Do cholinergic nerves innervating rat mesenteric arteries regulate vascular tone?

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Do cholinergic nerves innervating rat mesenteric arteries regulate vascular tone? Am J Physiol Regul Integr Comp Physiol 303: R1147–R1156, 2012. First published October 10, 2012; doi:10.1152/ajpregu.00317.2012.—Vascular blood vessels have various types of cholinergic acetylcholine receptors (AChR), but the source of ACh has not been confirmed. Perivascular adrenergic nerves and nonadrenergic calcitonin gene-related peptide (CGRP)-containing (CGRPergic) nerves innervate rat mesenteric arteries and regulate vascular tone. However, function of cholinergic innervation remains unknown. The present study investigated cholinergic innervation by examining effects of cholinesterase inhibitor (neostigmine), a muscarinic AChR antagonist (atropine), and a nicotinic AChR antagonist (hexamethonium) on adrenergic nerve-mediated vasconstriction and CGRPergic nerve-mediated vasodilation in rat mesenteric vascular beds without endothelium. In preparations treated with capsaicin (CGRP depletor) or in the presence of Nω-nitro-L-arginine methyl ester (nonselective nitric oxide synthase inhibitor), perivascular nerve stimulation (PNS; 2–12 Hz) evoked a frequency-dependent vasconstriction. In the same preparations, exogenous norepinephrine induced a concentration-dependent vasoconstriction. Atropine, hexamethonium, and neostigmine had no effect on vasoconstrictor responses to PNS and norepinephrine injections. In denuded preparations, these cholinergic agents did not affect the PNS (12 Hz)-evoked release of norepinephrine in perfusate. In preconstricted preparations without endothelium in the presence of guanethidine (adrenergic neuron blocker), PNS (1–4 Hz) induced a frequency-dependent vasodilation, which was not affected by atropine, hexamethonium, and neostigmine. In denuded preparations treated with capsaicin and guanethidine, PNS did not induce vascular responses, and atropine, neostigmine, and physostigmine had no effect on PNS. Immunohistochemistry study showed choline acetyltransferase-immunopositive fibers, which were resistant to capsaicin and 6-hydroxydopamine (adrenergic toxin). These results suggest that rat mesenteric arteries have cholinergic innervation, which is different from adrenergic and capsaicin-sensitive nerves and not associated with vascular tone regulation.

Perivascular cholinergic innervation; cholinergic nerve function; rat mesenteric artery
fibers have been identified in various regions of the rat gut including submucosal perivascular nerve fibers using pHAT-immunopositive nerve fibers (20). However, it is unknown whether cholinergic nerves innervate rat mesenteric arteries and function in regulating vascular tone.

Therefore, the present investigations were conducted to find cholinergic innervation and its function in rat mesenteric vascular beds.

**METHODS**

Perfusion of mesenteric vascular beds and measurement of perfusion pressure. The animal experiment was performed in accordance with the Ethics Review Committee for Animal Experimentation of the Graduate School of Medicine, Dentistry and Pharmaceutical Science, Okayama University.

Male Wistar rats weighing 250 to 350 g were anesthetized with pentobarbital sodium (50 mg/kg ip), and the mesenteric vascular bed without the intestine was isolated and prepared for perfusion as described previously (13, 14). The isolated mesenteric vascular bed was perfused with Krebs solution at a constant flow rate of 5 ml/min with a peristaltic pump (model AC-2120; ATTO, Tokyo, Japan) and superfused with the same solution at a rate of 0.5 ml/min to prevent drying. The Krebs solution was bubbled with a mixture of 95% O2 and 5% CO2. The Krebs solution had the following composition (in mM): 119.0 NaCl, 4.7 KCl, 2.4 CaCl2, 1.2 mM MgSO4, 25.0 mM NaHCO3, 1.2 mM KH2PO4, 0.03 EDTA-2Na, and 11.1 glucose (pH 7.4).

Changes in the perfusion pressure were measured with a pressure transducer (model TP-400T; Nihon Kohden, Tokyo, Japan) and recorded using a pen recorder (model U-228; Nippon Denshi Kagaku, Tokyo, Japan).

Chemical removal of vascular endothelium. To remove the vascular endothelium, preparations with resting tone were perfused with a 1.80 mg/ml solution of sodium deoxycholate (SD) in saline for 30 s as described previously (21, 23). The preparations were then rinsed with SD-free Krebs solution for 30 min. After the 30-min washout, Krebs solution containing methoxamine (α1-adrenoceptor agonist) and guanethidine (adrenergic neuron blocker) was used to produce vascular tone and to block adrenergic neurotransmission. Chemical removal of the endothelium was assessed by the lack of a relaxant effect after a bolus of 1 nmol acetylcholine (ACh) was injected directly into the perfusate proximal to the arterial cannula with an infusion pump (model 975; Harvard Apparatus, Holliston, MA). A volume of 100 μl was injected over a period of 12 s.

Periarterial nerve stimulation and injection of agonists. Periarterial nerve stimulation (PNS) at 2, 4, 8, and 12 Hz was applied at 7-min intervals using bipolar platinum ring electrodes placed around the superior mesenteric artery. Rectangular pulses of 1 ms and supramaximal voltage (50 V) were applied for 30 s using an electronic stimulator (model SEN 3301; Nihon Kohden). Krebs solution containing ACh, NE, or CGRP was directly injected into the perfusate proximal to the arterial cannula with an infusion pump (model 975; Harvard Apparatus). A volume of 100 μl was injected during a period of 12 s.

Experimental protocols for vasoconstriction in preparations with resting tone. After the endothelium removal with SD, capsaicin (5 μM; CGRP depletor) was perfused for 20 min to eliminate CGRPergic function according to a method described previously (21, 24). In another series of experiments, to eliminate nitrergic nerve function (7, 17), Krebs solution containing Nω-nitro-L-arginine methyl ester (L-NAME; nonsselective NOS inhibitor) at 100 μM was perfused throughout the experiment. After rinsing with capsaicin-free Krebs solution for 30 min was completed, PNS (S1) at 2–12 Hz and NE injections (I1) at 0.5 and 1.0 nmol were performed to observe adrenergic nerve- and α-adrenoceptor-mediated vasoconstrictor responses as the controls, respectively. Thereafter, the Krebs solution was switched to one containing a cholinergic agent including atropine (10 and 100 nM; muscarinic ACh receptor antagonist), hexamethonium (10 μM; nicotinic ACh receptor antagonist), or neostigmine (1 mM; cholinesterase inhibitor), and then the second PNS (S2) and NE injection (I2) were carried out. Perfusion of these cholinergic agents started 30 min before and continued throughout the PNS or NE injections. To estimate the effects of the cholinergic agents tested, changes in perfusion pressure in response to PNS or NE were expressed as the ratio between the vasoconstriction induced by S2 and I2 and S1 and I1, respectively. At the end of each experiment, the preparations were perfused with Krebs solution containing 2 μM methoxamine to elevate vascular tone and 5 μM guanethidine to block adrenergic neurotransmission. The elimination of endothelium function and CGRPergic nerve function was assessed by verifying the lack of a relaxant effect after the injection of 1 nmol ACh and PNS at 1 Hz.

Experimental protocols for vasodilation in preparations with active tone. The effects of cholinergic agents on CGRPergic nerve-mediated and exogenous CGRP-induced vasodilation were examined in denuded mesenteric vascular beds with active tone. After responses to the first PNS (S1: 1, 2, and 4 Hz) and CGRP (I1, 50 pmol) injection were obtained as the control, the Krebs solution containing 2 μM methoxamine and 5 μM guanethidine was switched to Krebs solution containing 2 μM methoxamine, 5 μM guanethidine, and atropine (10 nM), hexamethonium (1 μM), or neostigmine (100 nM), and then the second PNS (S2) and CGRP injection (I2) were carried out. After the perfusion pressure returned to preinjection (I2) levels, the Krebs solution was switched to one containing 2 μM methoxamine, 5 μM guanethidine, and a higher concentration of atropine (100 nM), hexamethonium (10 μM), or neostigmine (1 μM), and then the third PNS (S3) and CGRP injection (I3) were carried out. To quantify the effects of the cholinergic agents, changes in perfusion pressure in response to PNS or CGRP were expressed as the ratio between the vasodilation induced by S2 and I2 or I1, or S3 and I3 or I1, respectively. At the end of each experiment, 100 μM papaverine was perfused to produce complete relaxation. Vasodilation was expressed as the percentage of the perfusion pressure at maximum relaxation induced by papaverine.

Experimental protocols for vascular responses without adrenergic and CGRPergic nerve function. The preparation with intact endothelium at resting tone was perfused with Krebs solution containing 5 μM methoxamine for 20 min to eliminate CGRPergic function, rinsed with capsaicin-free Krebs’ solution for 30 min, and perfused with Krebs solution containing 5 μM guanethidine for 30 min to block adrenergic nerve function. Thereafter, the Krebs solution containing 5 μM guanethidine was switched to one containing 7 μM methoxamine and 5 μM guanethidine to produce active tone. After the perfusion pressure had stabilized, vascular responses to the first ACh (1 nmol) injection and PNS (1, 2, 4, 8, and 12 Hz) were measured as controls and a switch was made to normal Krebs solution to produce resting tension. The normal Krebs solution was then changed to Krebs solution containing methoxamine (7 μM), guanethidine (5 μM), and atropine (10 nM) or phystostigmine (10 μM), and the second ACh (1 nmol) injection and PNS (1, 2, 4, 8, and 12 Hz) were carried out.

Measurement of NE in the perfusate. The amount of NE in the perfusate was measured as described by Hatanaka et al. (7). In denuded preparations with resting tone, the perfusate was collected before and after the first PNS (S1, 12 Hz) for 3 min. Thereafter, the Krebs solution was switched to one containing atropine (10 nM), hexamethonium (10 μM), or neostigmine (1 μM), and perfusate was collected before and after the second PNS (S2). NE in the perfusate was adsorbed to the alumina, and the extract was obtained with acetic acid was assayed by high-performance liquid chromatography with an electrochemical detector (model HTEC-500; Eicom, Kyoto, Japan).

3,4-Dihydroxybenzylamine hydrobromide (Sigma-Aldrich, St. Louis, MO) was used as an internal standard.

Immunohistochemical analysis. The animals were anesthetized with a large dose of pentobarbital sodium (50 mg/kg ip). The mesenteric artery was removed together with the intestine as described.
previously (8, 9). The superior mesenteric artery was cannulated with polyethylene tubing and infused and fixed with Zamboni solution (2% paraformaldehyde and 15% picric acid in 0.15 M phosphate buffer).

The second or third branch of the mesenteric artery proximal to the intestine was removed and immersion fixed in the Zamboni solution for 48 h. The artery was repeatedly rinsed in phosphate-buffered saline (PBS), immersed in PBS containing 0.5% Triton X-100 overnight, and incubated with PBS containing 0.1% skim milk for 60 min. It was then incubated with guinea pig polyclonal anti-CGRP antiserum (American Research Products, Belmont, MA), anti-tyrosine hydroxylase (TH) mouse IgG (clone LNC1) (Invitrogen, Carlsbad, CA) at a dilution of 1:200, and rabbit polyclonal anti-nerve terminal acetyltransferase (pChAT) antiserum (26) at a dilution of 1:200 for 72 h at 4°C. The artery was washed in PBS, and sites of the antigen-antibody reaction were detected by incubation with AlexaFluor 488-conjugated goat anti-guinea pig IgG (1:200), AlexaFluor 488-conjugated goat anti-mouse IgG (1:1,000), or Alexa Fluor 555-conjugated goat anti-rabbit IgG (1:1,000) (Invitrogen, Carlsbad, CA) for 60 min. Thereafter, the artery was thoroughly washed in PBS, mounted on a slide, cover-slipped with glycerol-PBS (2:1 vol/vol), and observed under a confocal laser scanning microscope (CLSM510, Carl Zeiss, Tokyo, Japan) for 30 min twice with a 30-min interval in 6-OHDA-free Krebs solution, and subjected to immunohistochemistry.

In another series of experiments, in vitro adrenergic denervation was carried out by incubation with 6-hydroxydopamine (6-OHDA) as described previously (28). The isolated mesenteric vascular bed was incubated in Krebs solution containing 6-OHDA (Sigma-Aldrich, Japan) at the Okayama University Medical School Central Research Laboratory.

In another series of experiments, in vitro depletion of CGRPergic nerves was performed according to the method described by Kawasakiet al. (13, 16). The isolated mesenteric vascular bed was perfused with Krebs solution containing capsaicin (20 μM) for 30 min and then rinsed with capsaicin-free Krebs solution. The small arteries were removed, immersion fixed with Zamboni solution, and subjected to immunohistochemistry.

In another series of experiments, in vitro depletions of CGRPergic nerves was performed according to the method described by Kawasakiet al. (13, 16). The isolated mesenteric vascular bed was perfused with Krebs solution containing capsaicin (20 μM) for 30 min and then rinsed with capsaicin-free Krebs solution. The small arteries were removed, immersion fixed with Zamboni solution, and subjected to immunohistochemistry.

**RESULTS**

**Effects of atropine, hexamethonium, and neostigmine on vasoconstrictor responses to PNS and NE injections.** As shown in Fig. 1A, PNS (2, 4, 8, and 12 Hz) of rat mesenteric vascular bed without an endothelium and CGRPergic nerve function and with resting tone induced a frequency-dependent increase in the perfusion pressure due to vasoconstriction: 2 Hz, 3.5 ± 0.9 mmHg; 4 Hz, 8.5 ± 2.1 mmHg; 8 Hz, 44.2 ± 9.0 mmHg and 12 Hz, 105.2 ± 8.5 mmHg (n = 6). The injection of NE (0.5 and 1 nmol) into the perfusate produced concentration-dependent vasoconstriction: 0.5 nmol, 81.7 ± 6.9 mmHg and 1 nmol, 97.8 ± 7.8 mmHg (n = 6). Repeated PNS and NE injections caused reproducible vasoconstrictor responses. In the control response, the ratios of S1 and S2 at 2, 4, 8, and 12 Hz and I1 and I2 at 0.5 and 1 nmol were 1.17 ± 0.18, 1.35 ± 0.11, 1.24 ± 0.11, 1.05 ± 0.04, and 1.06 ± 0.04, respectively (Fig. 1, B and C).

Atropine, hexamethonium, and neostigmine had no effect on the perfusion pressure in preparations without an endothelium under the basal tone condition. As shown in Fig. 1, B and C, neither low nor high concentrations of atropine altered the vasoconstrictor response to the PNS and NE injections. Additionally, vasoconstrictor responses were not affected by hexamethonium and neostigmine. There was no significant difference in vasoconstrictor responses to the PNS and NE injections between the control and atropine, hexamethonium, or neostigmine treatment groups (Fig. 1, B and C).

As shown in Fig. 1A, in preparations with active tone produced by methoxamine perfusion, the injection of 1 nmol ACh and PNS (2 Hz) did not cause vasodilation, confirming the effective removal of the endothelium and CGRPergic nerve function.

**Effects of atropine, hexamethonium, and neostigmine on vasoconstrictor responses to PNS and NE injection in the presence of L-NAME.** To assess the involvement of NO, we investigated the effects of a nonselective NO synthase inhibitor; L-NAME, on PNS- and exogenous NO-mediated vasoconstrictions in mesenteric vascular beds without endothelium. The perfusion of L-NAME (100 μM) after removal of the endothelium did not alter the baseline pressure (Fig. 2A). In preparations with resting tone in the presence of L-NAME, PNS frequency dependently induced vasoconstriction: 2 Hz, 3.5 ± 0.9 mmHg; 4 Hz, 8.5 ± 2.1 mmHg; 8 Hz, 44.2 ± 9.0 mmHg; and 12 Hz, 105.2 ± 8.5 mmHg (n = 6). The injection of NE (0.5 and 1 nmol) produced concentration-dependent vasoconstriction: 0.5 nmol, 81.7 ± 6.9 mmHg; and 1 nmol, 97.8 ± 7.8 mmHg (n = 6). Repeated PNS and NE injections caused reproducible vasoconstrictor responses. In the control response, the ratios of S1 and S2 at 2, 4, 8, and 12 Hz and I1 and I2 at 0.5 and 1 nmol were 1.17 ± 0.18, 1.35 ± 0.11, 1.24 ± 0.11, 1.05 ± 0.04, and 1.06 ± 0.02, 1.04 ± 0.04, respectively (Fig. 2, B and C).

As shown in Fig. 2B, in denuded preparations in the presence of L-NAME, atropine (10 nM), and hexamethionium (10 μM), but not neostigmine (10 μM), reduced slightly the ratio (S2/S1) of PNS- but not NE-induced vasoconstriction (Fig. 2B). However, there was no significant difference in responses to the PNS or NE injection between the control and atropine or hexamethionium-treated groups (Fig. 2, B and C).

**Effect of atropine, hexamethionium, and neostigmine on PNS-evoked norepinephrine release.** In perfused mesenteric vascular beds without an endothelium and with resting tone, the spontaneous release of NE was detected in the perfusate (8.32 ± 2.02 pg/ml, n = 6). Perfusion of atropine (10 nM), hexamethionium (10 μM), or neostigmine (1 μM) did not affect the spontaneous release of NE in the perfusate. PNS at 12 Hz markedly increased the amount of NE in the perfusate (141.38 ± 33.97 pg/ml, n = 6, P < 0.01, compared with pre-PNS). Ratios of net NE release (PNS-evoked release minus spontaneous release) for the control, atropine (10 nM), hexa-
Effects of atropine, hexamethonium, and neostigmine on vasodilation in response to PNS and CGRP injections. As shown in Fig. 3A, to observe vasodilator responses, active tone in the perfused mesenteric vascular bed without an endothelium was produced by perfusion of Krebs solution containing 2 μM methoxamine and 5 μM guanethidine. In this preparation, PNS (1, 2, and 4 Hz) induced a frequency-dependent and a long-lasting decrease in perfusion pressure due to vasodilation. The injection of CGRP (50 pmol) into the perfusate also caused vasodilation in the same manner to PNS. Repeated PNS and CGRP injections

methonium (10 μM), or neostigmine (1 μM) treatment were 1.04 ± 0.160, 1.21 ± 0.13, 1.11 ± 0.12, and 1.23 ± 0.18, respectively. There was no significant difference between the control and treated groups.

Effects of atropine, hexamethonium, and neostigmine on vasodilation in response to PNS and CGRP injections. As shown in Fig. 3A, to observe vasodilator responses, active tone in the perfused mesenteric vascular bed without an endothelium was produced by perfusion of Krebs solution containing 2 μM methoxamine and 5 μM guanethidine. In this preparation, PNS (1, 2, and 4 Hz) induced a frequency-dependent and a long-lasting decrease in perfusion pressure due to vasodilation. The injection of CGRP (50 pmol) into the perfusate also caused vasodilation in the same manner to PNS. Repeated PNS and CGRP injections
caused reproducible vasodilator responses. In the control response, ratios of S2/S1 and S3/S1 were 1.12 ± 0.12 and 1.51 ± 0.22 at 1 Hz; 1.15 ± 0.16 and 1.17 ± 0.16 at 2 Hz, and 1.08 ± 0.02 and 1.07 ± 0.03 at 4 Hz, respectively. Ratios of I2/I1 and I3/I1 were 1.09 ± 0.09 and 1.00 ± 0.08 at 50 pmol, respectively (Fig. 3, B–E). There were no significant differences in responses to the PNS and CGRP injections between control S2/S1 and control S3/S1 groups or control I2/I1 and control I3/I1 groups.

As shown in Fig. 3B, vasodilator responses to PNS at 1, 2, and 4 Hz were not affected by a low concentration of atropine
As shown in Fig. 3D, perfusion of a high concentration of atropine (100 nM), hexamethonium (100 µM), or neostigmine (10 µM) did not significantly alter vasodilator responses to PNS at 1, 2, and 4 Hz. Additionally, atropine, hexamethonium, and neostigmine did not affect vasodilator responses to the CGRP injection at any concentration (Fig. 3, C and E).

Effect of atropine, neostigmine, and physostigmine on vascular responses to PNS and ACh injections in preparations without adrenergic and CGRPergic nerve function. As shown in Fig. 4A, in the preparations with an intact endothelium and without adrenergic and CGRPergic nerve function, the injection of ACh (1 nmol) induced a sharp and transient vasodilation, which was abolished in the presence of atropine (10 nM, P < 0.01) (Fig. 4, A and B). However, in this preparation, PNS did not induce vasodilation (Fig. 4, A and B). Furthermore, PNS did not cause vascular responses in the presence of atropine, neostigmine, and physostigmine (10 µM) (Fig. 4B).

Immunohistochemical analysis of perivascular cholinergic nerves. Immunohistochemical staining of the mesenteric artery showed dense innervation of adrenergic tyrosine hydroxylase (TH)-like immunoreactive (LI) fibers (Fig. 5A) and CGRP-LI fibers (Fig. 5B) surrounding the artery like a network. The density of TH-LI fibers was greater than that of CGRP-LI fibers (Fig. 5, A and B).
As shown in Fig. 5C, fibers immunopositive for pChAT (peripheral type of choline acetyltransferase) were observed in rat mesenteric arteries like a network. The pChAT-LI fibers were detected in the artery treated with 6-OHDA (Fig. 5E) or capsaicin (Fig. 5F) but not observed in the arteries treated with cold-storage denervation (Fig. 5D).

DISCUSSION

Our current and previous (15) immunohistochemical studies showed that rat mesenteric arteries were densely innervated by adrenergic TH (NE synthase enzyme)-immunopositive and CGRP-immunopositive nerve fibers, which reciprocally control the vascular tone and blood flow. The previous report presented evidence of few or no ChAT-immunopositive fibers in rat mesenteric arteries (15). Recently, two additional markers, pChAT and cChAT, that identify cholinergic elements have been characterized within the ENS (2, 3, 20, 26). Cholinergic nerve fibers in various regions of rat gut, which were labeled by pChAT-immunopositive fibers, have been shown to include submucosal perivascular nerve fibers (20). The present study demonstrated that pChAT-immunopositive fibers were distributed in rat mesenteric arteries and were abolished by cold-storage denervation (Fig. 5D) but not 6-OHDA (adrenergic destroyer) and capsaicin (CGRP depletor) (Fig. 5, E and F). These findings provide evidence that pChAT-LI cholinergic nerves, which are different from adrenergic and CGRPergic nerves, innervate the rat mesenteric artery.

In the present study, to investigate the function of cholinergic nerves, effects of cholinergic agents including atropine (muscarinic ACh receptor antagonist), hexamethonium (nicotinic ACh receptor antagonist), and neostigmine (cholinesterase inhibitor) on adrenergic vasoconstrictor responses to the PNS and the injection of NE were examined in preparations without an endothelium and CGRPergic nerve function. In these preparations, PNS and NE induced intrinsic vasoconstriction mediated by adrenergic nerves and postsynaptic $\alpha$-adrenoceptors, since CGRPergic nerves and endothelium, which release endothelium derived-relaxing factors (EDRF), inhibited adrenergic vasoconstriction (10). Additionally, adrenergic nerves have been shown to endow presynaptic muscarinic (24) and nicotinic (4) ACh receptors whose activation modulates adrenergic neurotransmission (11). Therefore, it is expected that PNS stimulates cholinergic nerves innervating the mesenteric arteries to release the transmitter ACh, which activates muscarinic or nicotinic receptors to modulate adrenergic neurotransmission. However, atropine and hexamethonium did not alter adrenergic vasoconstrictor responses to PNS and NE, suggesting that muscarinic and nicotinic ACh

Fig. 4. Typical recording (A) and bar graph (B) showing effects of atropine (10 nM), neostigmine (10 $\mu$M), and physostigmine (10 $\mu$M) on vascular responses to PNS (1, 2, 4, 8, and 12 Hz) and injections of ACh (1 nmol) in perfused mesenteric vascular beds with active tone and pretreated with capsaicin (5 $\mu$M) and guanethidine (5 $\mu$M). Solid circle, injection of ACh. Open triangles, PNS. SD, the perfusion of sodium deoxycholate. In B, bars represent the means ± SE. **$P < 0.01$, compared with the control.
Receptors are not activated by cholinergic nerve stimulation. Additionally, the cholinesterase inhibitor neostigmine, which facilitates cholinergic effects by inhibiting ACh degradation, had no effect on adrenergic vasoconstrictor responses to PNS and the injection of NE. It seems unlikely that perivascular cholinergic nerves act to modulate adrenergic neurotransmission. This notion is supported by the present finding that atropine, hexamethonium, and neostigmine did not affect the neurogenic release of NE by PNS.

We have reported that perivascular nitrergic nerves suppressed adrenergic neurotransmission in rat mesenteric arteries (7, 17). Therefore, it is possible that perivascular

Fig. 5. Confocal laser micrographs showing tyrosine hydroxylase (TH)-like immunoreactive (LI) fibers (A) and CGRP-LI fibers (B) in rat mesenteric arteries. Images in C–F show the peripheral type of cholineacetyltransferase-like immunoreactive fibers (pChAT-LI) in intact, cold-storage denervation, 6-hydroxydopamine (6-OHDA), and capsaicin-treated arteries, respectively. The scale bar in the right lower corner of each image indicates 50 µm.
cholinergic nerves exert nitrergic neurotransmission to modulate adrenergic vasoconstrictor responses. Therefore, to exclude the influence of nitrergic nerves, adrenergic vasoconstrictor responses to PNS and the injection of NE were examined in the presence of L-NAME, a nonselective inhibitor of NO synthase, which has no antagonistic effect on cholinergic receptors. However, atropine, hexamethonium, and neostigmine had no significant effect on vasoconstrictor responses to PNS and NE, suggesting that atropine, hexamethonium, and neostigmine do not affect nitrergic nerve function. These results strongly suggest that perivascular cholinergic nerves have little or no effect on the adrenergic nerve function in rat mesenteric arteries.

Our previous study demonstrated that ACh induces the endothelium-independent vasodilation of denuded mesenteric vascular beds, which is mediated by endogenous CGRP released from CGRPeric nerves via direct stimulation of muscarinic ACh receptors located on CGRPeric nerves (22, 24). A high concentration of ACh induces adrenergic and CGRPeric-dependent vasodilation via nicotinic ACh receptors by the same mechanisms as nicotine-induced vasodilation (22). The present study demonstrated that in perfused mesenteric artery without an endothelium and with active tone produced by methoxamine in the presence of guanethidine (adrenergic neuron blocker), vasodilator responses to PNS or the injection of CGRP were not affected by low and high concentrations of atropine, hexamethonium, and neostigmine. These findings suggest that CGRPeric nerve function is not modulated by cholinergic nerves in rat mesenteric arteries. It is unlikely that the PNS-induced vasodilation includes cholinergic nerve-mediated vasodilation.

In the present study, to investigate direct vascular effects induced by cholinergic nerve stimulation, PNS was applied to preparations with an intact endothelium and active tone and without adrenergic and CGRPeric nerve function. In these preparations, ACh induced endothelium-dependent vasodilation, while PNS did not cause vascular responses. Additionally, atropine abolished the ACh-induced vasodilation, while no vascular response to PNS was observed in the presence of atropine. Furthermore, PNS did not cause vascular responses in the presence of the cholinesterase inhibitors neostigmine and physostigmine. These findings suggest that cholinergic nerves innervating mesenteric arteries do not mediate direct vascular responses.

**Perspectives and Significance**

In conclusion, the present results suggest that the rat mesenteric artery has cholinergic innervation, which is different from adrenergic and capsaicin-sensitive nerves and plays no role in the neurogenic regulation of vascular tone. It is speculated that cholinergic nerves innervating rat mesenteric arteries may participate regulation of function of perivascular nerves, which are different from adrenergic and CGRPeric nerves.

**AUTHOR CONTRIBUTIONS**

Author contributions: P.T. performed experiments; P.T., S.T., and Y.Z. analyzed data; P.T., prepared figures; P.T. and P.S. drafted manuscript; P.T. and F.T. edited and revised manuscript; Y.Z., M.G., P.F., T.T., and H.K. interpreted results of experiments; P.P. and Y.K. conception and design of research; F.T. and H.K. approved final version of manuscript.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

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