary body were dissected from the surrounding tissue. These segments (one per cow) (internal diameter approximately 400 μm) were mounted as ring preparations on an isometric myograph, allowing direct determination of the vessel wall force while controlling the internal circumference. The vessel segments were then equilibrated in oxygenated (5% CO2 in O2) physiological saline solution at 37°C, pH 7.4, for 30 minutes and stretched to a lumen diameter close to where they develop maximal active force. Initially in each experiment, the ability of the vessels to contract was tested by stimulation with noradrenaline (1 · 10−5 M). Noradrenaline was chosen as the test agonist instead of potassium to avoid depletion of neurotransmitters caused by depolarization during the stimulation period.

Nerve Stimulation

Electrical field stimulation consisting of constant-current pulses (35 mA, 0.3 msec duration, 20 sec trains, 0.5 to 32 Hz) of alternating polarity were passed between two platinum electrodes placed on either side of the vessel at a distance of approximately 0.5 mm from the vessel wall, thus creating an electrical field in the transverse direction of the vessel lumen.

Frequency–Response Curves

Frequency–response curves were constructed by increasing the frequency in steps from 0.5 Hz to 32 Hz, doubling the frequency in each step. Vessels lacking spontaneous tone were contracted with PGF2α (3 · 10−6 M or 1 · 10−5 M) before EFS. Stimulation at each step was started when the vessel had regained its tone or when a steady-state level in tone was reached. Vessel responses (t = 20 sec) are expressed as the fraction of the vessel tone just before stimulation (t = 0 sec) at each frequency. The vessels were relaxed with papaverine (1 · 10−4 M) at the end of the experiment to determine the level of tone throughout the experiment. The sensitivity of the vessels to EFS is expressed as an EF50 value, which is the frequency (in Hz) required to give half-maximal response. For each frequency–response experiment, the EF50 was determined by nonlinear regression using the equation: R/Rmax = F/Fn + EF50), where R/Rmax and F is the relative response and frequency (in Hz) of stimulation, respectively, and n is a parameter determining the steepness of the curve (Hill coefficient). Rmax is the maximal observed response. NANCmax denotes the average of Rmax values.

Pharmacology

Postjunctional neural receptor blockade of the sympathetic and parasympathetic nerves was obtained by preincubating the vessels with phentolamine (3 · 10−6 M), propranolol (1 · 10−6 M), and atropine (1 · 10−7 M)
FIGURE 1. Effect on responses in isolated intraocular segments of bovine long posterior ciliary arteries to EFS of phentolamine (3 \cdot 10^{-6} M) (triangles) and the combination of phentolamine (3 \cdot 10^{-6} M), atropine (1 \cdot 10^{-7} M), and propranolol (1 \cdot 10^{-6} M) (squares). Circles represent response in vessels in control conditions. Points represent means of seven vessels, and vertical bars show \pm SEM.

for 20 minutes and keeping the vessels exposed to these receptor antagonists throughout the experiment.

Endogenous CGRP and SP (substance P) in sensory nerve endings were eliminated by treating the vessels in vitro with capsaicin (1 \cdot 10^{-6} M) for 1 hour. Capsaicin is a selective excitotoxin for sensory nerves containing CGRP and SP\textsuperscript{27} (see below). To confirm that the vessels were in fact depleted of CGRP/SP, a repeated exposure of the vessels to capsaicin was performed at the end of the hour. This had no effect on vessel tone.

Desensitization of the vessels to SP was achieved by exposing the vessels to SP (1 \cdot 10^{-7} M) for 20 minutes. Pilot experiments have shown complete desensitization of the vessels to SP using this procedure.

To analyze further the nature of the released transmitters during EFS, we tested the effect of inhibition of nitric oxide synthase\textsuperscript{28,29} and soluble guanylate cyclase with L-NOARG and methylene blue,\textsuperscript{30} respectively. The effect of CGRP(8-37) fragment was studied in vessels pretreated with L-NOARG to eliminate the vasodilatory effect of the neurogenic nitric oxide. When used, methylene blue, CGRP(8-37) fragment, and L-NOARG were applied to the organ bath at least 20 minutes before EFS. The antagonistic effect of the CGRP(8-37) fragment is expressed as the -log(K\textsubscript{d}) value\textsuperscript{31}(= pK\textsubscript{d}). This value is calculated as log(CR-1) = log([ antagonist], (M)), where CR is the ratio between EF\textsubscript{50}(+ antagonist) and EF\textsubscript{50}(control)\textsuperscript{32}.

The endothelium was removed by gently rubbing the inside of the vessel with a human scalp hair inserted through the lumen of the vessel. Successful denudation of the vessel was indicated by the lack of effect of 1 \cdot 10^{-7} M SP or by a contractile response rather than a relaxant response to acetylcholine (1 \cdot 10^{-5} M) in vessels precontracted with PGF\textsubscript{2\alpha} (1 \cdot 10^{-5} M). The contractile response to depolarization with potassium (125 mM) was preserved in these vessels as an indication of an intact underlying smooth muscle cell layer.

Drugs
The following drugs were used: acetylcholine chloride, atropine sulphate, papaverine sulphate, and methylene blue (Danish Pharmacy Labs, Copenhagen, Denmark); phentolamine mesylate (Regitine, Ciba, Basel, Switzerland); prostaglandin F\textsubscript{2\alpha} (PGF\textsubscript{2\alpha}, Dinoprost, Upjohn, Kalamazoo, MI); human \alpha-CGRP(8-37) frag-

FIGURE 2. Tracings showing the NANC response in two different intraocular segments of bovine long posterior ciliary arteries contracted with 3 \cdot 10^{-6} M PGF\textsubscript{2\alpha} at 4 Hz before (left) and after (right) treatment with 1 \cdot 10^{-4} M L-NOARG. Upper trace shows the response in a vessel with a relatively slow recovery phase. Lower trace shows the response in a vessel rapidly regaining tone after stimulation. Vertical scale shows active force in millinewtons (mN). Horizontal scale shows time.

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A

B

FIGURE 3. Relationship between maximal vessel tone and maximal NANC response in intraocular segments of bovine long posterior ciliary arteries. Open circles represent vessels with spontaneous tone. Closed circles represent vessels precontracted with PGF<sub>2α</sub> (3 • 10<sup>−6</sup> M or 1 • 10<sup>−5</sup> M). (A) Plot of the NANC response expressed in absolute values (N/m) against maximal vessel tone (N/m). The slope of the line fitted by linear regression is 0.52, which is significantly different from zero (P < 0.001); correlation coefficient = 0.87. The dashed lines represent the 95% confidence interval. (B) Plot of the NANC response expressed as percentage of tone before EFS against maximal vessel tone (N/m). The slope of the line fitted by linear regression does not vary significantly from zero. Dashed lines represent the 95% confidence interval. The average maximal response in these 19 vessels is 55 ± 3%, whereas the total average of all 40 vessels was 58.4 ± 2.7%.

Statistics

The vessel responses are expressed either as active wall tension (N/m) or as a percentage of active tension. The results are given as the mean ± SEM (number of vessels/one per cow). All comparisons are paired within the same vessel, except for the data using endothelium-denuded vessels. Differences between means were tested using Student’s t-test for paired or un-

TABLE 1. NANC Relaxation and EF<sub>50</sub>

<table>
<thead>
<tr>
<th>Drug</th>
<th>NANC&lt;sub&gt;MAX&lt;/sub&gt; (%)</th>
<th>EF&lt;sub&gt;50&lt;/sub&gt; (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>73.9 ± 7.0</td>
</tr>
<tr>
<td>Capsaicin</td>
<td>4</td>
<td>86.5 ± 5.5</td>
</tr>
<tr>
<td>CGRP (8-37) fragment</td>
<td>6</td>
<td>30.5 ± 6.8</td>
</tr>
<tr>
<td>SP</td>
<td>4</td>
<td>59.5 ± 4.4</td>
</tr>
<tr>
<td>L-NOARG 1 • 10&lt;sup&gt;−8&lt;/sup&gt; M</td>
<td>4</td>
<td>62.0 ± 5.5</td>
</tr>
<tr>
<td>L-NOARG 1 • 10&lt;sup&gt;−4&lt;/sup&gt; M</td>
<td>4</td>
<td>44.5 ± 7.3</td>
</tr>
<tr>
<td>L-NOARG + capsaicin</td>
<td>6</td>
<td>66.7 ± 3.3</td>
</tr>
<tr>
<td>Methylene blue 3 • 10&lt;sup&gt;−5&lt;/sup&gt; M</td>
<td>6</td>
<td>54.7 ± 1.0</td>
</tr>
<tr>
<td>Methylene blue 3 • 10&lt;sup&gt;−4&lt;/sup&gt; M</td>
<td>5</td>
<td>53.4 ± 6.6</td>
</tr>
</tbody>
</table>

Summary of all paired comparisons. NANC<sub>MAX</sub> (%) is the average of R<sub>MAX</sub>-values ± SEM within each group. EF<sub>50</sub> (Hz) is the averaged mean ± SEM within each group. N is the number of vessels in each group. Columns 1 and 2 are NANC<sub>MAX</sub> or EF<sub>50</sub> before and after addition of drug, respectively. For the group treated with CGRP (8-37) fragment, column 3 shows the values after washout of fragment. For L-NOARG experiments, column 3 shows the NANC<sub>MAX</sub> and EF<sub>50</sub> values after addition of 3 • 10<sup>−5</sup>M l-arginine. Paired t-tests were made within all groups for comparison of values in column 2 versus column 1 and column 3 versus column 1. *P < 0.05; †P < 0.01.
RESULTS

Responses to EFS

The mounted vessels frequently developed spontaneous tone. All vessels, whether in the presence of spontaneous tone or precontracted with PGF2α, relaxed in response to EFS at frequencies 4 Hz or above \( (n = 8) \). At frequencies lower than 4 Hz, three out of eight vessels contracted whereas the rest relaxed irrespective of level of tone. The relaxation was increased, and any contractions were abolished when the vessels were treated with \( 1 \cdot 10^{-6} \) M phentolamine. Low-frequency relaxations were further increased when \( 1 \cdot 10^{-7} \) M atropine and \( 3 \cdot 10^{-6} \) M propranolol also were included in the bathing medium (Fig. 1). To characterize the nonadrenergic noncholinergic vasodilation (the NANC response) of the ciliary arteries, all the following series of experiments were made in the presence of the adrenergic and cholinergic receptor antagonists. All responses to EFS were abolished by \( 1 \cdot 10^{-7} \) M TTX.

The NANC Response

The character of the NANC response to EFS varied between vessels, as seen in Figure 2 showing the NANC response of a vessel with a relatively slow recovery phase (upper left) and a vessel rapidly regaining tone after stimulation (lower left), respectively. The character of the recovery phase is not illustrated in the figures showing average values for the NANC responses because these values were measured as the change in tension from the beginning \( (t = 0 \text{ sec}) \) and to the end \( (t = 20 \text{ sec}) \) of the stimulating period.

The relationship between the vessel tone and the maximal NANC response is illustrated in Figure 3. There was a significant \( (P < 0.01) \) linear correlation between the vessel tone and the NANC response when expressed in absolute values \( (\text{N/m}) \) (Fig. 3A). However, if the NANC response is expressed as percentage of tone before EFS and plotted against maximal vessel tone (Fig. 3B), the slope of the line fitted by linear regression did not vary significantly from zero. Because of this, the NANC responses were expressed in terms of their relative values when comparisons were made. During the experiments drifting of basal tone was the rule, as illustrated in the shifts in basal tone observed in Figures 2, 5, and 9 rendering calculations using relative values necessary.

FIGURE 4. NANC response in endothelium-denuded (open circles) and endothelium-intact (closed circles) intraocular segments of bovine long posterior ciliary arteries. Points represent means of four endothelium-denuded and 40 endothelium-intact vessels, respectively, and vertical bars show ±SEM where this value exceeds the size of the symbol.

FIGURE 5. Effect of capsaicin on NANC response in intraocular segments of bovine long posterior ciliary arteries. Open circles represent vessels in control conditions. Closed circles represent vessels treated with capsaicin. Points represent means of four vessels, and vertical bars show ±SEM where this value exceeds the size of the symbol. Inset traces show the NANC response to EFS at 4 Hz before (left trace) and after (right trace) treatment with capsaicin in an isolated intraocular segment of bovine long posterior ciliary artery. Vertical scale shows active force in millinewtons (mN). Horizontal scale shows time. The corresponding 20 sec values for this vessel are 48% and 56% relaxation, respectively. Note the faster regaining of tone after treatment with capsaicin.
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Figure 6. Effect of CGRP(8-37) fragment on NANC response in intraocular segments of bovine long posterior ciliary arteries treated with L-NOARG (1 \times 10^{-4} M). Open circles represent vessels in control conditions. Closed circles represent vessels after incubation with CGRP(8-37) fragment. Open squares represent vessels after washout of CGRP8(8-37) fragment. Points represent means of five to six vessels and vertical bars show ± SEM.

The maximal NANC response, \( \text{NANC}_{\text{max}} \), obtained in the first control curve of all vessels during adrenergic and cholinergic receptor blockade was 58.4 ± 2.7% of vessel tone before EFS, and \( EF_{50} \) was 2.32 ± 0.15 Hz (n = 40). The results of all paired comparisons are summarized in Table 1. The second frequency response curve for the NANC response without any intervention was shifted slightly to the right (\( \Delta EF_{50} = 0.31 ± 0.12 \), n = 7, \( P < 0.05 \)) without any change in \( \text{NANC}_{\text{max}} \).

Removal of the endothelium did not affect the NANC response (\( \text{NANC}_{\text{MAX}} = 65.8 ± 11.6\% \), n = 4, \( P = \text{NS} \), and \( EF_{50} = 3.65 ± 1.53 \) Hz, n = 4, \( P = \text{NS} \)) if compared with endothelium intact vessels (Fig. 4).

The Capsaicin-Sensitive NANC Response

Part of the NANC response is sensitive to capsaicin. Because it was found that the remaining response was eliminated by L-NOARG, the capsaicin-sensitive component could be isolated by preincubation of the vessels with L-NOARG. Treatment of vessels with capsaicin in the absence of L-NOARG affected the character of the recovery phase after EFS so that all vessels regained tone faster (Fig. 5 inset), and capsaicin treatment also significantly reduced the response by 16 ± 2% at 16 Hz (n = 6) (Table 1, Fig. 5).

The NANC\(_{\text{max}}\) response (30.5 ± 6.8%, n = 6) was significantly (\( P < 0.01 \)) lower in the presence of 1 \times 10^{-4} M L-NOARG than in the control condition (70.9 ± 7.0, n = 7). In the presence of 1 \times 10^{-4} M L-NOARG, the specific CGRP, receptor antagonist, CGRP(8-37) fragment (1 \times 10^{-6} M), competitively antagonized the NANC response (Fig. 6, Table 1). The frequency response curve was shifted to the right with an unaffected maximal response, and the \( EF_{50} \) value was increased 1.93 ± 0.34 times (n = 6). The affinity of the fragment (\( -\log(K_d) \) value) was 5.83 ± 0.15 (n = 6). The NANC response was not affected by desensitization of the vessels to substance P (Table 1, Fig. 7).

The L-NOARG-Sensitive NANC Response

As mentioned above, the NANC response was also affected by treatment with L-NOARG. The change in response depended on the character of the NANC response in control conditions as shown in Figure 2. If the vessel had a slow recovery phase after EFS, the maximal response was reduced to some extent but the recovery was still slow. If the vessel had a rapid recovery phase after EFS, it was much more sensitive to L-NOARG, and the NANC response was almost abolished.

The inhibition of the NANC response induced by 1 \times 10^{-5} M was partly reversed by a surplus of l-arginine (3 \times 10^{-3} M). A tenfold increase in the concentration of L-NOARG to 1 \times 10^{-4} M augmented the inhibition of the NANC response, and the addition of a surplus of

l-arginine (3 \times 10^{-5} \text{ M}) reversed the inhibition induced by 1 \times 10^{-4} \text{ M} to a lesser degree (Fig. 8).

Simultaneous treatment with capsaicin and L-NOARG (1 \times 10^{-4} \text{ M}) almost extinguished the relaxations to EFS in all vessels (Fig. 9).

**Endothelium and the L-NOARG Sensitive NANC Response**

The capsaicin-resistant/L-NOARG sensitive component of the NANC response was not significantly inhibited by endothelium denudation. If compared with capsaicin-treated endothelium-intact vessels, NANC-max was 49.3 ± 5.2\% (n = 4) and 62.7 ± 6.0\% (n = 15) (P = 0.28), and EF_{50} was 3.82 ± 0.72 Hz (n = 4) and 2.82 ± 0.18 Hz (n = 15) (P = 0.06), respectively (Fig. 10).

**Second Messenger in the L-NOARG Sensitive NANC Response**

Methylene blue (3 \times 10^{-6} \text{ M} and 3 \times 10^{-5} \text{ M}) inhibited the NANC response resistant to capsaicin and sensitive to L-NOARG in a concentration-dependent manner (Fig. 11). The inhibition at 16 Hz was 23 ± 4\% (n = 6) when the vessels were preincubated with 3 \times 10^{-6} \text{ M} methylene blue and 59 ± 13\% (n = 5) when they were preincubated with 3 \times 10^{-5} \text{ M} methylene blue.

**DISCUSSION**

The main findings in this study are that the anterior intraocular segment of the bovine long posterior ciliary artery is innervated by NANC nerves and that CGRP and nitric oxide generated from nerve fibers in the vessel wall mediate the vasodilation.

It has been shown that the ocular circulation is innervated by sensory and other autonomic nerves containing a multitude of neurotransmitters. It has been shown that the ocular circulation is innervated by sensory and other autonomic nerves containing a multitude of neurotransmitters.16 Trigeminal fibers in the eye contain SP, CGRP, and cholecystokinin. Parasympathetic nerve fibers from the facial nerve contain VIP and peptide histidine/isoleucine besides acetylcholine.20 Sympathetic nerves contain neuropeptide Y and ATP besides noradrenaline.20 The ocular innervation seems to be similar among different mammalian species, and the only main difference appears to be the nerve fiber density.33

In spite of the striking similarity in the type of nerves innervating mammalian eyes, functional studies have revealed a remarkable difference in the ocular response to neuropeptides between different species. The different action of neuropeptides in the eye and...
elsewhere in the body can now be attributed to heterogeneity in receptor distribution and receptor type in the target cells of the innervating nerve fibers.

Intracranial stereotactic stimulation of the facial nerve in rabbits causes a largely nonmuscarinic rise in intraocular pressure due to a marked vasodilation in the uvea. Immunohistochemical studies indicate that parasympathetic nerve fibers from the facial nerve contain VIP, which is a potent vasodilator in the uvea in rabbits but which has no observed effect in cats. However, Bill and coworkers failed to observe a rise in VIP content in the blood from the vorticose veins during facial nerve stimulation and, thus, questioned the function of VIP in the vasculature of at least the rabbit eye. This observation could, therefore, indicate that another neurotransmitter in the facial nerve mediates the uveal vasodilation.

The present experiments show that the arteries are innervated with contractile and dilatory nerves because blockade of the postjunctional adrenergic and cholinergic receptors on the smooth muscle cells antagonized the contraction seen at the lower frequencies of stimulation, and the relaxations were potentiated. Because we aimed in this work to investigate the nonadrenergic noncholinergic vasodilatory innervation of the ciliary arteries, we have not further analyzed the type of neurotransmitters involved in the contractile response to EFS.

The NANC response of the ciliary arteries differed markedly between vessels; the recovery phase after EFS was rapid in some and slow in other vessels, indicating a heterogeneous NANC innervation. The difference in the character of the NANC response could be due to strain differences among the cows or to age and sex differences, but, according to our data, there was no relation between any of these parameters and the character or shape of the NANC response in the ciliary arteries.

Capsaicin significantly reduced the NANC response and shortened the recovery phase after EFS. Because capsaicin is a selective excitotoxin for sensory nerves containing CGRP and SP, this strongly indicates that one of these neuropeptides participates in the NANC response in ciliary arteries. To determine whether CGRP or SP, or the two in combination, were responsible for the capsaicin-sensitive part of the NANC response, we used different approaches. We have found that the ciliary arteries are desensitized to SP by a single exposure to \(1 \times 10^{-7}\) M and that the relaxation induced by SP, but not by CGRP, is totally dependent on an intact endothelium in these vessels (results not shown). In the present experiments, desensitization of the ciliary arteries to SP had no effect on the NANC response, and the NANC response was also unchanged by endothelium removal. Thus, both results indicate that CGRP but not SP is involved in the NANC response in ciliary arteries.
To confirm the role of CGRP as a mediator of vasorelaxation, we used the specific CGRP1 receptor antagonist, the CGRP(8-37) fragment in vessels treated with L-NOARG to block the effect of NO from nerves and endothelium (see below). Previously, we found that the CGRP(8-37) fragment in these vessels competitively inhibits the vasodilatory response to exogenously applied CGRP with a -log(KD) value of 6.7 (results not shown). In the present experiments, the CGRP(8-37) fragment inhibited the capsaicin-sensitive part of the NANC response, shifting the frequency–response curve to the right in a competitive fashion. The inhibition was reversible after washout of the fragment. We can, therefore, conclude that the capsaicin-sensitive part of the NANC response must be mediated by endogenously released CGRP probably coming from trigeminal sensory nerve fibers in the vessel wall.

L-NOARG, an inhibitor of nitric oxide synthase* significantly reduced the NANC response. The inhibition could either be due to a block of release of endothelium-derived relaxing factor (EDRF, NO) mediated by neurotransmitters released by EFS in the vessel wall, or it could be due to a block of NO generation in perivascular nerves. If the former explanation is true, the removal of the endothelium should have an equal inhibitory effect as L-NOARG on the relaxation induced by EFS. Because the relaxation of capsaicin-treated, endothelium-denuded vessels was similar to that obtained in capsaicin-treated, endothelium-intact vessels, it seems reasonable to believe that most of the capsaicin-resistant NANC response is mediated by NO or a NO-containing compound released from nerve fibers in the vessel wall. Further evidence that NO is responsible for the capsaicin-resistant NANC response in the ciliary arteries comes from the finding that the inhibitory effect of L-NOARG was antagonized by L-arginine, the substrate of the NO synthase.**

Vasodilation induced by NO (EDRF) is mediated by activation of the soluble guanylate cyclase, causing a rise in the intracellular cGMP concentration in the smooth muscle cells.*** We have also tested this pathway for the capsaicin-resistant part of the NANC response in the ciliary arteries using methylene blue as an inhibitor of the soluble guanylate cyclase.**** In our experiments, methylene blue inhibited the capsaicin-resistant NANC response concentration dependently. However, the highest concentration of methylene blue used, 3 · 10⁻⁵ M, was not able to inhibit the NANC response completely, suggesting that a rise in the intracellular cGMP concentration only partly explains the vasodilatory mechanism. Therefore, this may suggest the involvement of another non-cGMP-dependent messenger system, as has been reported in other cells, but the nature of this messenger must be determined in future experiments.

It is not possible from our experiments to say whether all NANC vasodilating responses in the anterior intraocular segment of the bovine long posterior ciliary artery in vivo can be explained by NO and CGRP alone. Even though about 90% of the NANC response disappeared in the presence of capsaicin and L-NOARG, we cannot exclude that other transmitters, such as VIP, may be released. Based on immunohistological studies on the parasympathetic innervation of the penile vasculature and on neurons in the pterygopalatine ganglion, it seems likely that the neurogenic NO synthase is colocalized with VIP. Even so, we have presented evidence strongly suggesting that neurally evoked vasorelaxation in the anterior intraocular segment of the bovine long posterior ciliary artery in the presence of adrenergic and cholinergic receptor blockade is mediated by neurogenic NO and by CGRP released from sensory nerve endings. The size of the NANC response in these vessels suggests it may be relevant in the control of ocular blood flow in vivo; thus, the demonstration of NO as a "new" neurotransmitter in the ocular circulation may lead to revision of the current concepts of mechanisms and factors involved in the control of ocular blood flow in health and disease.

Key Words
nitric oxide, CGRP, transmural nerve stimulation, ciliary artery, bovine

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References


