Modulation by Neurogenic Acetylcholine of Nitroxidergic Nerve Function in Porcine Ciliary Arteries

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Purpose. To determine whether nitroxidergic, cholinergic, and vasoactive intestinal polypeptide (VIP)-mediated nerves participate in the regulation of porcine ciliary arterial tone and to analyze the mechanisms underlying the neuronal interaction.

Methods. Changes in isometric tension were recorded in helical strips of the arteries, which were stimulated by transmurally applied electrical pulses or nicotine. The presence of perivascular nerve fibers containing reduced nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase, acetylcholinesterase, and VIP immunoreactivity were determined histologically.

Results. Transmural electrical stimulation (2, 5, and 20 Hz) and nicotine produced a relaxation of the arterial strips denuded of the endothelium and contracted with prostaglandin F2α. The response was not influenced by timolol but was abolished by oxyhemoglobin and methylene blue. N⁶-nitro-L-arginine, a nitric oxide (NO) synthase inhibitor, abolished the neurogenic relaxation, and L-arginine restored the response. Physostigmine inhibited, but atropine potentiated, the neurogenic response. The relaxation was attenuated by acetylcholine but was not influenced by VIP. There were nerve fibers and bundles containing NADPH diaphorase, acetylcholinesterase, and VIP immunoreactivity in the adventitia of ciliary arteries.

Conclusions. Porcine ciliary arteries are innervated by NO synthase-containing nerves that liberate NO, possibly as a neurotransmitter on excitation to produce muscular relaxation. Nitroxidergic nerve function is inhibited by acetylcholine released from cholinergic nerve, possibly because of impaired production or release of NO. VIP does not seem to function as a neurotransmitter or a modulator. Invest Ophthalmol Vis Sci. 1997; 38:2261-2269.

It is widely recognized that nitric oxide (NO) plays important roles in the regulation of blood flow by dilating the vasculature in various regions of primate and subprimate mammals and also in protection from atherosclerosis by inhibiting platelet aggregation and adhesion or smooth muscle proliferation.¹² Functional studies on ocular arteries and arterioles or blood flow have revealed evidence that the ocular vascular tone or circulation is also controlled by NO synthesized from L-arginine. Intravenous or topical application of NO synthase inhibitors vasoconstricts ocular arteries and arterioles or decreases ocular blood flow.³⁻⁷ Many of the authors have suggested that suppressed synthesis of NO in the endothelium is a mechanism underlying the inhibitors’ action. However, we have demonstrated the possible release of NO as a neurotransmitter from perivascular nerves in cerebral and ocular (ophthalmic, retinal, and ciliary) arteries from primate and subprimate mammals,¹⁷⁻¹⁸; thus, it is postulated that impaired NO synthesis in nerve terminals by NO synthase inhibitors is also involved.’¹

Histochemical studies have demonstrated the presence of neurons containing not only acetylcholinesterase but also NO synthase and vasoactive intestinal polypeptide (VIP) in pterygopalatine ganglia,¹⁶⁻¹⁸ from which NO synthase-immunoreactive nerve fibers innervating canine retinal and cerebral arteries originate.¹⁰ Pharmacologic studies so far reported have indicated that acetylcholine and VIP do not act as vasodilator neurotransmitters in canine cerebral,¹⁷,¹⁸ ophthalmic, and retinal arteries.¹⁴,¹¹ However, these compounds, if liberated by nerve stimulation, may play some role in modulating the ocular arterial smooth muscle tone.

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Supported in part by a Grant-in-Aid for Scientific Research (B) from the Ministry of Education, Science, Culture and Sports, and a Research Grant for Cardiovascular Diseases (64-3) from the Ministry of Health and Welfare, Japan.

Submitted for publication March 20, 1997; revised June 5, 1997; accepted June 9, 1997.

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The present study was undertaken to determine vasodilator innervation in porcine ciliary arteries, to analyze mechanisms underlying the relaxation induced by nerve stimulation by electrical pulses and nicotine, and to clarify modifications by cholinergic and VIPergic nerves of the vasodilator nerve function.

METHODS

Preparation
All experimental procedures that involved animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The institutional review board at our university approved the use of animal blood vessels in this study. Pigs of both sexes, weighing 12 to 29 kg, were anesthetized with intramuscular injections of ketamine (40 mg/kg) and intravenous injections of sodium thiopental (20 mg/kg) and were killed by bleeding from the carotid arteries. Eyeballs, along with the optic nerves and extraocular tissue, were removed from the orbital cavities. Posterior ciliary arteries were isolated and cut into helical strips approximately 15 mm long. The endothelium was removed by gently rubbing the intimal surface with a cotton ball; endothelial denudation was verified by abolishment of the relaxation caused by 10^{-7} M Ca^{2+} ionophore A23187.

The specimens were fixed vertically between hooks in a muscle bath (20-ml capacity) containing modified Ringer–Locke solution maintained at 37°C ± 0.3°C aerated with a mixture of 95% oxygen and 5% carbon dioxide. The hook anchoring the upper end of the strip was connected to the lever of a force-displacement transducer (Nihonkohden Kogyo, Tokyo, Japan). The resting tension was adjusted to 0.7 g, which is optimal for inducing maximal contraction. The composition of the bathing medium was as follows: 120 mM NaCl, 5.4 mM KCl, 2.2 mM CaCl_2, 1.0 mM MgCl_2, 25.0 mM NaHCO_3, and 5.6 mM dextrose. The pH of the solution was 7.36 to 7.42. Before the experiments began, all the specimens were allowed to equilibrate for 60 to 90 minutes in the bathing media, during which time the medium was replaced three times every 10 to 15 minutes.

Tension Recording
Isometric contractions and relaxations were recorded on an ink-writing oscillograph. The contraction induced by 3 × 10^{-5} M K+ was first obtained, and the arterial strips were repeatedly washed by the fresh medium and equilibrated. Only one specimen per pig per side was used. The arteries were partially contracted with prostaglandin F_20 (5 × 10^{-7} to 3 × 10^{-6} M); the contraction ranged from 25% to 35% of the contraction induced by 3 × 10^{-2} M K+. At the end of each experiment, papaverine (10^{-4} M) was added to obtain the maximal relaxation. Relaxations and contractions induced by test drugs were presented as relative values to the relaxation caused by 10^{-4} M papaverine and the contraction caused by 3 × 10^{-2} M K+, respectively. Most of the arteries were placed between platinum electrodes to stimulate nerve terminals transmurally by the application of electrical square pulses of 0.2-msec duration at frequencies of 2, 5, and 20 Hz for periods of 100, 40, and 10 sec, respectively (total pulse number = 200). The concentration–response curves for nicotine (5 × 10^{-6} to 5 × 10^{-4} M) were obtained by applying a single concentration in each series to avoid tachyphylaxis. Nicotine and NO in single concentrations were successively applied. The strips had been exposed for 20 to 30 minutes to blocking agents before the responses to agonists or electrical stimulation were obtained.

Histologic Analysis
Isolated ciliary arteries were fixed in ice-cold 10^{-1} M phosphate-buffered saline (PBS, pH 7.4) containing 0.3% glutaraldehyde and 4% paraformaldehyde for 7 minutes and were then postfixed overnight in PBS with 4% paraformaldehyde, followed by cryoprotection in 15% sucrose. Thin sections (20-μm thick) were cut on a cryostat (−18°C). For reduced nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase staining, the tissue sections were mounted onto glass slides coated with gelatin, chrome, and alum and incubated with 10^{-1} M PBS (pH 8.0) containing 10^{-5} M β-NADPH (reduced form) (Kohjin, Tokyo, Japan), 2 × 10^{-3} M nitro blue tetrazolium (Sigma Chemical, St. Louis, MO), and 0.3% Triton X-100 at 37°C. After several washes with distilled water, the sections were coveredslipped with xylene and alkylacylates. Free-floating thin sections were stained for acetylcholinesterase according to the method described by Tago et al.19 In brief, the sections were fixed by 10^{-1} M PBS containing 4% paraformaldehyde and 5% glutaraldehyde. Pseudocholinesterase was eliminated by the use of tetraisopropyl pyrophosphoramide (10^{-5} M).

Other thin sections, cut on a cryostat, were exposed to polyclonal rabbit anti-VIP (porcine) antibody (Incstar, Wokingham, Berkshire, United Kingdom) (1:1000) in PBS with 0.3% Triton X-100 for 2 days at 4°C. Subsequently, biotinylated goat antirabbit immunoglobulin G antibody and avidin-biotinylated peroxidase complex (Vector Laboratories, Burlingame, CA) were conjugated to the primary antibody at 4°C for 12 hours each. Immunolabeled peroxidase was visualized by incubation at room temperature for 2 minutes with 5.6 × 10^{-4} M 3'3'-diaminobenzidine tetrahydrochloride, 1.3 × 10^{-3} M hydrogen peroxide, and 10^{-2} M nickel ammonium sulfate under a dissecting microscope with ×8 magnification. The sections were air-dried onto glass slides coated with gelatin, chrome,
and alum and coverslipped with Entellan (Merck, Darmstadt, Germany).

Statistics and Drugs Used

The results shown in the text, table, and figures are expressed as mean values ± standard error. Statistical analyses were made using the Student’s paired and unpaired t-tests for two groups and the Tukey’s method after one-way analysis of variance for three or more groups. Drugs used were nicotine, L- and D-arginine, hexamethonium bromide, methylene blue (Nacalai Tesque, Kyoto, Japan), indomethacin, timolol maleate, physostigmine sulfate, [D-p-Cl-Phe6,Leu17] VIP (Sigma), tetrodotoxin (Sankyo, Tokyo, Japan), prazosin hydrochloride (Wako Pure Chemical Industries, Osaka, Japan), prostaglandin F2 alpha (Pharmacia Upjohn, Tokyo, Japan), VIP, N6-nitro-L-arginine (L-NA), N6-nitro-D-arginine (D-NA), [8-37] calcitonin gene-related peptide ([8-37] CGRP) (Peptide Institute, Osaka, Japan), acetylcholine chloride (Daichi, Tokyo, Japan), Ca2+ ionophore A23187 (C.H. Boehringer Ingeheim, Elmsford, NY), and papaverine hydrochloride (Dainippon Pharmaceutical, Osaka, Japan). Responses to NO were obtained by adding the NaNO2 solution adjusted at pH 2.20 Oxyhemoglobin was prepared according to the method of Martin et al.21 with the use of hemoglobin (Sigma).

RESULTS

Effect of Transmural Electrical Stimulation

In ciliary arterial strips partially contracted with prostaglandin F2 alpha from which the endothelium was removed, transmural electrical stimulation at frequencies of 2, 5, and 20 Hz for 100, 40, and 10 seconds, respectively, produced frequency-related relaxations. Stimulation of perivascular nerves by electrical pulses was verified by abolishment of the response by treatment with 3 X 10^-7 M tetrodotoxin. Treatment with 10^-5 M L-NA abolished the stimulation-induced relaxation. L-arginine (10^-3 M), but not D-arginine (10^-3 M), reversed the response (Fig. 1). The results are quantitatively compared in Figure 2. D-NA (10^-5 M) did not alter the response to nerve stimulation (n = 4).

Relaxant responses to transmural electrical stimulation (5 Hz) were not influenced by timolol (10^-7 M), [8-37] CGRP (10^-7 M), a CGRP antagonist, and [D-p-Cl-Phe6,Leu17] VIP (10^-6 M), a VIP receptor antagonist, but were abolished by treatment with oxyhemoglobin (10^-5 M) and methylene blue (10^-5 M) (Table 1). Treatment with VIP (10^-3 and 10^-8 M) did not significantly alter the response, although the peptide in these concentrations relaxed the arteries (13.8 ± 4.1% and 32.0 ± 4.0%, n = 7, relative to the relaxation caused by 10^-4 M papaverine).

Effect of Nicotine

Nicotine (5 X 10^-6 to 5 X 10^-4 M), applied in a single concentration in each series, produced a dose-dependent relaxation in ciliary arterial strips denuded of endothelium. The concentration–response curve of nicotine is shown in Figure 3. The maximal relaxation was attained at 10^-4 M. The response at this concentration was consistent and reproducible; therefore, 10^-4 M nicotine was used to analyze the mechanism of action in the remainder of this study. Nicotine (10^-4 M) and NO (acidified NaNO2, 10^-7 and 10^-6 M) elicited moderate relaxations (Fig. 4). Treatment with D-NA did not alter the responses, but L-NA abolished only a relaxation induced by nicotine. The relaxation was restored by L-arginine (10^-3 M). Effects of L-NA and D-NA and L- and D-arginine are compared in Figure 5.

Treatment with timolol (10^-7 M) did not inhibit the nicotine-induced relaxation; it was, however, abolished by hexamethonium (10^-5 M) (see Table 1). Oxyhemoglobin (10^-5 M) and methylene blue (10^-5 M) also abolished the response to nicotine.

Modification by Acetylcholine of the Response to Electrical Nerve Stimulation

The relaxation elicited by electrical nerve stimulation at 5 Hz was inhibited with physostigmine (10^-7 M) and was reversed or potentiated by atropine (10^-7 M). Typical responses are shown in Figure 6. Quantitative data are summarized in Figure 7. Paired comparisons between the responses under control and atropine-treated conditions indicate that atropine produced significant potentiation (10.3 ± 3.8% increase in the response, n = 7, P < 0.05). The stimulation-induced response was also inhibited by acetylcholine (10^-6 and 10^-5 M) in a dose-dependent manner (Fig. 8). Atropine (10^-7 M) reversed the inhibition and potentiated the response, as compared with that in control media. The relaxation induced by exogenously applied NO was not significantly reduced by acetylcholine; mean values of the response at 10^-7 M NO before and after acetylcholine (10^-6 and 10^-5 M) were 33.1 ± 6.4%, 27.3 ± 5.9%, and 25.4 ± 5.1% (n = 7), respectively. Those at 10^-6 M NO were 54.0 ± 6.5%, 52.3 ± 6.3%, and 55.3 ± 4.5% (n = 7), respectively.

In the arterial strips in which the relaxant response to 5-Hz stimulation was reversed to a contraction by treatment with L-NA (10^-6 M), the effects of physostigmine (10^-7 M) and atropine (10^-7 M) were evaluated. The contraction (55 ± 15 mg, n = 6) was inhibited by 23.7 ± 8.8% (P < 0.05, paired t-test) by physostigmine, and the inhibition was reversed by atropine (−2.7 ± 6.2% versus control). The atropine-resistant contraction was depressed by 10^-5 M prazosin (53.0 ± 5.6% inhibition, n = 6, P < 0.001, paired t-test), and the remaining response was abolished by...
FIGURE 1. Recordings of the response to transmural electrical stimulation (5 Hz) of a ciliary arterial strip before and after treatment with N°-nitro-D-arginine (D-NA, 10^{-5} M), N°-nitro-L-arginine (L-NA, 10^{-5} M), D-arginine (10^{-3} M), L-arginine (10^{-3} M), and tetrodotoxin (TTX, 3 \times 10^{-7} M). The strip was partially contracted with 3 \times 10^{-7} M prostaglandin F_{2α}. PA represents 10^{-4} M papaverine, which produced the maximal relaxation. Dots denote the application of electrical stimulation. Upward arrows indicate the addition of supplemental dose of prostaglandin F_{2α}.

PORCINE CILIARY ARTERY

FIGURE 2. Relaxant responses to transmural electrical stimulation at different frequencies of ciliary arterial strips before (filled circle) and after treatment with either N°-nitro-L-arginine (L-NA, 10^{-3} M; open circle) or L-NA plus l-arginine (X, 10^{-3} M). The arterial strips were contracted with prostaglandin F_{2α}. Relaxations induced by 10^{-4} M papaverine were taken as 100%. Significantly different from control, aP < 0.01, bP < 0.05; significantly different from the value with L-NA plus l-arginine, cP < 0.01 (Tukey’s method). Seven strips from different pigs were used. Vertical bars = standard error.

Histology

Nerve fibers and bundles containing NADPH diaphorase and acetylcholinesterase were histochemically demonstrated in the adventitia of the ciliary arteries (Figs. 9A and 9B). VIP immunoreactivity was also seen in perivascular nerve fibers innervating the artery (Fig. 9C). Similar results were also obtained in the arteries from two additional pigs. In particular, the artery is

TABLE 1. Modifications by Antagonists of the Relaxant Response to Transmural Electrical Stimulation (TES, 5 Hz) or Nicotine (10^{-8} M) of Porcine Ciliary Arterial Strips Denuded of the Endothelium

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Control (%)</th>
<th>Experimental (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TES</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Timolol, 10^{-7} M</td>
<td>4</td>
<td>38.5 ± 4.7</td>
<td>39.5 ± 4.9</td>
</tr>
<tr>
<td>[8-37]CGRP, 10^{-7} M</td>
<td>7</td>
<td>33.0 ± 2.9</td>
<td>33.6 ± 2.6</td>
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<td>VIP antagonist, 10^{-6} M</td>
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<td>28.0 ± 4.4</td>
<td>28.6 ± 4.3</td>
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<td>Oxymethemoglobin, 10^{-7} M</td>
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<td>38.2 ± 5.0</td>
<td>38.7 ± 5.4</td>
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<tr>
<td>Methylene blue, 10^{-5} M</td>
<td>7</td>
<td>37.7 ± 4.7</td>
<td>36.9 ± 3.9</td>
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<td>37.3 ± 4.3</td>
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<td>36.8 ± 4.6</td>
<td>37.6 ± 4.2</td>
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<td>Nicotine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Timolol, 10^{-7} M</td>
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<td>66.3 ± 2.9</td>
<td>66.9 ± 4.1</td>
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<tr>
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<td>70.0 ± 3.6</td>
<td>54.2 ± 2.3</td>
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<td>61.2 ± 6.5</td>
<td>73.3 ± 3.7</td>
</tr>
<tr>
<td>Methylene blue, 10^{-9} M</td>
<td>5</td>
<td>66.2 ± 4.0</td>
<td>52.6 ± 3.6</td>
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</table>

n = number of strips from separate pigs; VIP antagonist = [D-p-Glu-Phe6,Leu17]VIP. Relaxations were expressed as relative values to those induced by 10^{-4} M papaverine. *P < 0.02 vs control (unpaired t-test). †P < 0.001 vs control (unpaired t-test).
Nerve-Derived NO and ACh in Ciliary Artery

FIGURE 3. Concentration–relaxation curve of nicotine in ciliary arterial strips contracted with prostaglandin F$_{2\alpha}$. Nicotine in one of the concentrations from $5 \times 10^{-6}$ to $5 \times 10^{-4}$ M was applied in each series to avoid tachyphylaxis. Relaxations induced by $10^{-4}$ M papaverine were taken as 100%. Five strips from different pigs were used. Vertical bars = standard error.

surrounded by abundant, cholinesterase-containing neurons.

DISCUSSION

Transmural electrical stimulation (2 to 20 Hz) or nicotine ($10^{-4}$ M) relaxed isolated, endothelium-denuded porcine ciliary arteries in a frequency- or dose-dependent manner. The responses to stimulation by electrical pulses and nicotine were abolished by tetrodotoxin and hexamethonium, respectively, suggesting the involvement of perivascular nerve activation. Treatment with L-NA, an NO synthase inhibitor, abolished the neurogenic response, which was restored by the addition of L-, but not D-, arginine. D-NA was without effect. L-NA did not influence the relaxation induced by exogenous NO. Similar findings have also been obtained in canine ophthalmic and retinal arteries and human ciliary arteries. The relaxations elicited by nerve stimulation and exogenous NO were abolished by treatment with oxyhemoglobin, an NO scavenger, and methylene blue, an inhibitor of soluble guanylate cyclase. Networks of the nerve fibers containing NO synthase, demonstrated by the NADPH diaphorase staining method, were found in porcine ciliary arteries, as in canine retinal arteries. These findings strongly support the hypothesis that NO synthesized from L-arginine and liberated as a neurotransmitter from the nerve acts on soluble guanylate cyclase in smooth muscle and promotes the production of 3',5' cyclic guanosine monophosphate, resulting in the relaxation of porcine ciliary arteries. The nerve is called “nitroxidergic.”

According to Wiencke et al., NO and CGRP participate in the neurogenic relaxation of bovine ciliary arteries. However, CGRP does not seem to be involved in the porcine ciliary artery, because treatment with

FIGURE 4. Typical tracings of the response to nicotine ($10^{-4}$ M) and nitric oxide (acidified NaNO$_{2}$, $10^{-7}$ and $10^{-6}$ M) of a ciliary artery before and after treatment with N$^\omega$-nitro-D-arginine (D-NA, $10^{-5}$ M), N$^\omega$-nitro-L-arginine (L-NA, $10^{-5}$ M), and L-arginine ($10^{-3}$ M). The strip was partially contracted with $5 \times 10^{-7}$ M prostaglandin F$_{2\alpha}$. PA represents $10^{-4}$ M papaverine, which produced the maximal relaxation.
the CGRP receptor antagonist in a concentration sufficient to suppress the response to exogenous CGRP did not inhibit the response to nerve stimulation. Treatment with timolol, indomethacin, and atropine did not inhibit the response to nerve stimulation, suggesting that norepinephrine, vasodilator prostanoids, and acetylcholine do not act as vasodilator neurotransmitters.

Relaxations induced by electrical nerve stimulation were attenuated by acetylcholine (10^{-6} and 10^{-5} M) in a concentration-dependent manner, and the effect was abolished by atropine. Acetylcholine did not inhibit the relaxation induced by exogenous NO.

**FIGURE 5.** Modifications by NO-nitro-D-arginine (D-NA, 10^{-5} M), NO-nitro-L-arginine (L-NA, 10^{-5} M), L-NA plus D-arginine (D-Arg, 10^{-5} M), and L-NA plus L-arginine (L-Arg, 10^{-5} M) of the response to nicotine and nitric oxide (acidified NaNO2) in ciliary arterial strips contracted with prostaglandin F2α. Relaxations induced by 10^{-4} M papaverine were taken as 100%. Significantly different from control (C), *P < 0.01; significantly different from the value with D-NA, **P < 0.01; significantly different from the value with L-NA plus L-arginine, †P < 0.01 (Tukey's method). Nine strips from different pigs were used. Vertical bars = standard error.

**FIGURE 6.** Typical tracing of the response to transmural electrical stimulation (5 Hz) in a porcine ciliary arterial strip before and after treatment with physostigmine (10^{-7} M), atropine (AT, 10^{-7} M), and tetrodotoxin (TTX, 3 × 10^{-7} M). The strip was partially contracted with 4 × 10^{-7} M prostaglandin F2α. PA represents 10^{-4} M papaverine, which produced the maximal relaxation. Dots denote the application of electrical stimulation.

**FIGURE 7.** Modifications by physostigmine (ES, 10^{-7} M) and atropine (AT, 10^{-7} M) of the response to transmural electrical stimulation (5 Hz) of ciliary arterial strips contracted with prostaglandin F2α. Relaxations induced by 10^{-4} M papaverine were taken as 100%. Significantly different from the value with atropine, *P < 0.05 (Tukey's method). Numbers in the columns indicate the values for paired comparisons (34% inhibition by physostigmine and 10% potentiation by atropine). Significantly different from control, *P < 0.01; **P < 0.02; significantly different from the value with atropine, †P < 0.01 (paired t-test). Seven strips from different pigs were used. Vertical bars = standard error.
Stimulation of prejunctional muscarinic receptors in adrenergic nerves and parasympathetic nerves has been reported to inhibit the release of neurotransmitter norepinephrine and VIP, respectively. This would be the case in nitroxidergic nerves.

Despite the clear demonstration of prejunctional actions of exogenous acetylcholine, there is a paucity of evidence concerning the functional role of endogenous acetylcholine in blood vessels. Contrasting results have been reported in canine and bovine cerebral arteries; neurogenic acetylcholine is prejunctionally effective only in the bovine artery.

Physostigmine in a concentration (10^-7 M) sufficient to significantly potentiate the vascular action of acetylcholine inhibited the neurogenic relaxation; in contrast, atropine potentiated the response. Perivascular nerve fibers containing acetylcholinesterase were histochemically demonstrated in the porcine ciliary artery. Electrical train pulses applied would stimulate nerve terminals, including nitroxidergic and cholinergic, innervating the arterial wall. Physostigmine does not seem to potentiate the action of acetylcholine liberated from cholinergic nerves on the endothelium, because all of the arterial strips used were denuded of endothelium. However, it is expected that the cholinesterase inhibitor augments the action of neurogenic acetylcholine on prejunctional muscarinic receptors responsible for reduced release of NO from nitroxidergic nerves, as speculated above for the inhibitory effect of exogenous acetylcholine. A possible mechanism of prejunctional actions is to interfere with the influx of Ca^2+ like those of Cd^2+ and Mg^2+; although analytic studies could not be carried out in the present study. The hypothesis on the prejunc-

![Graph of Relaxation (% vs. Concentration](image)

**Figure 8.** Attenuation by acetylcholine (ACh, 10^-6 and 10^-5 M) and reversal of attenuation by atropine (AT, 10^-7 M) of the relaxation response to transmural electrical stimulation of ciliary arterial strips contracted with prostaglandin F_2alpha. Relaxations induced by 10^-4 M papaverine were taken as 100%. Significantly different from control \( * P < 0.01 \), \( ** P < 0.05 \); significantly different from the value with atropine, \( \triangle P < 0.01 \) (Tukey's method). Numbers in the columns indicate the values for paired comparisons (36% and 54% inhibition by ACh 10^-6 and 10^-5 M, respectively, and 28% potentiation by AT). Significantly different from control, \( \triangle P < 0.01 \), \( ** P < 0.05 \); significantly different from the value with atropine, \( \triangle P < 0.01 \) (paired test). Seven strips from different pigs were used. Vertical bars = standard error.

**Figure 9.** Histochemical demonstration of perivascular nerves containing NADPH diaphorase (A), acetylcholinesterase (B), and vasoactive intestinal polypeptide (C) in porcine ciliary arteries. There are some nerve bundles (arrows) and plenty of nerve fibers (arrowheads) in the adventitia close to the adventitiomedial border. Bar = 50 μm.
tional action of endogenous acetylcholine is supported by the data obtained with atropine, which potentiated the vasodilator response to nerve stimulation, possibly by an antagonism to presynaptic muscarinic actions of acetylcholine.

The other possibility for the inhibitory effect of physostigmine is that it potentiates the contractile action of acetylcholine seen in canine cerebral arteries and portal and mesenteric veins. In the arterial strips treated with L-NA in which the neurogenic contraction was obtained, the response was not potentiated but rather inhibited by physostigmine, suggesting that acetylcholine from cholinergic nerves does not participate in the stimulation-induced contraction. Endogenous acetylcholine appears to act prejunctionally on vasoconstrictor nerves and attenuate the release of neurotransmitters, as seen in nitroxidergic nerves. On the basis of results with prazosin, an alpha-1 adrenoceptor antagonist, and α, β-methylene ATP, a P2x receptor antagonist, the neurogenic vasoconstriction caused by acetylcholine from cholinergic nerves does not participate in the basis of results with prazosin, an alpha-1 adrenoceptor antagonist, and α, β-methylene ATP, a P2x receptor antagonist, the neurogenic vasoconstriction is expected to be mediated mainly by norepinephrine and also by ATP.

In our preliminary study with endothelium-intact strips, atropine did not reduce but rather potentiated the response to electrical stimulation or nicotine, and the neurogenic relaxation was not reduced by endothelium denudation. No difference has been seen in the neurally induced relaxation of the strips with and without the endothelium in canine cerebral, retinal, and internal ophthalmic arteries. However, VIP in concentrations sufficient to produce significant relaxations of the ciliary artery did not influence the response to nerve stimulation; this excludes the possibility of its neuromodulator action. The concentrations used were sufficient to develop tachyphylaxis to this peptide. The neurogenic relaxation was also unaffected by treatment with a VIP receptor antagonist but was abolished by NO synthase inhibitors in deendothelialized strips. VIP-induced relaxations were independent of endothelial status (data not shown). Therefore, VIP, even though released by nerve stimulation, does not seem sufficient to act as a neurotransmitter.

The present study revealed that strong vasodilator and weak vasoconstrictor nerves function in the porcine ciliary artery under the experimental conditions used. It appears that vasodilatation due to nerve stimulation is mediated by NO produced from L-arginine by NO synthase, whereas the neurogenic vasoconstriction is associated with stimulation of alpha-1 adrenoceptors and P2x purinoceptors. The nitroxidergic nerve function would be impaired by activation of prejunctional muscarinic receptors caused by acetylcholine liberated from cholinergic nerves. Although neurons containing VIP immunoreactivity innervate ciliary arteries, vascular actions of nerve-derived VIP so far tested could not be observed. Porcine ocular circulation is expected to be maintained by tonic discharges of nitroxidergic vasodilator nerves from the vasomotor center and by nitroxidergic–cholinergic nerve interactions in neuromuscular transmission.

**Key Words**

cholinergic nerve, ciliary artery, neurogenic nitric oxide, presynaptic inhibition

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