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Adrenergic nerves mediate acetylcholine-induced endothelium-independent vasodilation in the rat mesenteric resistance artery

Hinako Shiraki [a], Hiromu Kawasaki [a,*], Satoko Tezuka [a], Akira Nakatsuma [a], Hideki Nawa [a], Hiroaki Araki [b], Yutaka Gomita [b], Yuji Kurosaki [a]

[a] *Department of Clinical Pharmaceutical Science, Graduate School of Natural Science and Technology, Okayama University, 1-1-1 Tsushima-naka, Okayama 700-8530, Japan*

[b] *Department of Hospital Pharmacy, Okayama University Medical School, 2-5-1 Shikata-cho, Okayama 700-8558, Japan*

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Abstract

Mechanisms underlying acetylcholine-induced endothelium-independent vasodilation were studied in the rat mesenteric vascular bed isolated from Wistar rats. In preparations without endothelium, and contracted by perfusion with Krebs solution containing methoxamine (2–7 μM), perfusion of acetylcholine (1–100 μM) for 1 min produced a concentration-dependent vasodilation. Denervation of denuded preparations by cold storage (4°C for 72 h) abolished the acetylcholine-induced vasodilation; 10 and 100 nM atropine abolished 1 and 10 μM acetylcholine-induced vasodilation, but it inhibited only 20% of vasodilation by 100 μM acetylcholine. The acetylcholine-induced atropine-resistant vasodilation was inhibited by 10 and 100 μM hexamethonium, 5 μM guanethidine, 50 μM bretylium, in vitro 6-hydroxydopamine (2 mM for 20 min, twice), 1 μM capsaicin and 0.5 μM calcitonin gene-related peptide (CGRP)-(8-37) (CGRP receptor antagonist). These findings suggest that the acetylcholine-induced endothelium-independent nicotinic vasodilation requires the presence of intact adrenergic nerves, and is mediated by endogenous CGRP released from CGRP-containing nerves.

Keywords: Vasodilation, acetylcholine-induced; Vasodilation, endothelium-independent; Nicotinic acetylcholine receptor; Adrenergic nerves; CGRP-containing nerve

1. Introduction

It is widely accepted that acetylcholine, a muscarinic and nicotinic acetylcholine receptor agonist, induces vasorelaxation, which is absolutely dependent on an intact endothelium and is mediated by endothelium-derived relaxing factors (EDRF) (Furchgott and Zawadzki, 1980). Accumulating evidence shows that EDRF is nitric oxide (NO) or an NO-containing compound (Moncada et al., 1991). However, some studies have shown that acetylcholine has endothelium-independent vasorelaxation, because the relaxation is not fully antagonized by the NO synthase inhibitor, N^{w}-nitro-L arginine (L-NA) (Ralevic et al., 1992), the potent inactivator of EDRF, methylene blue (Khan et al., 1992), or mechanical and chemical removal of vascular endothelium (Brayden and Bevan, 1985; Fukushima and Ohhashi, 1993; Takenaga et al., 1995).

Although the tone of peripheral blood vessels is mainly regulated by vascular adrenergic nerves, recent studies have demonstrated that many blood vessels have innervation of non-adrenergic non-cholinergic (NANC) nerves (Bevan and Brayden, 1987; Kawasaki et al., 1988). Previously, we reported that the rat mesenteric resistance blood vessels are innervated by NANC vasodilator nerves, which are sensitive to tetrodotoxin (a neurotoxin, TTX) and capsaicin (a depletor for primary sensory neurons). Calcitonin gene-related peptide (CGRP), a potent vasodilator neuropeptide (Brain et al., 1985; Kawasaki et al., 1988), acts as a neurotransmitter in NANC vasodilator nerves (Kawasaki et al., 1988) and is widely distributed in

* Corresponding author. Tel.: +81-86-251-7970; fax: +81-86-251-7970.

E-mail address: kawasaki@pheasant.pharm.okayama-u.ac.jp (H. Kawasaki).

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perivascular nerves throughout the vascular system (Mulderry et al., 1985; Kawasaki et al., 1988; Del Bianco et al., 1991). Thus, we proposed that the tone of the peripheral resistance vascular bed is controlled not only by sympathetic adrenergic nerves, but also by CGRP-containing vasodilator nerves (CGRPergic nerves) (Kawasaki et al., 1988, 1990a; Takenaga and Kawasaki, 1999).

We reported that acetylcholine induces endothelium-independent vasodilation in the rat mesenteric artery (Takenaga et al., 1995). In that study, we revealed that acetylcholine activates muscarinic acetylcholine receptors located on CGRPergic nerves to release transmitter CGRP, which then acts at CGRP receptors on vascular smooth muscles to cause vasodilation (Takenaga et al., 1995). Furthermore, our recent study demonstrated that in the rat mesenteric artery, nicotine, a nicotinic acetylcholine receptor agonist, induces the endothelium-independent vasodilation, which is mediated by endogenous CGRP (Shiraki et al., 2000). In that study, we showed that the nicotine-induced vasodilation requires intact adrenergic nerve function (Shiraki et al., 2000). However, it is unclear whether nicotinic receptors are involved in the acetylcholine-induced endothelium-independent vasodilation.

Therefore, the present study was undertaken to further investigate the mechanisms underlying acetylcholine-induced endothelium-independent vasodilation in the mesenteric artery of the rat.

2. Materials and methods

2.1. Perfusion of the mesenteric vascular bed

Male Wistar rats, weighing 250–350 g, were used in this study. Animals were given food and water ad libitum. They were housed in the Animal Research Center of Okayama University at a controlled ambient temperature of 22°C with 50 ± 10% relative humidity and with a 12-h light/12-h dark cycle (light on at 8:00 a.m.).

The animals were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally) and the mesenteric vascular bed was isolated and prepared for perfusion as described previously (Kawasaki et al., 1988, 1990a). The superior mesenteric artery was cannulated and flushed gently with Krebs-Ringer bicarbonate solution (Krebs solution) to eliminate blood from the vascular bed. After removal of the entire intestine and associated vascular bed, the mesenteric vascular bed was separated from the intestine by cutting close to the intestinal wall. Only four main arterial branches from the superior mesenteric trunk running to the terminal ileum were perfused. All other branches of the superior mesenteric artery were tied off. The isolated mesenteric vascular bed was then placed in a water jacketed organ bath, maintained at 37°C and perfused with a modified (see below) Krebs solution, at a constant flow rate of 5 ml/min with a peristaltic pump (model AC-2120, ATTO, Tokyo, Japan). The preparation was also 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% O_2–5% CO_2 before passage through a warming coil maintained at 37°C. The modified Krebs solution has the following composition (in mM): NaCl 119.0, KCl 4.7, $CaCl_2$ 2.4, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2, disodium EDTA 0.03 and dextrose 11.1 (pH 7.4). Changes in the perfusion pressure were measured with a pressure transducer (model TP-400T, Nihon Kohden, Tokyo, Japan) and recorded on a pen recorder (model U-228, Nippon Denshi Kagaku, Tokyo, Japan).

2.2. Perfusion of acetylcholine and periarterial nerve stimulation

After the basal perfusion pressure had been allowed to stabilize, the preparation was perfused with Krebs solution containing methoxamine (α_1-adrenoceptor agonist) at concentrations of 2–7 μM to induce submaximal vasoconstriction. After stabilization of the elevated perfusion pressure, the preparation was subjected to perfusion of acetylcholine and periarterial nerve stimulation. The final concentration of acetylcholine at concentrations of 0.01–100 μM, which was made by dilution of Krebs solution containing methoxamine, was perfused for 1 min at 10–30 min-intervals.

Periarterial nerve stimulation was applied for 30 s using bipolar platinum ring electrodes placed around the superior mesenteric artery. Rectangular pulses of 1 ms and supramaximal voltage (50 V) were applied at 2, 8 and 12 Hz using an electronic stimulator (model SEN 3301, Nihon Kohden).

2.3. Chemical removal of vascular endothelium

To remove the vascular endothelium, the preparation with resting tone was perfused with a 1.80 mg/ml solution of sodium deoxycholate in saline for 30 s as described previously (Takenaga et al., 1995). This caused a transient increase (20–30 mm Hg) in perfusion pressure. Then, the preparation was rinsed with sodium deoxycholate-free Krebs solution for 60 min. After the preparation was contracted by perfusion with Krebs solution containing methoxamine (2 μM), chemical removal of the endothelium was assessed by the lack of a relaxant effect after a bolus injection of 1 nmol acetylcholine, which was injected directly into the perfusate proximal to the arterial cannula with an infusion pump (model 975, Harvard Apparatus, S. Natick, MA). Volumes were 100 μl for 10 s.

2.4. In vitro treatment with capsaicin

In vitro depletion of CGRPergic nerves was performed according to the method described by Kawasaki et al. (1988, 1990a). First, the isolated mesenteric vascular bed was perfused with Krebs solution containing capsaicin (1 μM) for 20 min and then rinsed with capsaicin-free Krebs solution for 60 min. Thereafter, sodium deoxycholate solution was perfused for 30 s to remove the vascular endothelium. After rinsing for 60 min with sodium deoxycholate-free Krebs solution, the preparation was contracted by perfusion with Krebs solution containing methoxamine (2 μM) and subjected to perfusion of acetylcholine. After the elevated perfusion pressure had stabilized, a bolus injection of acetylcholine (1 nmol) and periarterial nerve stimulation at 2 Hz was performed to check for the presence of the endothelium and CGRPergic nerves. Successful depletion of CGRPergic nerves was confirmed by the lack of a relaxant effect by periarterial nerve stimulation (2 Hz). In some preparations treated with capsaicin, the vascular effect of acetylcholine was examined in the intact endothelium.

2.5. Cold-storage denervation

Isolated mesenteric vascular bed was stored in cold Krebs solution at 4°C for 72 h to achieve cold-storage denervation, as described previously (Kawasaki et al., 1991; Shiraki et al., 2000). After being perfused with Krebs solution at 37°C for 60 min, chemical denudation of the preparation was performed with perfusion of sodium deoxycholate solution for 30 s. Sixty minutes later, the preparation was contracted by perfusion of Krebs solution containing methoxamine (7 μM), and to determine the intact responsiveness of the smooth muscle, a bolus injection of CGRP (100 pmol) was carried out to cause vasodilation. Successful denervation of periarterial nerves was confirmed by the lack of the periarterial nerve stimulation-induced vasoconstriction (8 and 12 Hz) at a resting tone, and vasodilation (2 Hz) at an active tone.

2.6. In vitro chemical adrenergic denervation with 6-hydroxydopamine

In vitro adrenergic denervation was carried out by incubation with 6-hydroxydopamine as described by Shiraki et al. (2000). The isolated mesenteric vascular bed was incubated in Krebs solution containing 6-hydroxydopamine (2 mM) for 20 min twice, with a 30-min interval, in 6-hydroxydopamine-free Krebs solution. After being perfused with 6-hydroxydopamine-free Krebs solution for 60 min, sodium deoxycholate solution was perfused for 30 s to remove the endothelium. Successful denervation of adrenergic nerves was confirmed by the lack of the periarterial nerve stimulation-induced vasoconstriction (8 and 12 Hz) and noradrenaline release in the perfusate (12 Hz) at the resting tension.

2.7. Experimental protocols

The endothelium-dependent vasodilator response to perfusion of acetylcholine (0.01–100 μM) was examined in capsaicin-treated or non-treated preparations with intact endothelium and precontracted with methoxamine (7 μM). In another series of experiments, the vascular response to acetylcholine perfusion was studied in chemically denuded preparations, which were treated by cold storage, 6-hydroxydopamine or capsaicin. In the endothelium removal experiments, the active tone of preparation was produced by perfusion with Krebs solution containing methoxamine (2 μM). After the elevated perfusion pressure was allowed to stabilize, periarterial nerve stimulation (2 Hz) or a bolus injection of acetylcholine (1 nmol) was performed, to determine the presence of adrenergic nerves and CGRPergic nerves and successful removal of vascular endothelium. Subsequently, perfusion of the final concentration of acetylcholine at 1, 10 and 100 μM, which was diluted with Krebs solution containing methoxamine, was carried out for 1 min.

To assess the underlying mechanisms of the endothelium-independent vasodilator response to acetylcholine, the effects of various agents were examined in the preparation without endothelium and with an active tone. After the perfusion pressure elevated by methoxamine (2 μM) had stabilized, Krebs solution containing the final concentration of acetylcholine and 10 or 100 nM atropine (muscarinic acetylcholine receptor antagonist) was perfused. In another series of experiments, the preparation without endothelium was perfused with Krebs solution containing methoxamine (2 μM), atropine (10 nM) and the final concentration of acetylcholine and hexamethonium (nicotinic acetylcholine receptor antagonist, 100 μM), guanethidine (adrenergic neuron blocker, 5 μM), bretylium (adrenergic neuron blocker, 50 μM) or CGRP-(8-37) (CGRP receptor antagonist, 0.5 μM).

In another series of experiments, the vascular responses of acetylcholine in the preparations without endothelium and treated with capsaicin or 6-hydroxydopamine were carried out in the presence of atropine (10 nM).

At the end of each experiment, the preparation was perfused with 100 μM papaverine to cause complete relaxation. Vasodilator activity was expressed as a percentage of the perfusion pressure at maximum relaxation induced by papaverine.

2.8. Catecholamine assay

The perfusate was collected for 3 min before and during periarterial nerve stimulation (12 Hz) in perfused mesen-

teric vascular beds which were treated by guanethidine, bretylium or in vitro 6-hydroxydopamine incubation. Activated alumina (30 mg) and 100 μl internal standard (3,4-dihydroxybenzylamine, 100 ng/ml) were added to each sample to absorb catecholamines and the internal standard. The alumina collected was washed with pure water, and both catecholamines and the internal standard were eluted with 0.2 M perchloric acid. Noradrenaline and the internal standard in the elute was assayed by high performance liquid chromatography with an electrochemical detector (model ECD-300, Eicom, Kyoto, Japan).

2.9. Statistical analysis

Data are presented as the mean ± S.E.M. Statistical analysis was evaluated using Student's unpaired or paired *t*-test and one-way analysis of variance followed by Tukey's test. A value of $P < 0.05$ was considered statistically significant.

2.10. Drugs

The following drugs were used: acetylcholine chloride (Daiichi Pharmaceutical, Tokyo, Japan), atropine sulfate (Ishizu Seiyaku, Tokyo, Japan), capsaicin (Sigma, St. Louis, MO), bretylium tosylate (Sigma), guanethidine sulfate (Sigma), hexamethonium bromide (Sigma), 6-hydroxydopamine hydrobromide (Sigma), human CGRP-(8-37) (Peptide Institute, Osaka, Japan), methoxamine hydrochloride (Nihon Shinyaku, Kyoto, Japan), papaverine hydrochloride (Dainippon Pharmaceutical, Tokyo, Japan), rat CGRP (Peptide Institute), sodium deoxycholate (Ishizu Seiyaku). All drugs, except capsaicin and sodium deoxycholate, were dissolved in distilled water and diluted with Krebs solution containing 2–7 μM methoxamine, when perfused and injected directly. Capsaicin was dissolved in 50% ethanol and diluted with Krebs solution (final alcohol concentration, 0.4 mg/ml). Sodium deoxycholate was dissolved in 0.9% saline.

3. Results

3.1. Vascular responses to perfusion of acetylcholine

In the perfused mesenteric vascular bed with intact endothelium and contracted by 7 μM methoxamine, periarterial nerve stimulation at 2 Hz caused an initial transient increase in perfusion pressure due to vasoconstriction, followed by a long-lasting decrease in perfusion pressure due to vasodilation, as shown in Fig. 1A. The initial vasoconstriction and subsequent long-lasting vasodilation have been shown to result from stimulation of vascular adrenergic nerves and CGRPergic nerves, respectively (Shiraki et al., 2000). In this preparation, perfusion of acetylcholine at lower concentrations of 0.01–0.1 μM for 1 min produced a rapid and short-lived vasodilation. Higher

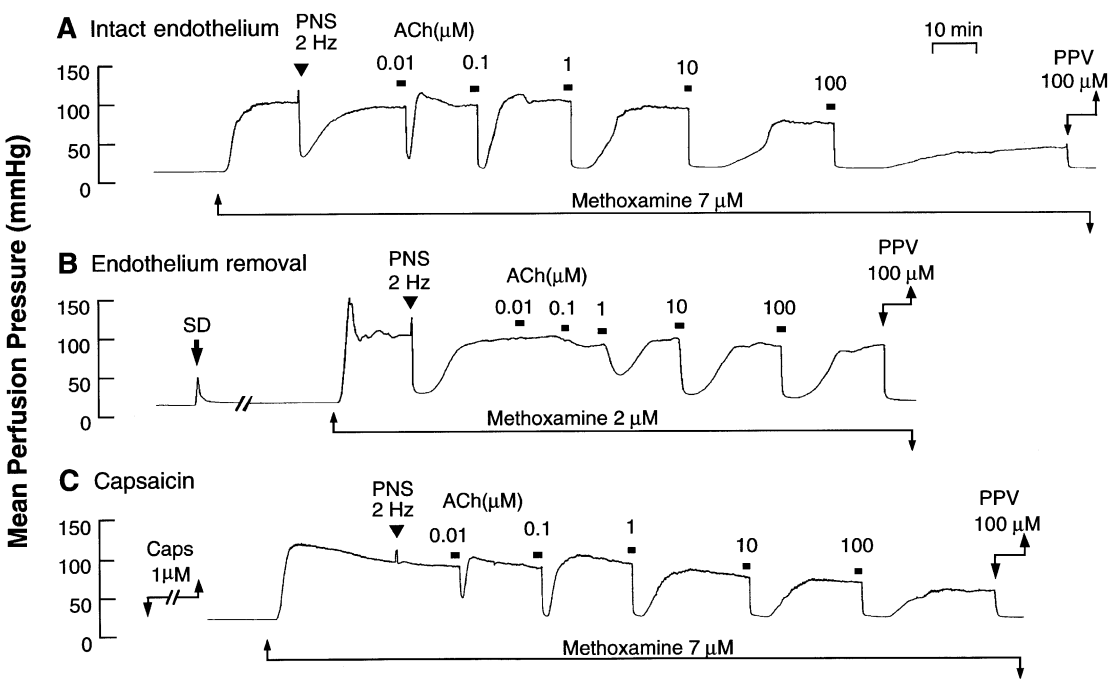

Fig. 1. Typical records showing vascular responses to acetylcholine (ACh) perfusion (A) and effects of endothelium removal (B) and capsaicin treatment (C, 1 μM) in rat perfused mesenteric vascular beds, with an active tone produced by methoxamine (2–7 μM). Acetylcholine (0.01–100 μM) was perfused for 1 min at the horizontal bars. PNS, periarterial nerve stimulation (2 Hz). SD, perfusion of sodium deoxycholate for 30 s. PPV, perfusion of papaverine (100 μM).

concentrations of acetylcholine at 1–100 μM produced a rapid vasodilation and subsequent long-lasting vasodilation, in which the decreased perfusion pressure returned to the preperfusion level over a 15–30-min course. The duration of vasodilator response to acetylcholine was prolonged in a concentration-dependent manner (Fig. 1A).

In the preparation denuded chemically with sodium deoxycholate, the initial rapid vasodilation induced by acetylcholine at lower concentrations of 0.01–0.1 μM disappeared as shown in Fig. 1B. However, higher concentrations of acetylcholine (1–100 μM) caused a concentration-dependent vasodilation, which was slow in onset and long-lasting. However, the initial rapid vasodilation disappeared after removal of the endothelium.

In the capsaicin-treated preparation with intact endothelium, periarterial nerve stimulation (2 Hz) caused only a transient vasoconstriction. In this preparation, the initial rapid vasodilation induced by acetylcholine was not affected and similar to the response in non-capsaicin treated preparations. However, the duration of acetylcholine-induced response was markedly shorten after capsaicin treatment (Fig. 1C).

3.2. Effect of cold-storage denervation on the vasodilator response to acetylcholine

Cold-storage denervation of the preparation without endothelium abolished both the periarterial nerve stimulation (8 and 12 Hz)-induced vasoconstriction at the resting tone and vasoconstriction followed by vasodilation (2 Hz) at the active tone (Fig. 2B and Tables 1 and 2). However, a bolus injection of CGRP induced long-lasting vasodilation

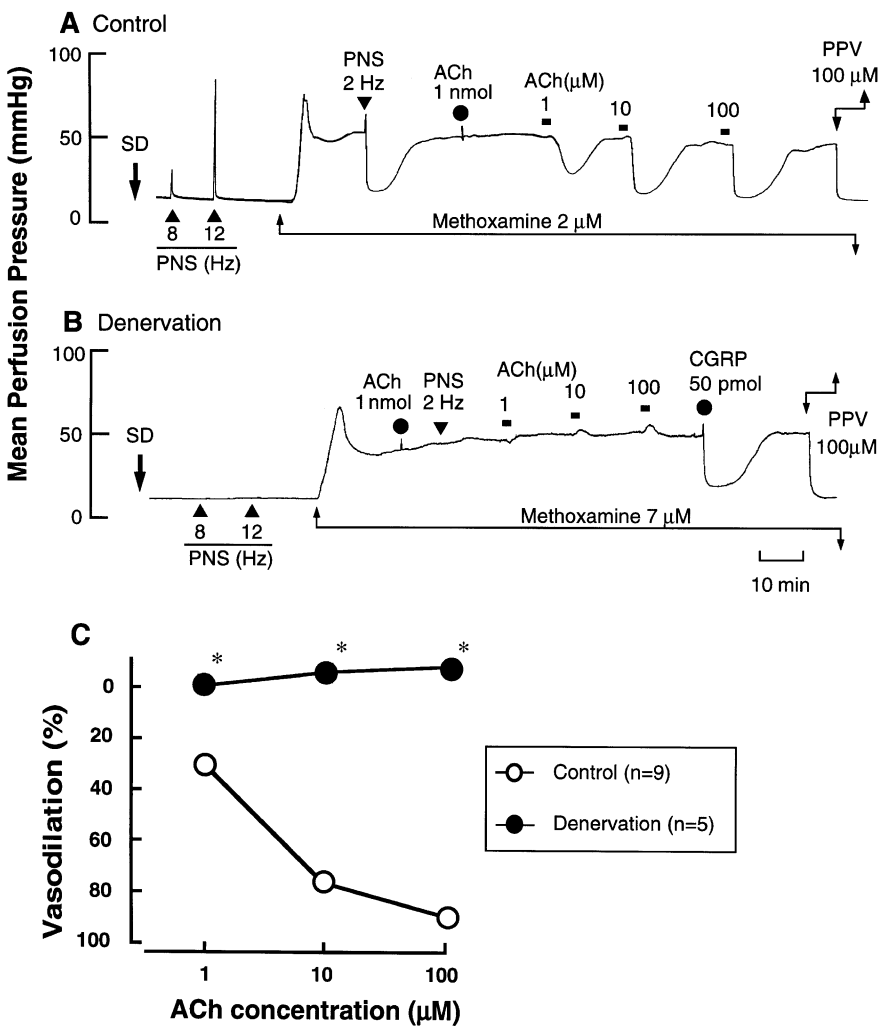

Fig. 2. Typical records and line-graph showing the effect of cold-storage denervation on vascular responses to acetylcholine (ACh) perfusion in rat perfused mesenteric vascular beds without endothelium and with active tone produced by methoxamine (2–7 μM). (A) Control vasoconstrictor responses in the non-treated preparation. (B) Responses after cold-storage denervation (4°C for 72 h). Acetylcholine (1–100 μM) was perfused for 1 min at the horizontal bars. Circles and triangles show bolus injections of acetylcholine (1 nmol) and calcitonin gene-related peptide (CGRP, 50 pmol) and periarterial nerve stimulation (PNS; 2, 8, 12 Hz), respectively. SD, perfusion of sodium deoxycholate for 30 s. PPV, perfusion of papaverine (100 μM). (C) Line-graph showing effect of cold-denervation on the acetylcholine-induced vasodilation. Each point indicates the mean ± S.E.M. $^{*}P < 0.01$, compared with control.

Table 1
Effects of various drugs and treatments on vasoconstriction and vasodilation, induced by periarterial nerve stimulation (2 Hz) in rat perfused mesenteric vascular beds, without endothelium and with active tone produced by methoxamine
Values show the mean ± S.E.M. of four to seven experiments.

Treatments	Vasoconstriction[a] (%)	Vasodilation (%)
Control	15.7 ± 2.9	71.5 ± 3.6
Cold-storage (4°C, 72 h)	0	0
Atropine (10 nM)	19.5 ± 2.0	76.4 ± 4.2
Atropine (10 nM) + hexamethonium (10 μM)	10.0 ± 3.3	70.9 ± 9.7
Atropine (10 nM) + guanethidine (5 μM)	0	82.2 ± 2.5
Atropine (10 nM) + bretylium (50 μM)	0	85.9 ± 2.3
Atropine (10 nM) + 6-hydroxydopamine (2 mM, ×2)	0	65.7 ± 5.1
Atropine (10 nM) + capsaicin (1 μM)	35.7 ± 9.4[b]	4.2 ± 3.1[c]
Atropine (10 nM) + CGRP-(8-37) (0.5 μM)	33.2 ± 3.5	3.2 ± 3.5[c]

[a] The perfusion pressure before periarterial nerve stimulation was taken as 100%.
[b] $P < 0.05$, compared with controls.
[c] $P < 0.01$, compared with controls.

(Fig. 2B and Table 1). In this preparation with active tone, perfusion of acetylcholine did not cause vasodilation, but induced a slight vasoconstriction (Figs. 2B,C).

3.3. Effects of atropine and hexamethonium on the vasodilator response to acetylcholine

In preparations without endothelium, as shown in Figs. 3A and 4A, vasodilator responses to low concentrations of acetylcholine (1 and 10 μM) were abolished in the presence of atropine (10 and 100 nM). However, atropine inhibited only 20% of the response to high concentration of acetylcholine (100 μM). This vasodilation, which remained in the presence of atropine, was termed as the acetylcholine-induced atropine-resistant vasodilation.

Hexamethonium (100 μM) significantly inhibited the acetylcholine (100 μM)-induced endothelium-independent vasodilation (Fig. 4B). Furthermore, the acetylcholine-induced atropine-resistant vasodilation was abolished by addition of hexamethonium (100 μM) as shown in Figs. 3A and 4B.

3.4. Effects of adrenergic neuron blockers on acetylcholine-induced atropine resistant vasodilation

In preparations without endothelium, the adrenergic neuron blocker, guanethidine (5 μM) and bretylium (50 μM) abolished vasoconstrictor responses to periarterial nerve stimulation (8 and 12 Hz) at the resting tone (Fig. 5A,B and Table 2) and to periarterial nerve stimulation (2 Hz) at the active tone (Fig. 5 and Tables 1 and 2). Also, noradrenaline overflow in the perfusate evoked by 12-Hz periarterial nerve stimulation was markedly reduced by guanethidine and bretylium (Table 2), indicating that adrenergic neurotransmission was successfully blocked by guanethidine and bretylium. However, neither agents affected the vasodilator response induced by periarterial nerve stimulation (2 Hz) at the active tone (Table 1 and Fig. 5).

As shown in Figs. 5 and 6, the acetylcholine-induced atropine-resistant vasodilation was markedly inhibited by guanethidine or bretylium treatment. Both guanethidine and bretylium significantly decreased the acetylcholine-induced endothelium-independent vasodilation (Fig. 6).

3.5. Effect of adrenergic denervation on acetylcholine-induced atropine-resistant vasodilation

In perfused mesenteric vascular beds treated with in vitro 6-hydroxydopamine incubation, periarterial nerve stimulation (8 and 12 Hz) at the resting tone did not cause vasoconstrictor responses. However, periarterial nerve stimulation (2 Hz) at the active tone induced a long-lasting vasodilation without the initial vasoconstriction (Tables 1 and 2). In addition, release of noradrenaline in the perfusate by periarterial nerve stimulation (12 Hz) was markedly decreased after in vitro 6-hydroxydopamine treatment (Table 2), indicating that adrenergic nerves were effectively denervated. In this preparation, the acetyl-

Table 2
Effects of adrenergic neuron blockers, in vitro 6-hydroxydopamine incubation and cold-storage denervation on periarterial nerve stimulation (8 and 12 Hz)-induced vasoconstriction and periarterial nerve stimulation (12 Hz)-evoked noradrenaline overflow in the perfusate, in rat perfused mesenteric vascular beds without endothelium
Values show the mean ± S.E.M. of three to six experiments. N.D., not determined.

Treatments	Vasoconstriction[a] (mm Hg)		Noradrenaline overflow (pg/ml)
	8 Hz	12 Hz	12 Hz
Control	9.6 ± 1.7	38.4 ± 4.9	164.7 ± 25.4
Guanethidine (5 μM)	1.9 ± 0.6[b]	3.0 ± 0.7[b]	23.8 ± 16.2[b]
Bretylium (50 μM)	1.8 ± 0.7[b]	4.5 ± 2.4[b]	53.3 ± 10.8[b]
6-Hydroxydopamine (2 mM, ×2)	0.4 ± 0.3[b]	0.3 ± 0.3[b]	61.8 ± 8.4[b]
Cold-storage (4°C, 72 h)	0.2 ± 0.2[b]	0.5 ± 0.3[b]	N.D.

[a] The vasoconstriction was expressed as the increase in perfusion pressure.
[b] $P < 0.01$, compared with controls.

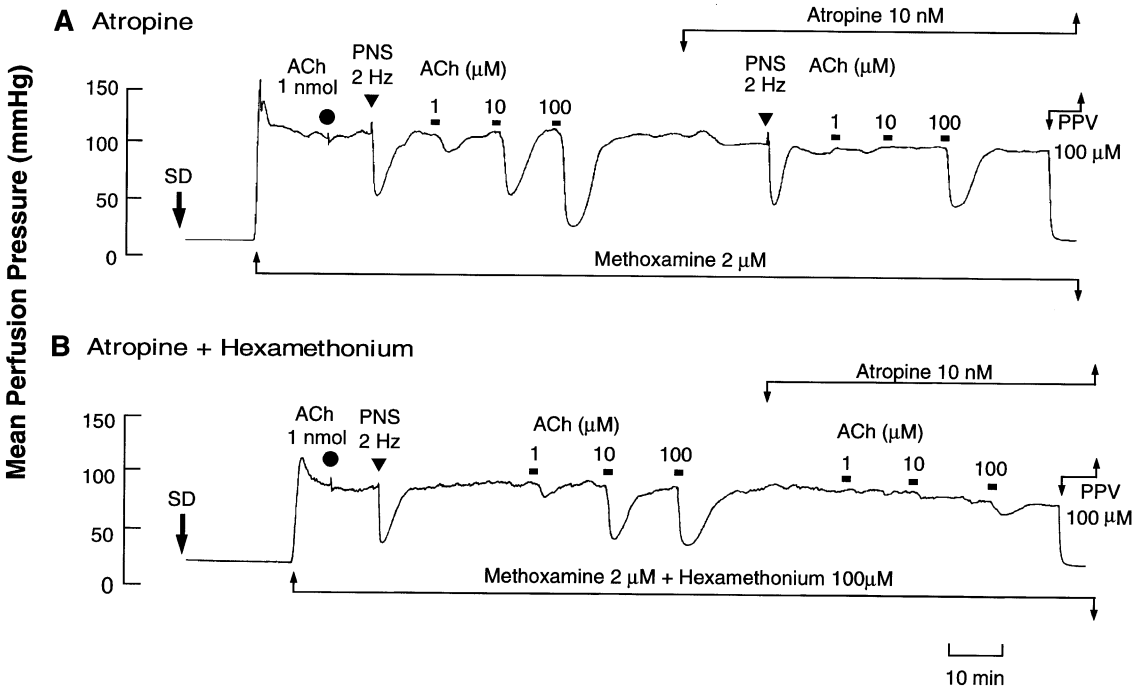

Fig. 3. Typical records showing effects of atropine (10 μM) (A) and atropine (10 μM) plus hexamethonium (10 μM) (B) on the acetylcholine (ACh)-induced vasodilation in rat perfused mesenteric vascular beds without endothelium, and with an active tone produced by methoxamine (2 μM). Acetylcholine was perfused for 1 min at the horizontal bars. SD, perfusion of sodium deoxycholate for 30 s. Circles and triangles indicate bolus injections of acetylcholine and periarterial nerve stimulation (PNS, 2 Hz), respectively. PPV, perfusion of papaverine (100 μM).

choline-induced atropine-resistant vasodilation was significantly inhibited, as shown in Fig. 6. Furthermore, 6-hydroxydopamine treatment significantly decreased the acetylcholine-induced endothelium-independent vasodilation (Fig. 6).

3.6. Effects of capsaicin and CGRP-(8-37) on acetylcholine-induced atropine resistant vasodilation

Capsaicin treatment in the endothelium-removed preparation abolished the vasodilator response to periarterial

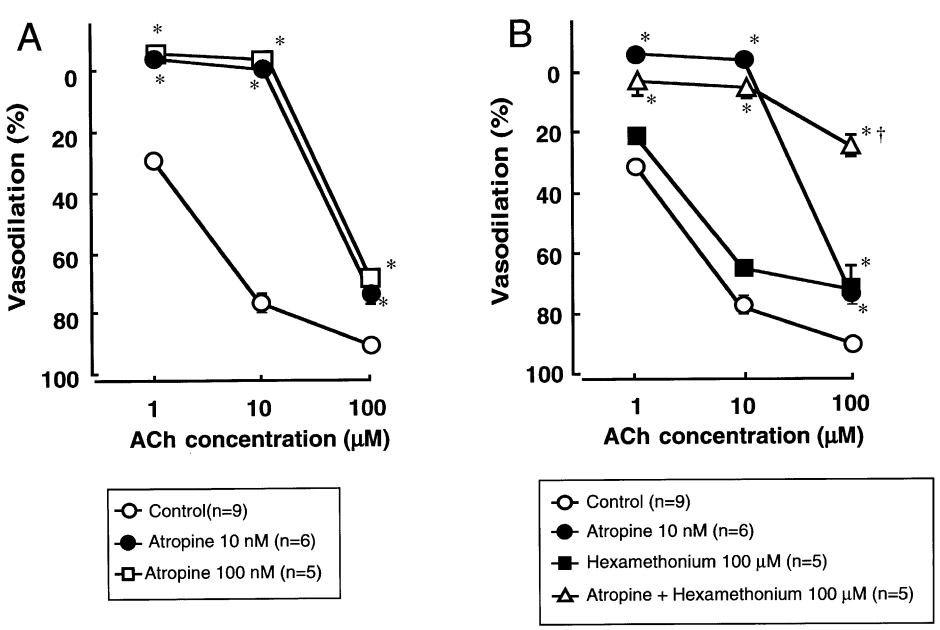

Fig. 4. Effects of atropine (10 and 100 μM) (A) and atropine (10 μM) plus hexamethonium (10 μM) (B) on the acetylcholine (ACh)-induced vasodilation in rat perfused mesenteric vascular beds without endothelium and with an active tone. Each point represents the mean ± S.E.M. * $P < 0.01$, compared with control. † $P < 0.01$, compared with atropine.

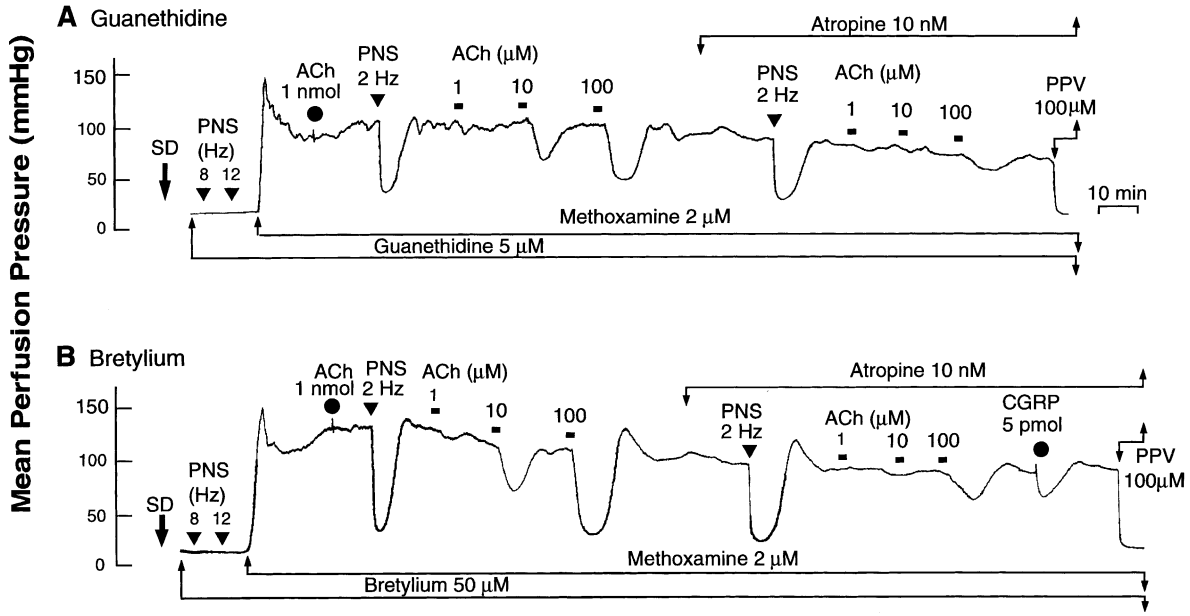

Fig. 5. Typical records showing effects of adrenergic neuron blockers, guanethidine (A) and bretylium (B), on vasodilator responses to acetylcholine (ACh) perfusion in rat perfused mesenteric vascular beds, without endothelium and with active tone produced by methoxamine (2 μM). Acetylcholine (1–100 μM) was perfused for 1 min at the horizontal bars. Circles and triangles show bolus injection of acetylcholine (1 nmol) and calcitonin gene-related peptide (CGRP, 50 pmol) and periarterial nerve stimulation (PNS; 2, 8, 12 Hz), respectively. SD, perfusion of sodium deoxycholate for 30 s. PPV, perfusion of papaverine (100 μM).

nerve stimulation (2 Hz) without affecting the initial vasoconstrictor response to periarterial nerve stimulation (2 Hz) (Fig. 7A and Table 1). In addition, the acetylcholine-induced atropine-resistant vasodilation was markedly attenu-

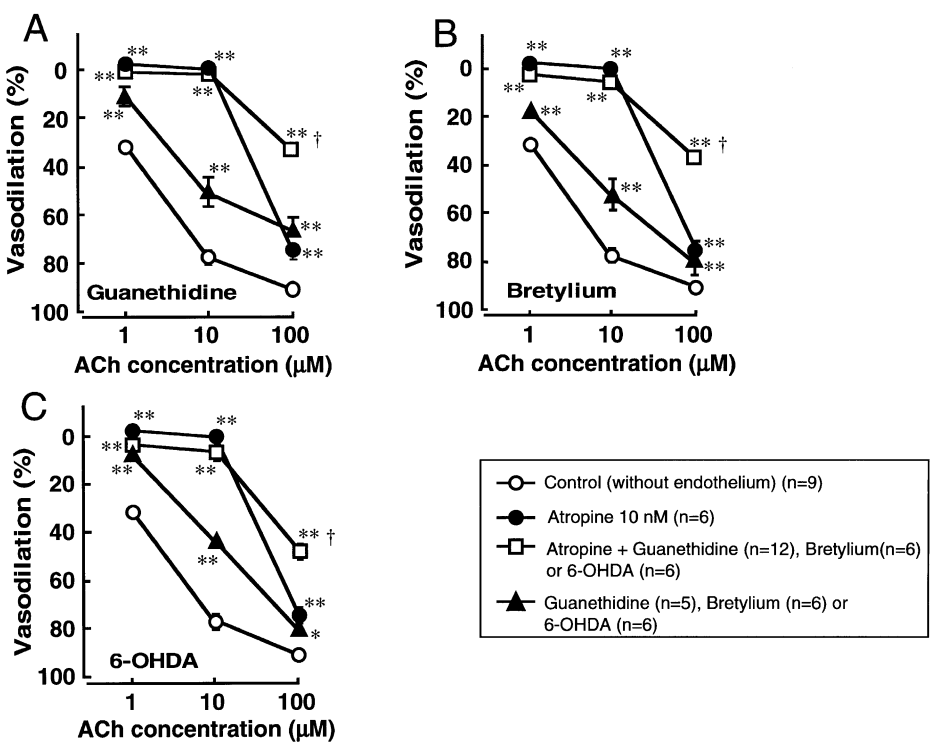

Fig. 6. Effects of adrenergic neuron blockers (A, guanethidine and B, bretylium) and in vitro 6-hydroxydopamine (6-OHDA) incubation (C) on vasodilator responses to acetylcholine (ACh) perfusion in rat perfused mesenteric vascular beds, without endothelium and with an active tone produced by methoxamine (2 μM). Each point represents the mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control. †$P < 0.01$, compared with atropine.

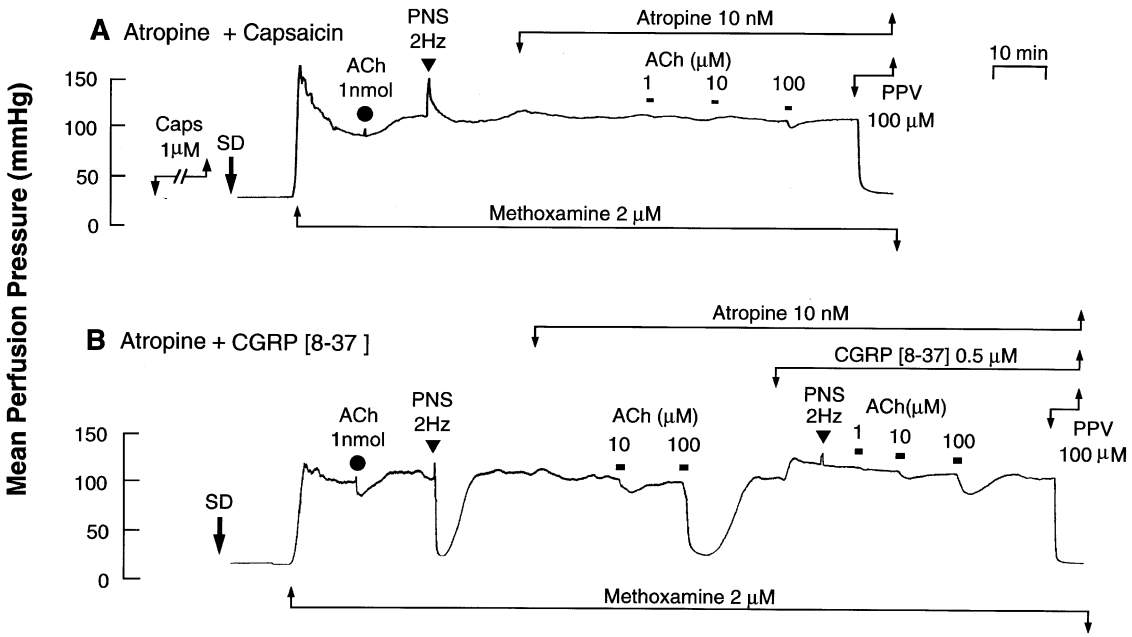

Fig. 7. Typical records showing effects of capsaicin (1 μM) (A) and CGRP-(8-37) (0.5 μM) (B) treatment on the acetylcholine (ACh)-induced vasodilation, in rat perfused mesenteric vascular beds without endothelium and with active tone produced by methoxamine (2 μM). Acetylcholine (1–100 μM) was perfused for 1 min at the horizontal bars. Circles and triangles show bolus injection of acetylcholine (1 nmol) and periarterial nerve stimulation (PNS; 2 Hz), respectively. SD, sodium deoxycholate perfusion for 30 s. PPV, perfusion of papaverine (100 μM).

ated after treatment with capsaicin, as shown in Figs. 7A and 8A.

In preparations without endothelium, perfusion of Krebs solution containing 0.5 μM CGRP-(8-37) resulted in a transient increase in perfusion pressure and markedly attenuated vasodilator response to periarterial nerve stimulation (2 Hz), without affecting the initial vasoconstrictor response to periarterial nerve stimulation (Fig. 7B and Table 1). In this preparation, the acetylcholine-induced vasodilation in the presence of atropine was significantly inhibited by addition of CGRP-(8-37) (Figs. 7B and 8B). After rinsing with CGRP-(8-37)-free Krebs solution, the

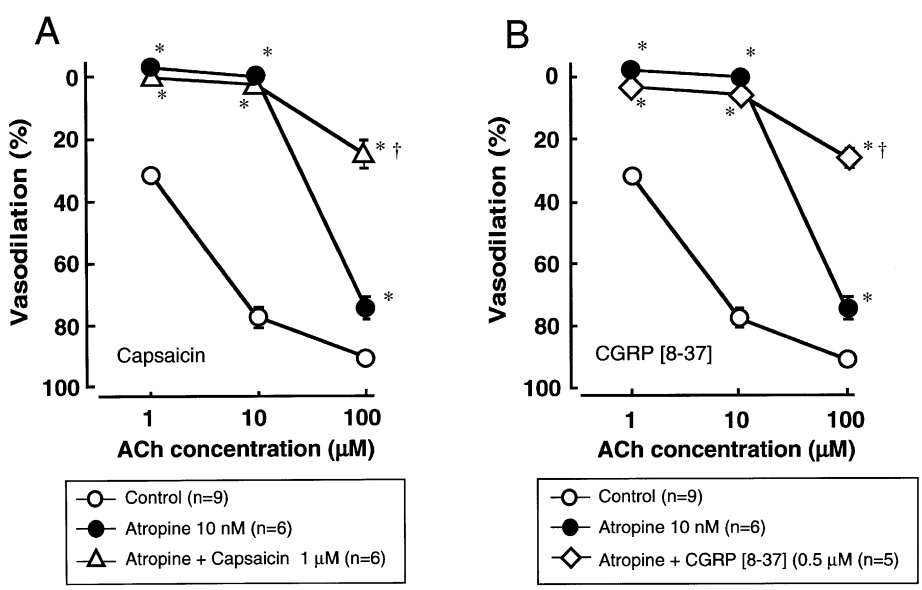

Fig. 8. Effects of capsaicin (1 μM) (A) and CGRP-(8-37) (0.5 μM) (B) treatments on the acetylcholine (ACh)-induced vasodilation in rat perfused mesenteric vascular beds without endothelium. Each point represents the mean ± S.E.M. $^{*}P < 0.01$, compared with control. †$P < 0.01$, compared with atropine.

acetylcholine-induced atropine-resistant vasodilation and periarterial nerve stimulation-induced vasodilation were reproduced (data not shown).

4. Discussion

The present study demonstrated that in the mesenteric artery with intact endothelium and with an active tone produced by methoxamine, perfusion of acetylcholine (0.01 to 100 μM) caused an initial rapid vasodilation followed by long-lasting vasodilation. Since endothelium removal abolished the initial rapid vasodilation induced by acetylcholine, the initial rapid vasodilation is endothelium-dependent. However, acetylcholine still caused long-lasting vasodilation even after endothelium removal, indicating that acetylcholine has endothelium-independent vasodilation in the rat mesenteric artery. In preparations with intact endothelium, the long-lasting vasodilation induced by high concentrations of acetylcholine was shortened by capsaicin treatment. Thus, the long-lasting vasodilation is likely to involve the periarterial nerves, probably capsaicin-sensitive neurons. Furthermore, cold-storage denervation of periarterial nerves (adrenergic and CGRPergic nerves) in the mesenteric vasculature without endothelium abolished the acetylcholine-induced endothelium-independent vasodilation. Taken together, it is likely that acetylcholine causes vasodilation by two mechanisms: one is an endothelium-dependent vasodilation, which is mediated by EDRF, and the other is an endothelium-independent vasodilation in which periarterial nerves are involved.

We have demonstrated in a previous study that acetylcholine induces endothelium-independent vasodilation, which is mediated by periarterial CGRPergic nerves (Takenaga et al., 1995). We suggested a vasodilator mechanism where acetylcholine activates muscarinic receptors located on CGRPergic neurons to release vasodilator transmitter CGRP, because the acetylcholine-induced endothelium-independent vasodilation was sensitive to both atropine and capsaicin and was antagonized by CGRP-(8-37) (Takenaga et al., 1995). The present study showed that the endothelium-independent vasodilation induced by lower doses of acetylcholine was blocked by atropine, indicating that the response was mediated by muscarinic acetylcholine receptors, as reported previously (Takenaga et al., 1995). However, the endothelium-independent vasodilator response to acetylcholine at 100 μM was resistant to atropine and was inhibited by the nicotinic acetylcholine receptor antagonist, hexamethonium. Thus, it is likely that the atropine-resistant vasodilation induced by high concentrations of acetylcholine resulted from stimulation of nicotinic acetylcholine receptors.

Takenaga et al. (1995) reported that the acetylcholine-induced endothelium-independent vasodilation in the rat mesenteric artery was not antagonized by hexamethonium They suggested that the nicotinic acetylcholine receptors were not responsible for the endothelium-independent vasodilation induced by acetylcholine. However, in that study, guanethidine, an adrenergic neuron blocker, was used to block adrenergic neurotransmission. Furthermore, Zhang et al. (1998) revealed striking evidence that the vasodilation evoked by stimulation of nicotinic acetylcholine receptors in the porcine basilar artery is blocked by either guanethidine or chemical destruction of adrenergic nerves by 6-hydroxydopamine. In addition, our recent study demonstrated that vasodilation of the rat mesenteric artery induced by nicotine, a nicotinic acetylcholine receptor agonist, is endothelium-independent and is inhibited by blocking adrenergic neurotransmission caused by guanethidine and 6-hydroxydopamine treatment (Shiraki et al., 2000). Taken together, these studies strongly suggest that nicotinic acetylcholine receptor-mediated vasodilation requires the presence of intact adrenergic nerves. In the present study, the acetylcholine-induced endothelium-independent vasodilation, which was resistant to atropine and was blocked by hexamethonium, was markedly inhibited not only by the adrenergic neuron blockers, guanethidine and bretylium, but also by chemical adrenergic destruction produced by 6-hydroxydopamine treatment. The treatments with adrenergic neuron blockers and in vitro 6-hydroxydopamine incubation abolished the adrenergic vasoconstriction and noradrenaline overflow evoked by periarterial nerve stimulation. Therefore, it is very likely that periarterial adrenergic nerves are responsible for the acetylcholine-induced atropine-resistant vasodilation, which is mediated by nicotinic acetylcholine receptors. In the present study, hexamethonium, adrenergic neuron blockers or 6-hydroxydopamine treatment significantly attenuated the endothelium-independent vasodilation of acetylcholine. This indicates that acetylcholine-induced endothelium vasodilation is involved in adrenergic nerves via activation of nicotinic acetylcholine receptors.

Evidence has accumulated that many blood vessels are innervated by NANC nerves (Bevan and Brayden, 1987; Kawasaki et al., 1988; Toda and Okamura, 1991; Lee et al., 1996). We reported previously that the mesenteric resistance arteries of the rat were innervated by CGRPergic nerves and periarterial nerve stimulation induces neurogenic vasorelaxation, which is sensitive to the neurotoxic effect of TTX, and is abolished by capsaicin (Kawasaki et al., 1988, 1990a). Capsaicin has been shown to deplete neuropeptides such as CGRP, substance P and vasoactive intestinal polypeptide (VIP) from primary sensory neurons, which leads to depleting capsaicin-sensitive primary nerves (Fujimori et al., 1989; Holzer, 1991). Furthermore, the periarterial nerve stimulation-induced neurogenic vasorelaxation in the rat mesenteric artery is also inhibited by human CGRP-(8-37), a C-terminal fragment of CGRP and a CGRP receptor antagonist (Han et al., 1990; Kawasaki et al., 1991; Takenaga et al., 1995). Thus, these studies indicate that the vasorelaxation is mediated by endogenous CGRP released from CGRPergic nerves. In the present

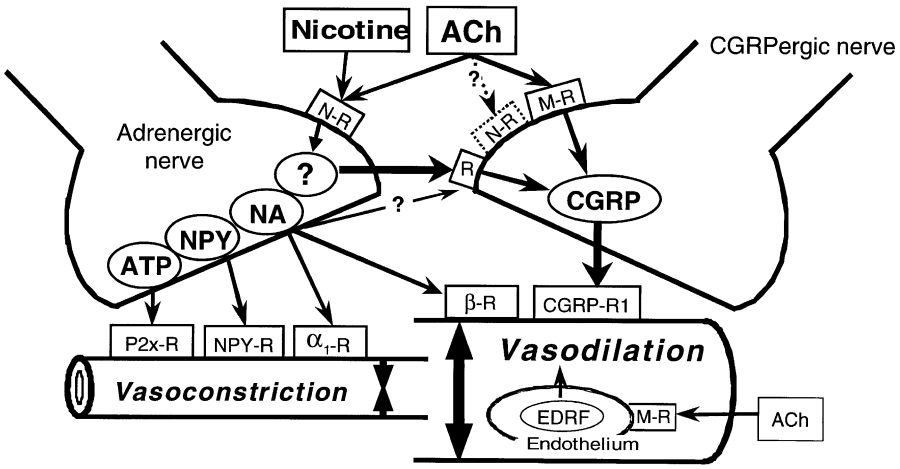

Fig. 9. Possible mechanisms underlying adrenergic mediated and endothelium-independent vasodilation induced by acetylcholine (ACh) in the rat mesenteric resistance artery. The N-R and M-R indicate nicotinic and muscarinic acetylcholine receptor, respectively. The α-R and β-R show α- and β-adrenoceptor, respectively. The P2x shows ATP P2X receptor. ATP, adenosine triphosphate; CGRP, calcitonin gene-related peptide; EDRF, endothelium derived relaxing factor; NA, noradrenaline; NPY, neuropeptide Y; R, receptor.

study, the endothelium-independent vasodilator response to acetylcholine perfusion in the presence of atropine was sensitive to the effect of capsaicin. Therefore, it is very likely that the capsaicin-sensitive peptidergic nerves are responsible for the vasorelaxation induced by acetylcholine. In addition, the present study showed that the acetylcholine-induced atropine-resistant endothelium-independent vasodilation was inhibited by the CGRP receptor antagonist, CGRP-(8-37), which also blocked the periarterial nerve stimulation-induced CGRPergic nerve-mediated vasodilation. These findings strongly suggest that CGRPergic vasodilator nerves in the mesenteric artery are involved in the nicotinic acetylcholine receptor-mediated vasodilation induced by acetylcholine. In the present study, either guanethidine, bretylium or 6-hydroxydopamine blocked the acetylcholine-induced atropine-resistant endothelium-independent vasorelaxation, but they did not affect the vasodilation mediated by CGRPergic nerves. Thus, it is unlikely that acetylcholine directory acts on nicotinic acetylcholine receptors in CGRPergic nerves to cause vasodilation. The acetylcholine-induced atropine resistant-vasodilation is likely to be elicited indirectly by neighboring neurons, probably adrenergic neurons, via neurotransmitters released from adrenergic nerve terminals.

Zhang et al. (1998) reported the possibility that the relaxation induced by stimulation of nicotinic acetylcholine receptors by nicotine in the porcine basilar artery may be mediated by noradrenaline, which is released from adrenergic nerve terminals by nicotine. However, we have demonstrated that noradrenaline inhibits the CGRP release from the CGRPergic nerve terminals of the mesenteric artery by presynaptic mechanisms via activation of α_2-adrenoceptors (Kawasaki et al., 1990b, 1991). In addition, the present study showed that perfusion of acetylcholine caused no vasoconstriction in the mesenteric artery, suggesting that only a small amount of noradrenaline or related vasoconstrictor transmitter may be released by acetylcholine. Thus, the involvement of neurally released noradrenaline is ruled out of the mechanisms underlying the acetylcholine-induced endothelium-independent and atropine-resistant vasodilation in the rat mesenteric artery. However, further studies are needed to clarify the identity of the neurotransmitter(s) involved.

In conclusion, the present study suggests that acetylcholine induces endothelium-independent vasodilation through nicotinic acetylcholine receptors located on adrenergic nerve terminals in mesenteric resistance blood vessels. As illustrated in Fig. 9, it is also suggested that the acetylcholine-induced endothelium-independent vasodilation is dependent on intact adrenergic nerves, and is mediated by endogenous CGRP released from CGRPergic nerves. It appears that acetylcholine acts on presynaptic nicotinic acetylcholine receptors to release adrenergic neurotransmitters or related substances, which then activate CGRPergic nerves to cause CGRP release and vasodilation (Fig. 9).

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