Cholinergic nerve function in monkey ciliary arteries innervated by nitroxidergic nerve

Toda, Noboru, Megumi Toda, Kazuhide Ayajiki, and Tomio Okamura. Cholinergic nerve function in monkey ciliary arteries innervated by nitroxidergic nerve. Am. J. Physiol. 274 (Heart Circ. Physiol. 43): H1582–H1589, 1998.—We sought to determine the control of ciliary arterial tone by neurogenic acetylcholine (ACh) acting directly on smooth muscle and in conjunction with vasodilator nerves. Isolated posterior ciliary arteries from monkeys responded to ACh (10⁻⁸–10⁻⁵ M) with dose-related contractions, which were endothelium independent. The response was not affected by cyclooxygenase inhibitors but was abolished by atropine. Relaxations induced at 10⁻⁴ M ACh in the atropine-treated arterial strips were abolished by hexamethonium and N⁵-nitro-L-arginine (L-NNA), and L-arginine (L-Arg) reversed the response suppressed by L-NNA. Similar results were also obtained on the nicotine (10⁻⁴ M)-induced relaxation. Contractions due to transmural electrical stimulation in the endothelium-denuded strips treated with L-NNA were potentiated by physostigmine and depressed by atropine; the remaining contraction in the presence of atropine was abolished by prazosin. Relaxations associated with electrical stimulation, sensitive to tetrodotoxin, were abolished or reversed to contractions by L-NNA and restored by L-Arg. Stimulation-induced relaxation was attenuated by exogenous ACh and physostigmine and was potentiated by atropine. ACh did not affect the relaxation caused by nitric oxide (NO). Nerve fibers and bundles containing NADPH diaphorase and acetylcholinesterase were histologically demonstrated in the adventitia of ciliary arteries. We conclude that 1) endogenous and exogenous ACh contracts monkey ciliary arteries by acting on muscarinic receptors in smooth muscle cell membranes, 2) vasodilatation elicited by nerve stimulation with electrical pulses or nicotine is mediated by NO synthesized from L-Arg, 3) neurogenic ACh seems to interfere with the nitroxidergic nerve function by acting on prejunctional muscarinic receptors, and 4) high concentrations of ACh stimulate nicotinic receptors in vasodilator nerve terminals and promote the synthesis and/or release of NO.

FUNCTIONAL ROLES of cholinergic nerve innervating blood vessels have not clearly been determined. Information for decreased vascular resistance mediated by neurogenic acetylcholine that liberates relaxing factor derived from the endothelium, possibly nitric oxide (NO), is limited (13, 15). Vasoconstriction due to acetylcholine from cholinergic nerve is observed only in canine isolated mesenteric and portal veins (36). Although prejunctional inhibition by exogenous acetylcholine of efferent autonomic nerve function in vasculatures is widely recognized (1, 12, 18, 33), little information is available concerning the effect of neuronally released acetylcholine on prejunctional receptors (4, 21).

Recent studies on cerebral and peripheral arteries in vitro and arterioles in vivo have provided evidence supporting the hypothesis that vasodilatation induced by nerve stimulation is mediated by NO synthesized from L-arginine in nerve terminals (20, 24, 26, 27, 29). Coexistence of NO synthase, acetylcholinesterase, and vasoactive intestinal polyepptide (VIP) in parasympathetic ganglionic cells, and possibly in neurons, that were histologically demonstrated (9, 11, 16) suggests interactions of NO, acetylcholine, and VIP on postjunctional sites and their synthesis and/or release in nerve terminals.

Acetylcholine produces contraction in canine and porcine cerebral arteries (3, 10, 32) and relaxation independent of the endothelium in monkey cerebral and canine retinal arteries (28, 32). Other canine, porcine, and monkey arteries, including coronary, mesenteric, and temporal, respond to acetylcholine with endothelium-dependent relaxation (14, 17, 32), as do many other blood vessels from various mammals (2, 7). In ocular and cerebral arteries, vasodilator innervation is dominant over vasoconstrictor nerve (25, 29); the opposite is true of most other blood vessels.

Aims of the study were 1) to analyze the action and the mechanism of action of acetylcholine derived from nerves and exogenously applied in monkey posterior ciliary arteries; and 2) to determine the influence of endogenous acetylcholine on vasodilator nerves, the information of which is transferred to smooth muscle, possibly via NO.

METHODS

The studies review board at our University approved the use of animal blood vessels in this study.

Preparation. Japanese monkeys (Macaca fascata) of either sex, weighing 6–10 kg, were killed by exsanguination from the common carotid arteries while anesthetized with intramuscular injection of ketamine (40 mg/kg) and intravenous injection of thiopental sodium (20 mg/kg). The eyeballs attached with optic nerves and extraocular tissues were rapidly removed from the orbital cavities. Branches of the short posterior ciliary artery (0.3–0.4 mm outside diameter) were isolated and cut into helical strips of ~15 mm in length. The endothelium was removed by gently rubbing the intimal surface with a cotton ball, unless otherwise mentioned. The specimens were vertically fixed between hooks in a muscle bath (20-ml capacity) containing the modified Ringer-Locke solution, which was maintained at 37 ± 0.3°C and aerated with a mixture of 95% O₂-5% CO₂. The hook anchoring the upper end of the strips was connected to the lever of a force-displacement transducer (Nihon-Kohden Kogyo, Tokyo, Japan). Some of the strips were placed between stimulating electrodes, and electrical pulses of 0.2 ms at frequencies of 2, 5, and 20 Hz were transmurally applied to stimulate perivascular nerves. Under these stimulus conditions, submaximal and reproducible relaxant responses were observed at 5 Hz in
monkey cerebral arteries (27), and the same was true with monkey ciliary arteries in our preliminary study. Therefore, the data obtained at 5 Hz were analyzed in the following study. The resting tension was adjusted to 1.0 g, which was optimal for inducing the maximal contraction. The composition of the bathing solution was as follows (mM): 120 NaCl, 5.5 KCl, 2.2 CaCl₂, 1.0 MgCl₂, 25.0 NaHCO₃, and 5.6 dextrose. The pH of the solution was 7.38–7.44. Before the start of experiments, all of the strips were allowed to equilibrate for 60–80 min in the bathing media, during which time the fluid was replaced every 10–15 min.

Tension recording. Isometric mechanical responses were displayed on an ink-writing oscillograph (Nihon-Kohden Kogyo). The contractile response to 30 mM K⁺ was first obtained, and the arterial strips were repeatedly washed with fresh media and equilibrated. The strips were partially contracted with prostaglandin (PGF₂ₐ) (7–45 × 10⁻⁸ M), with the contraction range between 28 and 46% of the contraction induced by 30 mM K⁺. Removal of the endothelium was determined by abolishment of the relaxation induced by substance P (10⁻⁸ M) or Ca²⁺ ionophore A-23187 (10⁻⁷ M). To obtain concentration-response relationships, acetylcholine was applied singly or cumulatively to the bathing media. Nicotine (10⁻⁴ M) and NO (acidified NaNO₂ solution) in one or two concentrations (10⁻⁷ and 10⁻⁶ M) were applied successively. In the preliminary study, nicotine produced a dose-dependent relaxation (2 × 10⁻⁵, 10⁻⁴, and 5 × 10⁻⁴ M). The concentration-response relationship was obtained by applying only one concentration in each trial to avoid the development of tachyphylaxis. Because submaximal and reproducible relaxations were obtained at 10⁻⁴ M, the concentration was used for the following analysis. Transmural electrical stimulation was applied every 10 min until the response was determined to be reproducible. At the end of each series of experiment, papaverine (10⁻⁴ M) was added to attain the maximal relaxation, which was taken as 100% for the relaxation induced by agonists or nerve stimulation. On the other hand, contractile responses were expressed as absolute values or values relative to those caused by 30 mM K⁺. The arterial strips had been treated for 20–30 min with blocking agents before the effects of agonists or electrical nerve stimulation were obtained. Histochemistry. Isolated monkey posterior ciliary arteries were fixed in ice-cold 0.1 M phosphate-buffered saline (PBS, pH 7.4) containing 0.3% glutaraldehyde and 4% paraformaldehyde for 7 min. The arteries were then postfixed overnight in 0.1 M PBS with paraformaldehyde, followed by cryoprotection in 15% sucrose. The fixed blocks were cut into sections (20-μm thick) in a cryostat. For NADPH diaphorase staining (34), the tissue sections were mounted onto gelatin/chrome-aluminum-coated glass slides and incubated with 0.1 M PBS at pH 8.0, containing 10⁻³ M β-NADPH (reduced form) (Kohjin, Tokyo), 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 cover-slipped with xylene and alkylacrylates.

Whole mount preparations fixed in 0.1 M PBS containing 4% paraformaldehyde and 0.5% glutaraldehyde were stained for acetylcholinesterase according to the method described by Tago et al. (19). Pseudocholinesterase was inhibited with tetraisopropylpyrophosphoramide (10⁻⁵ M) (Sigma).

Statistics and drugs used. The results shown in the text and figures are expressed as means ± SE. Statistical analyses were made using the Student’s paired and unpaired t-tests and the Tukey’s method after one-way analysis of variance. Drugs used were N⁵-nitro-L-arginine (L-NNA), N⁵-nitro-D-arginine (D-NNA), substance P (Peptide Institute, Minoh, J apan), L- and D-arginine, nicotine (base), hexamethonium bromide (Nacalai Tesque, Kyoto, Japan), acetylcholine chloride (Daichii, Tokyo, Japan), atropine sulfate (Tanabe, Osaka, Japan), physostigmine (eserine) sulfate (Sigma), prazosin hydrochloride (Pfizer, Tokyo, Japan), Ca²⁺ ionophore A-23187 (Boehringer Ingelheim, Elmsford, NY), PGF₂ₐ, (Pharmacia-Upjohn, Tokyo, Japan), tetrodotoxin (Sankyo, Tokyo, Japan), and papaverine hydrochloride (Dainippon, Osaka, Japan). Responses to NO were obtained by adding NaN₂O₃ solution adjusted at pH 2 (8), and the concentrations of NaN₂O₃ in the bathing media were expressed as those of NO.

RESULTS

Effects of acetylcholine. In monkey ciliary arterial strips partially contracted with PGF₂ₐ, the addition of acetylcholine in concentrations from 10⁻⁸ to 10⁻³ M produced a dose-dependent contraction, but a relaxation was elicited from the contracted level at 10⁻⁴ M (Fig. 1). The responses did not significantly differ in endothelium-intact and -denuded arterial strips (Fig. 2, left). The acetylcholine-induced contraction was not influenced by treatment with indomethacin (10⁻⁶ M;
Fig. 2. Concentration-response curves of Ach in monkey ciliary arterial strips with (E+) and without (E−) endothelium (left) and as affected by treatment with indomethacin (IM, 10−6 M, right) and atropine (10−7 M) in endothelium-denuded strips. Strips were partially contracted with PGF2α. Contractions of Ach are expressed as relative values to K+ (30 mM)-induced contraction, and relaxations are expressed as values relative to papaverine (10−4 M)-induced relaxation. Significantly different from control: aP < 0.01, bP < 0.05; significantly different from value with indomethacin: cP < 0.01, dP < 0.05 (Tukey’s method). Numbers in parentheses indicate number of strips from separate monkeys. Vertical bars denote means ± SE.

Fig. 2, right) and aspirin (5 × 10−5 M, n = 4) but was abolished by atropine (10−7 M; Figs. 1 and 2, right). With atropine treatment, the relaxation was induced by 10−4 M acetylcholine. Hexamethonium (10−5 M) abolished the relaxation or reversed it to a slight contraction (Fig. 1); mean values with 10−4 M acetylcholine before and after the ganglionic blockade were 35.1 ± 4.7% relaxation and 2.1 ± 1.4% contraction (n = 11), respectively.

Relaxations induced by 10−4 M acetylcholine in the endothelium-intact and -denuded strips treated with atropine were abolished or reversed to a slight contraction by treatment with L-NNA (10−5 M), and the response was restored by the addition of L-arginine (10−3 M) (Fig. 3). β-NNA (10−5 M, n = 3) and D-arginine (10−3 M, n = 3) were without effect. Typical tracings of the response to acetylcholine and exogenous NO are illustrated in Fig. 4. Relaxations by NO were not affected by L-NNA and L-arginine.

Effects of nicotine. Nicotine in a concentration of 10−4 M relaxed the ciliary arterial strip treated with prazosin (10−6 M) and partially contracted with PGF2α, as reported in our previous publications on monkey and dog retinal arteries (24, 30). The quantitative data are summarized in Fig. 5. The relaxation was abolished by treatment with L-NNA (10−3 M), and the inhibition was...
MONKEY CILIARY ARTERY (Atropine-treated)

Control

L-NNA

L-NNA + L-Arg

L-NNA + L-Arg + C6

MONKEY CILIARY ARTERY

reversed by L-arginine (10⁻³ M). The nicotine-induced relaxation was also abolished by 10⁻⁵ M hexamethonium (n = 8) but was unaffected by 10⁻⁷ M atropine (n = 4). NO-induced relaxations were not affected by L-NNA and L-arginine (Fig. 5). Mean values of the relaxation induced by 10⁻⁴ M acetylcholine (32.8 ± 3.6% relaxation, n = 19), 10⁻⁴ M nicotine (39.5 ± 3.7%, n = 8), and 5-Hz electrical stimulation (28.2 ± 4.8%, n = 12) averaged 1.9, 5.0, and 1.6 × 10⁻³ M, respectively.

Responses to transmural electrical stimulation. In PGF₂α-contracted ciliary arterial strips, transmural electrical stimulation at 5 Hz produced a slight contraction (3 of 13 strips) or a moderate relaxation (remaining 10). The responses were abolished by tetrodotoxin (3 × 10⁻⁷ M). Treatment with L-NNA (10⁻⁵ M) potentiated the contraction (Fig. 6) in the three strips. In 3 of 10 strips, in which electrical stimulation caused relaxations, L-NNA reversed the relaxation to a contraction, whereas in the remaining 7 strips, the relaxation was abolished by the inhibitor.

In the six strips treated with L-NNA that responded to electrical stimulation with contractions, effects of physostigmine (10⁻⁷ M), atropine (10⁻⁷ M), and prazosin (10⁻⁸ M) were evaluated. As demonstrated in Fig. 6, the stimulation-induced contraction was potentiated by physostigmine and suppressed by atropine. The remaining contraction was abolished by prazosin. Quantitative comparisons are made in Fig. 7.

The mean absolute contraction by 5-Hz stimulation before the addition of pharmacological antagonists was 27 ± 6 mg (n = 6), in which the atropine-sensitive response averaged 22 ± 6 mg. This value relative to the contraction induced by 30 mM K⁺ was 16.6 ± 2.8%, which was equivalent to that caused by 5.1 × 10⁻⁸ M of exogenous acetylcholine (estimated from the dose-response curve in endothelium-denuded strips in Fig. 2, left).
Modification by acetylcholine of the response to transmural electrical stimulation. In the arterial strips treated with $10^{-6}$ M prazosin, relaxations induced by electrical stimulation (5 Hz) were abolished by L-NNA ($10^{-5}$ M) and partially restored by L-arginine ($10^{-3}$ M) (Fig. 5, right). The stimulation-induced relaxation was also abolished by $3 \times 10^{-7}$ M tetrodotoxin.

We determined whether endogenous and exogenous acetylcholine modified the NO-mediated neurogenic response. The stimulation-induced relaxation was significantly attenuated by treatment with physostigmine ($10^{-7}$ M) and potentiated by atropine ($10^{-7}$ M) (Fig. 8). Figure 9 shows 1) physostigmine significantly inhibited the response to transmural electrical stimulation, 2) a reversal of the inhibition by atropine (left), and 3) a significant potentiation by atropine to the response with paired comparison (middle).

The stimulation-induced response was also reduced by treatment with acetylcholine ($10^{-6}$ and $10^{-5}$ M) in a concentration-dependent manner (Fig. 9, right). The inhibition was reversed by atropine. Relaxations induced by exogenous NO ($10^{-7}$ and $10^{-6}$ M) were not influenced by these concentrations of acetylcholine ($n = 4$).

Histochemical study. There are many fine nerve fibers and bundles containing NADPH diaphorase in the adventitia of a monkey ciliary artery (Fig. 10), as demonstrated in the monkey retinal central artery (30). Networks of nerve fibers containing acetylcholinesterase are demonstrated in a whole mount preparation of the artery (Fig. 11). Similar findings were obtained in two additional monkeys.

DISCUSSION

From the present study, it appears that up to $10^{-5}$ M of acetylcholine contracts monkey posterior ciliary artery by acting on muscarinic receptors located on smooth muscle cell membranes and that vasoconstrictor prostanoids and endothelium are not involved in the response. On the other hand, acetylcholine-induced contractions of atropine-sensitive canine cerebral arteries are endothelium dependent and are abolished or reversed to relaxations by treatment with indomethacin (10, 32), suggesting the involvement of endothelium-derived vasoconstrictor prostanoids. Acetylcholine induces relaxations in bovine retinal and porcine ophthalmic arteries, which are mediated possibly by NO derived from the endothelium (5, 35).

A high concentration of acetylcholine ($10^{-4}$ M) elicited a relaxation from the contracted level by the lower concentrations in the arteries denuded of endothelium. The relaxation was not affected by treatment with a muscarinic receptor antagonist but was abolished by a nicotinic receptor antagonist and a NO synthase inhibitor, as was the response to nicotine. Similar results were also observed in canine and monkey cerebral arteries in response to transmural electrical stimulation, nicotine, acetylcholine ($10^{-4}$ M) (26, 27, 32), and canine and monkey retinal arteries (24, 30). In canine cerebral arteries, relaxations associated with electrical stimulation and nicotine are dependent on the Ca$^{2+}$ influx into neurons (29, 31); NO, measured as NO$\mathrm{x}$, is

Fig. 6. Typical tracing of response to TES (5 Hz) of a monkey ciliary arterial strip before and after treatment with L-NNA ($10^{-5}$ M), physostigmine (eserine, $10^{-7}$ M), atropine ($10^{-7}$ M), and prazosin ($10^{-6}$ M). Strip was partially contracted with PGF$_2\alpha$. PA represents $10^{-4}$ M papaverine, which produced maximal relaxation.

Fig. 7. Modifications by physostigmine (Es, $10^{-7}$ M), atropine (At, $10^{-7}$ M), and prazosin (Pr, $10^{-6}$ M) of response to TES of monkey ciliary arterial strips treated with $10^{-5}$ M L-NNA. Strips were partially contracted with PGF$_2\alpha$. Ordinate indicates stimulation-induced contraction relative to K$^+$ (30 mM)-induced contraction. Numbers in columns represent paired comparison under control (100%) and experimental conditions. Significantly different from control (C): a$P < 0.01$; significantly different from value with physostigmine: b$P < 0.01$ (Tukey's method). Significantly different from control: t$P < 0.001$, *$P < 0.02$ (paired t-test). Numbers in parentheses indicate number of strips from separate monkeys. Vertical bars represent means ± SE.
Fig. 8. Typical tracing of response to TES of a monkey ciliary arterial strip treated with $10^{-6}$ M prazosin before and after treatment with eserine ($10^{-7}$ M), At ($10^{-7}$ M), L-NNA ($10^{-5}$ M), L-Arg ($10^{-7}$ M), and tetrodotoxin (TTX, $3 \times 10^{-7}$ M). Strip was partially contracted with PGF$_2\alpha$. PA represents $10^{-4}$ M papaverine, which produced the maximal relaxation. Induced relaxation was determined to derive from nitroxidergic nerve stimulation in this strip, on basis of abolishment of response by L-NNA and TTX and restoration by L-Arg from L-NNA-induced inhibition.

Fig. 9. Modifications by Es ($10^{-7}$ M), At ($10^{-7}$ M), and ACh of response to TES (5 Hz) of monkey ciliary arterial strips treated with $10^{-6}$ M prazosin and partially contracted with PGF$_2\alpha$. Ordinate represents stimulation-induced relaxation relative to that caused by $10^{-4}$ M papaverine. Numbers in columns indicate paired comparison under control (C; 100%) and experimental conditions. Significantly different from C: $aP < 0.01$, $bP < 0.05$; significantly different from value with atropine: $cP < 0.01$ (Tukey's method). Significantly different from C: $*P < 0.001$, $**P < 0.02$, $†P < 0.05$ (paired t-test). n, number of strips from separate monkeys. Vertical bars represent means $\pm$ SE.

Fig. 10. Histochemical demonstration of nerve fibers (arrowheads) and bundles (arrows) containing NADPH diaphorase in adventitia of a monkey ciliary artery. Bar, 50 $\mu$m.
liberated and tissue guanosine 3',5'-cyclic monophosphate is increased by electrical and chemical stimulation of nerves (26–28). The present study demonstrated the presence of nerve fibers and bundles containing NADPH diaphorase as reported in the monkey retinal central artery (30). Although the staining for NADPH diaphorase does not reflect the presence of NO synthase, colocalization of both enzymes has been reported in neural tissues (6). Therefore, the relaxations due to acetylcholine, nicotine, and electrical stimulation in the monkey ciliary artery are expected to derive from NO released from vasodilator nerve terminals that increases the production of guanosine 3',5'-cyclic monophosphate in smooth muscle, resulting in relaxation. On the basis of functional study, amounts of NO liberated from neurons by 10^{-8} M acetylcholine, 10^{-8} M nicotine, and 5 Hz for 40 s are estimated to be equivalent to exogenous NO of 1.9, 5.0, and 1.6 \times 10^{-7} M, respectively.

Transmural nerve stimulation produced contractions in some ciliary arterial strips, which were potentiated by treatment with l-NNA. In the strips responding to the stimulation with relaxations, l-NNA abolished the responses or reversed those to contractions. These results suggest that the observed response is a balance of neurogenic vasoconstriction in not all strips and of neurogenic vasodilatation in all strips so far tested. In the l-NNA-treated strips, the stimulation-induced contraction was potentiated by physostigmine and suppressed by atropine. Acetylcholine-induced contractions were abolished by atropine, and the concentration of physostigmine used here is sufficient to significantly potentiate the effect of acetylcholine in isolated arteries (22). Acetylcholinesterase-containing nerve fibers were histologically demonstrated in the adventitia of monkey ciliary arteries. It is thus concluded that electrical stimulation liberates acetylcholine from cholinergic nerves, acting on muscarinic receptors on smooth muscle to elicit contraction. Atropine did not always abolish the neurogenic contraction, but the remaining response was abolished by prazosin, suggesting the involvement of norepinephrine from adrenergic nerves in the response. On the basis of the dose-response curve of acetylcholine in endothelium-denuded strips and neurogenic contraction, the release of acetylcholine from the nerve is equivalent to \sim 5.1 \times 10^{-8} M of exogenously applied acetylcholine.

Electrical stimulation-induced relaxations, mediated by NO, were attenuated by acetylcholine in a dose-dependent manner, whereas the response to exogenous NO was not influenced. Atropine abolished the inhibition, suggesting the involvement of presynaptic muscarinic receptors. Physostigmine inhibited the neurogenic relaxation, and atropine potentiated it. Acetylcholine released from electrically stimulated perivascular nerves seems to participate in the modulation of nitroxidergic nerve functions. Endogenous and exogenous acetylcholine are expected to inhibit the synthesis and/or release of NO by a mediation of muscarinic receptors, as postulated with the action of acetylcholine. Inhibition by physostigmine of the response to nerve-derived NO would therefore be due to an impairment of acetylcholine degradation by cholinesterase and an accumulation of acetylcholine in the vicinity of muscarinic receptors, which increases the contraction by acting postjunctional sites and also augments the inhibitory action on prejunctional sites in nitroxidergic nerves.

For the first time, the present study revealed the functional roles of neurogenic acetylcholine in monkey posterior ciliary arteries: 1) prejunctional actions on nitroxidergic nerves mediated by activations of muscarinic receptors responsible for impaired nerve function and of nicotinic receptors responsible for the release of neurotransmitter, NO; and 2) postjunctional actions on arterial smooth muscle by activation of muscarinic receptors involved in muscle contraction. The muscarinic actions on the pre- and postjunctional sites are expected to physiologically participate in the neural regulation of arterial tone, whereas the action on prejunctional nicotinic site may not be considered to be involved in such a physiological regulation, since the
effective concentration is too high. It is hypothesized that cholinergic nerve contributes to induce vasoco-
striction by presynaptic actions particularly in blood vessels, such as monkey ciliary (present study) and
canine retinal, ophthalmic, and cerebral arteries (24–
29), in which nitroxidergic vasodilator nerve is
predominantly involved in the regulation of vascular
tone over noradrenergic vasoconstrictor nerve.

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