

HOSTED BY

Contents lists available at ScienceDirect

Journal of Pharmacological Sciences

journal homepage: www.elsevier.com/locate/jphs

Full paper

Muscarinic acetylcholine receptor M1 and M3 subtypes mediate acetylcholine-induced endothelium-independent vasodilatation in rat mesenteric arteries

Panot Tangsucharit ^{a, b}, Shingo Takatori ^{a, e, *}, Yoshito Zamami ^d, Mitsuhiro Goda ^{a, f}, Poungrat Pakdechote ^c, Hiromu Kawasaki ^{a, e}, Fusako Takayama ^a^a Department of Clinical Pharmaceutical Sciences, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama, Japan^b Department of Pharmacology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand^c Department of Physiology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand^d Department of Pharmaceutical Care and Health Science, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Japan^e Department of Clinical Pharmacy, College of Pharmaceutical Sciences, Matsuyama University, Matsuyama, Ehime, Japan^f Department of Biochemistry, Faculty of Pharmaceutical Sciences, Setsunan University, Osaka, Japan

ARTICLE INFO

Article history:

Received 23 September 2015

Received in revised form

15 December 2015

Accepted 20 December 2015

Available online 8 January 2016

Keywords:

Acetylcholine-induced vasodilatation

CGRPergic nerves

Muscarinic receptor subtypes

Rat mesenteric arteries

ABSTRACT

The present study investigated pharmacological characterizations of muscarinic acetylcholine receptor (AChR) subtypes involving ACh-induced endothelium-independent vasodilatation in rat mesenteric arteries. Changes in perfusion pressure to periarterial nerve stimulation and ACh were measured before and after the perfusion of Krebs solution containing muscarinic receptor antagonists. Distributions of muscarinic AChR subtypes in mesenteric arteries with an intact endothelium were studied using Western blotting. The expression level of M1 and M3 was significantly greater than that of M2. Endothelium removal significantly decreased expression levels of M2 and M3, but not M1. In perfused mesenteric vascular beds with intact endothelium and active tone, exogenous ACh (1, 10, and 100 nmol) produced concentration-dependent and long-lasting vasodilations. In endothelium-denuded preparations, relaxation to ACh (1 nmol) disappeared, but ACh at 10 and 100 nmol caused long-lasting vasodilations, which were markedly blocked by the treatment of pirenzepine (M1 antagonist) or 4-DAMP (M1 and M3 antagonist) plus hexamethonium (nicotinic AChR antagonist), but not methoctramine (M2 and M4 antagonist). These results suggest that muscarinic AChR subtypes, mainly M1, distribute throughout the rat mesenteric arteries, and that activation of M1 and/or M3 which may be located on CGRPergic nerves releases CGRP, causing an endothelium-independent vasodilatation.

© 2015 Japanese Pharmacological Society. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Many studies have revealed ACh-induced vasorelaxation is absolutely dependent on an intact endothelium and is mediated by endothelium-derived relaxing factors (EDRF) including nitric oxide (NO), prostanoids and endothelium-derived hyperpolarizing factor (EDHF) (1–3). In vascular beds from various species, the activation of muscarinic acetylcholine receptor (AChR) has been shown to induce a potent vasodilatation via the release of EDRF from the endothelium. Distribution of muscarinic AChR on vascular endothelial cells has been reported to be associated with ACh-induced vasodilatation (4).

Muscarinic AChR has been shown to be divided into 5 subtypes, M1, M2, M3, M4, and M5 (5). M1, M2, and M3 have been reported to

Abbreviations: ACh, acetylcholine; AChR, acetylcholine receptor; CGRP, calcitonin gene-related peptide; CGRPergic, calcitonin gene-related peptide-containing; 4-DAMP, 4-diphenyl-acetoxy-N-methyl-piperidine; EDRF, endothelium-derived relaxing factors; M1, muscarinic type 1 receptor; M2, muscarinic type 2 receptor; M3, muscarinic type 3 receptor; PNS, periarterial nerve stimulation; PPV, papaverine; SD, sodium deoxycholate.

* Corresponding author. Department of Clinical Pharmacy, College of Pharmaceutical Sciences, Matsuyama University, 4-2 Bunkyo-cho, Matsuyama, Ehime 790-8546, Japan.

E-mail address: stakator@cc.matsuyama-u.ac.jp (S. Takatori).

Peer review under responsibility of Japanese Pharmacological Society.

<http://dx.doi.org/10.1016/j.jphs.2015.12.005>

1347-8613/© 2015 Japanese Pharmacological Society. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

be distributed in the vasculature including artery, vein, and endothelium (6–8). It is well known the vascular endothelium has M1 and/or M3, and its activation induces a potent vasodilatation (9). Remarkably, the expression pattern of muscarinic AChR subtypes and their role in mediating vascular responses substantially differ according to individual vascular beds (10–15). Many studies confirmed that ACh-induced vasodilatation is partially mediated by M1, since selective M1 antagonist, pirenzepine, blunts the vasodilatation (10,16). However, Ren and coworkers reported, in isolated simian coronary arteries, ACh induced both vasodilatation and vasoconstriction via the activation of M3 on endothelium cells and smooth muscle cells, respectively (16). Additionally, M3 in the forearm vasculature has been shown to play a major role in ACh-induced endothelium-dependent vasodilatation (17). Endothelial M3 has been also reported to mediate ACh-induced vasodilatation in murine retinal arterioles via the activation of NO synthase and in cutaneous, skeletal muscle, and renal interlobar arteries (18). Moreover, Chiba and Tsukada demonstrated there are abundant functional M3 and a few M1 in the canine lingual artery in which these receptors mediate an endothelium-dependent vasodilatation (9). In contrast, neither M1 nor M5 subtypes appeared to be involved in ACh-induced responses of cutaneous, skeletal muscle, and renal interlobar arteries (19).

Takenaga et al. (20) reported interesting findings that ACh induced an initial transient endothelium-dependent vasodilatation followed by a secondary long-lasting endothelium-independent vasodilatation in rat mesenteric vascular beds. This study demonstrated the underlying mechanism of vasodilatation that ACh activated muscarinic receptors located on endothelium to cause an initial rapid vasodilatation and presynaptic muscarinic receptors on calcitonin gene-related peptide (CGRP)-containing neurons to release CGRP, which then acted at postsynaptic CGRP receptors on vascular smooth muscles to cause the endothelium-independent vasodilatation. Furthermore, Shiraki et al. reported ACh-induced endothelium-independent vasodilatation in rat mesenteric arteries is inhibited by the muscarinic AChR antagonist, atropine, and the part of atropine-resistant vasodilatation is abolished by hexamethonium (nicotinic AChR antagonist), guanethidine (adrenergic neuron blocker) and capsaicin (CGRP depletor), suggesting that adrenergic nerves via nicotinic AChR and CGRPergic nerves are involved in ACh-induced endothelium-independent vasodilatation (21). However, the detailed information on subtypes of muscarinic AChR associated with ACh-induced endothelium-independent vasodilatation remains unknown.

Therefore, the aim of this study was to investigate the distribution and pharmacological characterization of muscarinic AChR subtypes (M1, M2, and M3) involving in ACh-induced endothelium-independent vasodilatation in rat mesenteric arteries.

2. Materials and methods

2.1. Animals

A total of 60 male Wistar rats (Japan SLC Inc., Shizuoka, Japan) weighing 250–350 g were used in this study. All 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 ± 2 °C with $50 \pm 10\%$ relative humidity and with a 12-h light/12-h dark cycle (lights on at 8:00 a.m.). This study was performed in accordance with the Guidelines for Animal Experiments at Okayama University Advanced Science Research Center, Japanese Government Animal Protection and Management Law (No. 115), and Japanese Government Notification on Feeding and

Safekeeping of Animals (No. 6). Every effort was made to minimize the number of animals used and their suffering. All experiments conformed to international guidelines on the ethical use of animals.

2.2. Perfusion of mesenteric vascular beds

The animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) before the mesenteric vascular beds were isolated and prepared for perfusion as described previously (22). The mesenteric vascular bed was separated from the intestine by cutting close to the intestinal wall, and four main arterial branches from the superior mesenteric trunk running to the terminal ileum were perfused with modified Krebs solution at a constant flow rate of 5 ml/min with a peristaltic pump (model AC-2120; ATTO Co., Tokyo, Japan). Changes in 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).

2.3. Chemical removal of the vascular endothelium

To remove the vascular endothelium, preparations were perfused with sodium deoxycholate (1.80 mg/ml) for 30 s as described previously (20,23). In preparations precontracted by perfusion with Krebs solution containing 2 μ M methoxamine, successful removal of the endothelium was assessed by the lack of vasodilatation to 1 nmol ACh, which was injected directly into the perfusate proximal to the arterial cannula with an injection pump (model 975, Harvard Apparatus, Holliston, MA, USA). The volume injected was 100 μ L over 12 s.

2.4. Cold-storage denervation

Isolated mesenteric vascular beds were stored in cold Krebs solution at 4 °C for 72 h to achieve cold-storage denervation (24). To determine the intact responsiveness of smooth muscles, ACh or CGRP was carried out to cause vasodilatation. Successful denervation of periarterial nerves was confirmed by the lack of periarterial nerve stimulation (PNS)-induced vasoconstrictions (8 and 12 Hz) at resting tone and vasodilatation (2 Hz) at active tone.

2.5. Western blot analysis

The protein expression levels of M1, M2, and M3 were measured by western blot analysis. The mesenteric arteries were pulverized and homogenized in Tris-buffered saline (25 mM Tris–HCl (pH 7.4), 1 mM EDTA) containing 1% protease inhibitor cocktail. The tissue extracts were centrifuged, and total protein content was quantified using Lowry method. Proteins were diluted to 2.5 mg/ml, then mixed 1:1 with sample buffer (62.5 mM Tris–HCl, pH 6.8, containing 25% glycerol, 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol, and 0.01% bromophenol blue) and denatured at 95 °C for 5 min. Total proteins (12.5 μ g/protein 10 μ L) were separated in a 12% polyacrylamide gel (Bio-Rad Laboratories Inc., Hercules, CA, USA), and then transferred onto polyvinylidene fluoride membrane using a protein blotting equipment consisting of a Mini-PROTEAN Tetra Cell and a PowerPac HC (Bio-Rad Laboratories Inc.). The membranes were blocked with 5% nonfat milk dissolved in TBS-T buffer (25 mM Tris–HCl buffer, pH 7.4, containing 0.15 M sodium chloride (NaCl) and 0.1% Tween 20) for 1 h at room temperature, and incubated with primary antibodies against rabbit anti-M1, M2, or M3 (1:200) (Sigma–Aldrich, Tokyo, Japan) or anti- β -actin (1:2000) (Santa Cruz Biotechnology, Inc., TX, USA).

overnight at 4 °C, and then incubated with goat anti-mouse (1: 5000) (Santa Cruz Biotechnology) and goat anti-rabbit (1: 3000) secondary antibodies (Santa Cruz Biotechnology) for 0.5 h at room temperature. The membranes were washed and the blots were visualized using Chemiluminescence Luminol Reagent (Santa Cruz Biotechnology).

2.6. *In vitro* treatment with capsaicin

The depletion of CGRPergic nerves was performed according to the method described by Kawasaki et al. (22,25). Successful depletion of CGRPergic nerves was confirmed by the lack of vasodilatation in response to PNS (4 Hz).

2.7. Experimental protocols for PNS and pharmacological analysis

To prove ACh-induced endothelium-independent and CGRPergic nerve-mediated vasodilatation, the mesenteric vascular bed was perfused with Krebs solution containing methoxamine (7 μM). After the elevated perfusion pressure had stabilized, PNS (4 Hz) was applied for 30 s and then infusions of ACh (1, 10, and 100 nmol) were performed (control). After sodium deoxycholate or capsaicin was perfused in preparations with resting tone followed by washing with normal Krebs

solution, PNS and injections of ACh were carried out to examine involvement of CGRPergic nerves and endothelium, respectively.

In denuded preparations with active tone produced by perfusion of Krebs solution containing methoxamine (2 μM), the vascular response to the first PNS (4 Hz; R1) and injections of ACh (1, 10, and 100 nmol; I1) were obtained (control). The perfusion of Krebs solution containing pirenzepine (1 μM), methoctramine (100 nM) or 4-DAMP (10 nM) was begun 30 min before perfusion of Krebs solution containing methoxamine (2 μM). After the elevated perfusion pressure had stabilized, PNS (R2) and infusions of ACh (I2) were carried out. In another series of vasodilator response experiments, after the control responses to the first PNS (S1) and ACh injections (I1) were obtained in the presence of pirenzepine or 4-DAMP, the second PNS (S2) and ACh injections (I2) were carried out in the presence of hexamethonium and pirenzepine or 4-DAMP. The effects of pirenzepine, methoctramine, and 4-DAMP were expressed as the ratio between the vascular responses (R2/R1 or I2/I1).

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

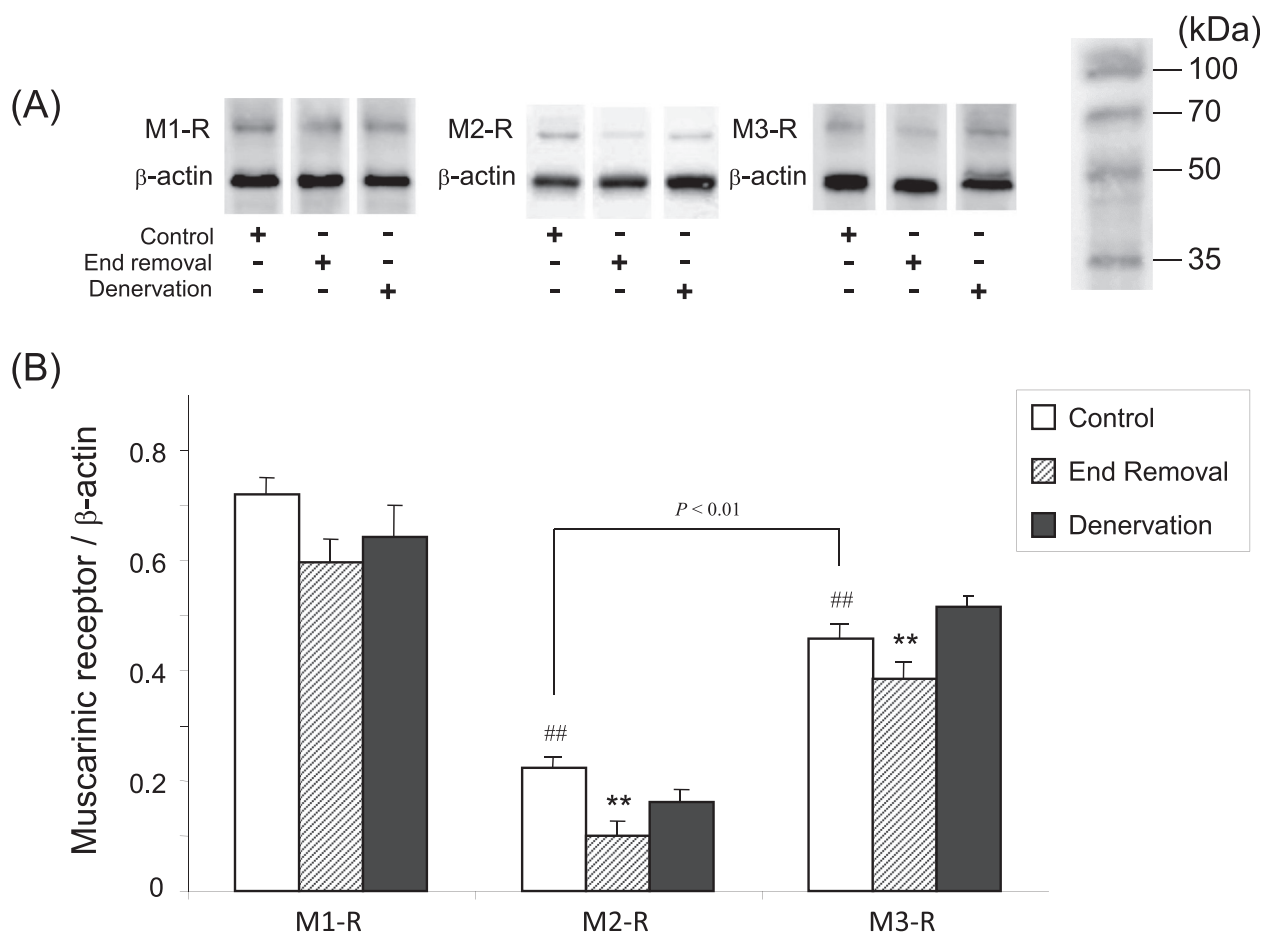


Fig. 1. Changes in muscarinic acetylcholine M1, M2, and M3 receptor (R) protein expression in rat mesenteric arteries isolated from control, sodium deoxycholate (SD)-treatment (endothelium removal) and cold-storage denervation groups ($n = 6$). In upper immunoreactive bands (A), molecular weights of M1, M2, and M3Rs and β -actin proteins indicated are about 55, 52, 67 kDa and 47 kDa, respectively. The quantitative value of M1, M2, and M3 expression shown in (B) was normalized to the expression levels of the housekeeping β -actin protein, by the densitometric analysis. Each column and bar indicates the mean \pm S.E.M. $**P < 0.01$ vs. Control. $##P < 0.01$ vs. M1 control.

2.8. Statistical analysis

Experimental results are each expressed as the mean ± S.E.M. Statistical analysis was performed by one-way analysis of variance followed by Tukey's test. A value of $P < 0.05$ was considered statistically significant.

2.9. Drugs

The following reagents were used: acetylcholine chloride (Daiichi Pharmaceutical, Tokyo, Japan), capsaicin (Sigma), hexamethonium bromide (Sigma), methocramine (Sigma), methoxamine hydrochloride (Nihon Shinyaku, Kyoto, Japan), papaverine hydrochloride (Dainippon Pharmaceutical, Tokyo, Japan), pirenzepine hydrochloride (Sigma), sodium deoxycholate (Ishizu

Seiyaku), 4-diphenyl-acetoxy-N-methyl-piperidine methiodide (4-DAMP) (Enzo Life Science). All drugs, except capsaicin and sodium deoxycholate, were dissolved in distilled water and diluted with Krebs solution. 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. Western blot analysis of muscarinic AChR subtypes in rat mesenteric arteries with intact endothelium

As shown in Fig. 1A, immunoreactive bands for M1, M2, and M3 subtypes were detected in rat mesenteric arteries with intact endothelium. Major bands were present at 55, 52, and 67 kDa for

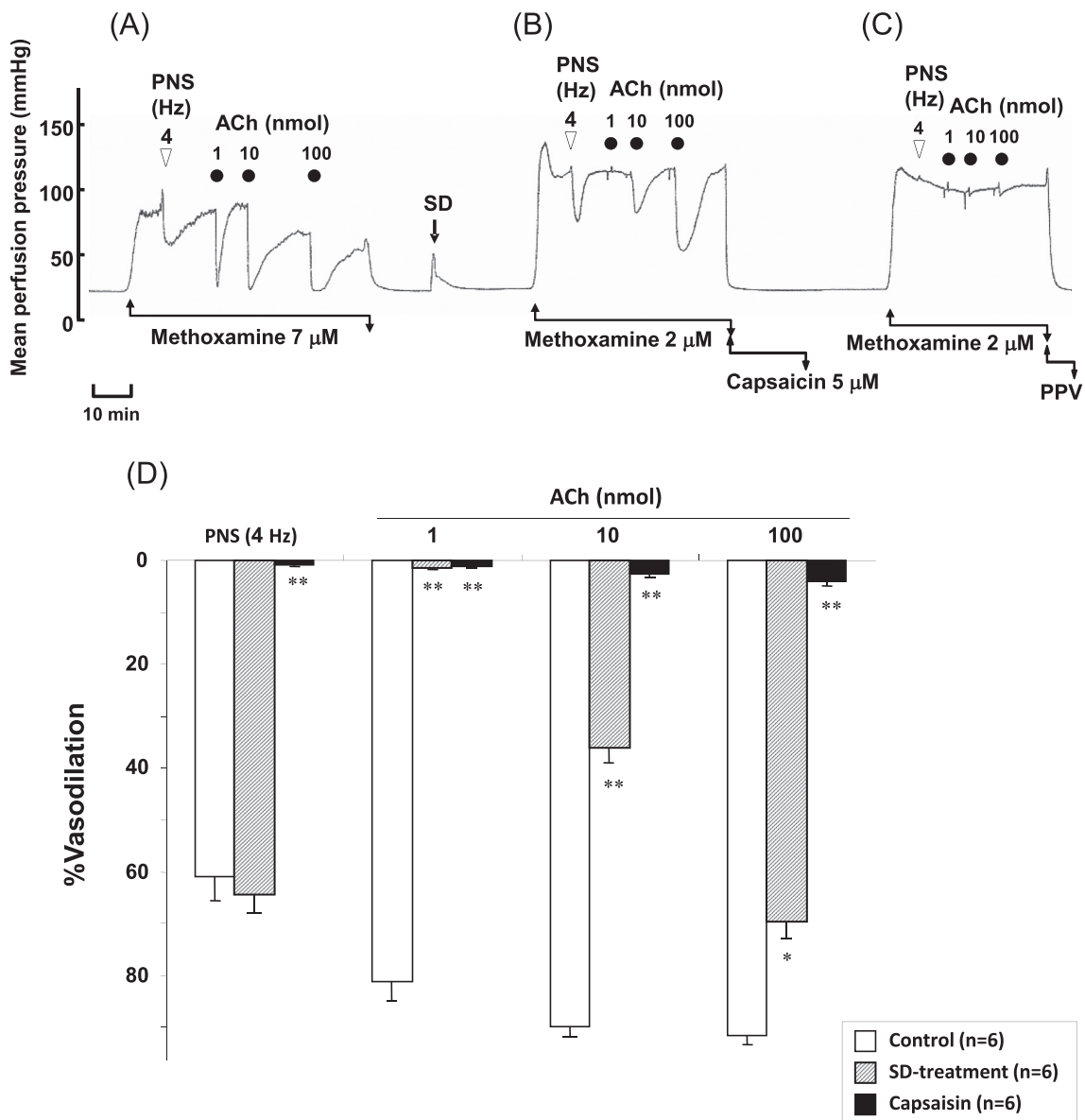


Fig. 2. A typical recording (A, B, and C) and bar graph (D) showing effects of sodium deoxycholate and capsaicin treatment on vasodilatation induced by PNS (4 Hz; open inverted triangles) and injections of acetylcholine (ACh; 1, 10, and 100 nmol; solid circles) in mesenteric vascular beds. A, B, and C show responses of control, after sodium deoxycholate (SD) and capsaicin treatments, respectively. SD, capsaicin and PPV indicate the perfusion of sodium deoxycholate for 30 s, capsaicin for 20 min and papaverine (100 μM), respectively. In D, ordinate indicates vasodilatation expressed as a percentage of the papaverine-induced maximum relaxation. Values represent the mean ± S.E.M. of six experiments. * $P < 0.05$, ** $P < 0.01$, compared with Control.

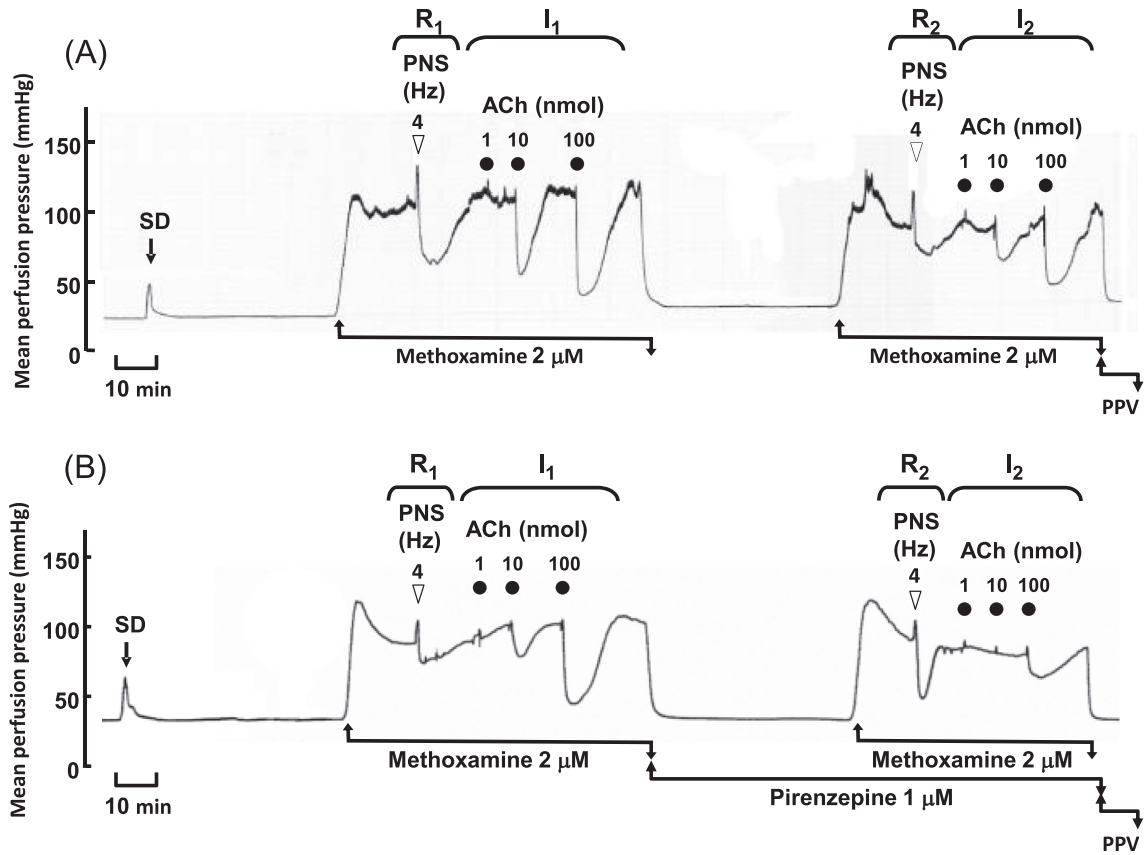


Fig. 3. Typical tracing showing vasodilator responses to PNS (4 Hz) and injections of acetylcholine (ACh; 1, 10, and 100 nmol) in mesenteric vascular beds with a denuded endothelium without (A) or with (B) pirenzepine. R1, I1 and R2, I2 show the first and second responses to PNS or injections of ACh, respectively. Solid circle, injection of ACh. Open inverted triangles, PNS. SD and PPV indicate the perfusion of sodium deoxycholate and papaverine, respectively.

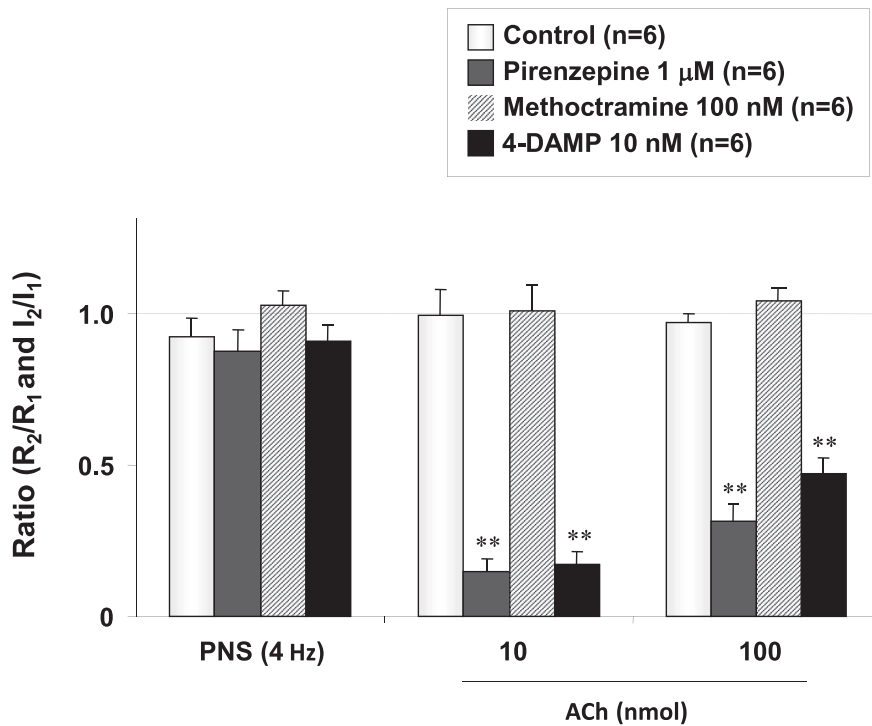


Fig. 4. Effects of pirenzepine (1 μM), methoctramine (100 nM) and 4-DAMP (10 nM) on vasodilator responses to periarterial nerve stimulation (PNS; 4 Hz) and acetylcholine (ACh; 10 and 100 nmol) injections. Ordinate shows the ratio of R1 and R2-and I1 and I2-induced vasodilatations. Each column and bar represents the mean ± S.E.M. of six experiments. **P < 0.01, compared with Control.

M1, M2, and M3 subtypes, respectively, as reported by the previous study (26). The expression level of M1 was 3.2 and 1.6 fold greater than that of M2 and M3 subtypes, respectively (Fig. 1B). The level of M3 expression was 2.0 fold greater than that of M2.

3.2. Changes in expression of muscarinic AChR subtypes after endothelium removal or cold-storage denervation

The expressions of M1 were detected in rat mesenteric arteries without endothelium and with cold-storage denervation, as observed in control group (Fig. 1). However, expression levels of M2 and M3 subtypes were significantly decreased in mesenteric arteries without endothelium, but not with denervation, as compared with control. The degrees of decrease were 55.3% and 15.8% for M2 and M3 subtype, respectively (Fig. 1B).

3.3. Effect of sodium deoxycholate and capsaicin treatment on ACh-induced vasodilatation

PNS (4 Hz) induced an initial increase in perfusion pressure due to vasoconstriction, which was followed by a long-lasting decrease in perfusion pressure due to vasodilatation. The injection of ACh (1 nmol) produced a rapid drop in perfusion pressure, which occurred in 1–2 s after ACh injection. However, higher doses of ACh (10 and 100 nmol) caused a rapid drop in perfusion pressure, followed by long-lasting vasodilatation in which the second phase was prolonged in a concentration-dependent manner, and the perfusion pressure gradually returned to the pre-injection level within 10–20 min.

As shown in Fig. 2B, since chemical denudation of vascular endothelium has been reported to augment the methoxamine-induced increase in the perfusion pressure (23), the concentration of methoxamine was decreased to 2 μM from 7 μM . The vasodilator response to PNS 4 Hz was not affected after removal of endothelium (Fig. 2B). However, chemical denudation abolished the initial rapid component of the vasodilatation in response to injections of ACh at 1, 10, and 100 nmol (Fig. 2B and D), and 10 and 100 nmol of ACh produced long-lasting vasodilator responses in a concentration-dependent manner.

As shown in Fig. 2C and D, after treatment with capsaicin at 5 μM for 20 min, vasodilator response to ACh at any doses and PNS (4 Hz) was also not produced.

3.4. Effects of pirenzepine, methoctramine, and 4-DAMP on endothelium-independent vasodilator responses to ACh

PNS (4 Hz; R1) induced an initial vasoconstriction followed by a long-lasting vasodilatation in denuded preparations with active tone (Fig. 3A and B). In this preparation, an injection of ACh (1 nmol) did not cause vasodilatation, while injections of ACh (10 and 100 nmol) induced a concentration-dependent and long-lasting vasodilatation (I1) (Fig. 3A). Repeated PNS (4 Hz; R2) and injections of ACh (10 and 100 nmol) caused reproducible vasodilator responses (I2). In the control response, ratios of R2/R1 were 0.92 ± 0.06 at 4 Hz and ratios of I2/I1 at 10 and 100 nmol were 0.99 ± 0.08 and 0.96 ± 0.13 , respectively (Fig. 4).

There were no significant differences in PNS-induced vasodilatation between the control and each antagonist-treated group. The

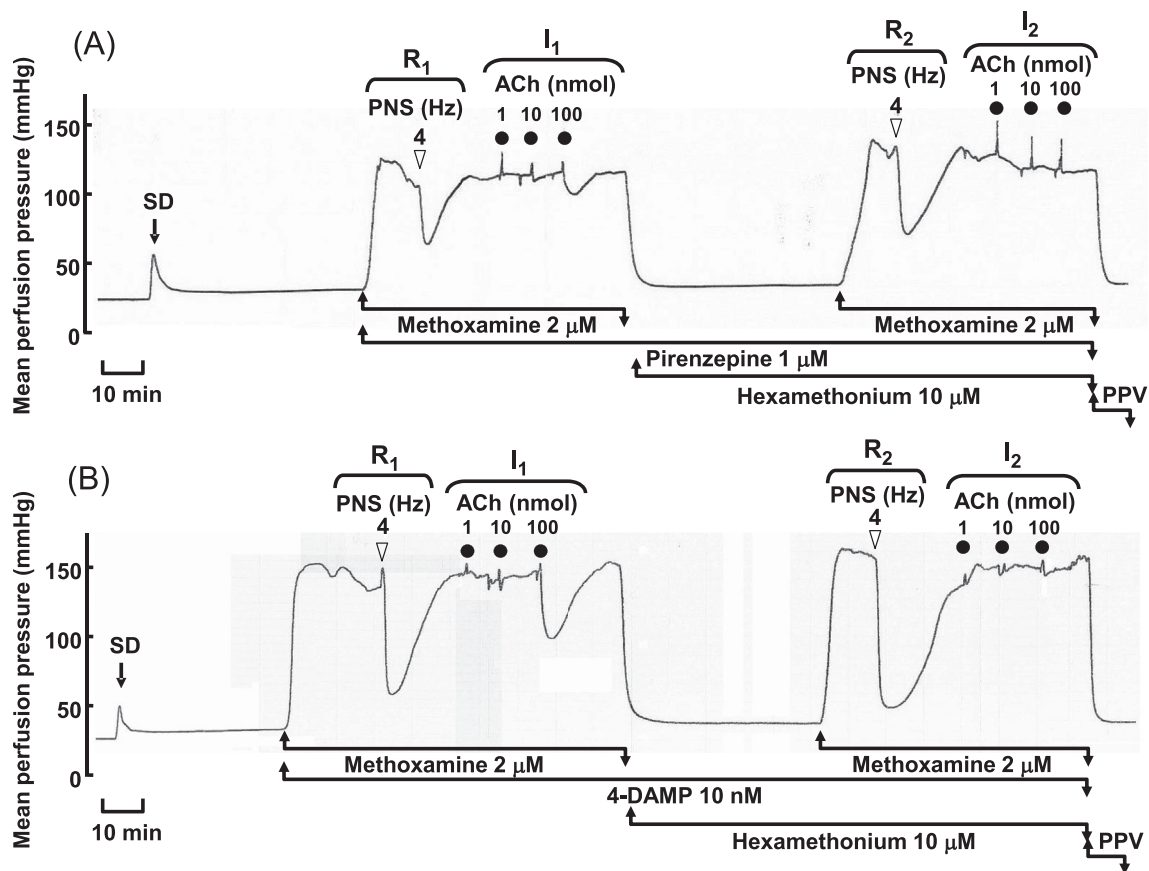


Fig. 5. Typical recordings showing the effect of pirenzepine (1 μM) (A) or 4-DAMP (10 nM) (B) and combination with hexamethonium (10 μM) on vasodilator responses to per-arterial nerve stimulation (PNS; 4 Hz) and injections of acetylcholine (ACh; 1, 10, and 100 nmol) in perfused mesenteric vascular beds without an endothelium. R1 and R2 or I1 and I2 show responses to the first and second PNS or injections of ACh, respectively. Solid circle, injections of ACh. Open inverted triangles, PNS. PPV, the perfusion of papaverine. SD, the perfusion of sodium deoxycholate.

vasodilatation to injections of ACh (10 and 100 nmol) was strongly abolished by the treatment with pirenzepine and 4-DAMP, but not methoctramine (Fig. 4).

3.5. Effect of hexamethonium on ACh-induced pirenzepine-resistant vasodilatation

In the presence of pirenzepine (1 μ M), the first PNS (4 Hz; R1) induced a long-lasting vasodilatation ($62.03 \pm 1.98\%$), and injections of ACh (10 and 100 nmol; I1) caused a significant diminished vasodilatation ($2.70 \pm 0.49\%$ and $6.65 \pm 1.04\%$) compared with control. Furthermore, combined treatment with hexamethonium (10 μ M) and pirenzepine pronouncedly inhibited the vasodilatation to an injection of ACh (100 nmol), but not to PNS (4 Hz), compared with control and pirenzepine treatment (Figs. 5A and 6A).

3.6. Effect of hexamethonium on ACh-induced 4-DAMP-resistant vasodilatation

In the presence of 4-DAMP (10 nM), PNS (4 Hz; R1) caused a long-lasting vasodilator ($81.33 \pm 3.84\%$), but responses to ACh (10 and 100 nmol; I1) were markedly diminished ($3.98 \pm 0.87\%$ and $6.04 \pm 1.07\%$) compared with control (Fig. 6B). In the combined presence of hexamethonium (10 μ M) and 4-DAMP, the 2nd PNS (R2) induced a similar vasodilator response ($87.82 \pm 6.73\%$) to the control response (R1). However, responses to ACh (10 and 100 nmol; I2) were almost abolished (1.63 ± 0.18 and $2.45 \pm 0.96\%$) (Figs. 5B and 6B).

4. Discussions

There have been few studies on pharmacological characterization, expression and distribution of the muscarinic receptor subtype mediating vascular responses in the rat mesenteric vascular bed. Therefore, the present study using Western blot analysis is the first report to clearly demonstrate that M1, M2, and M3 were detected in rat mesenteric arteries, and M1 and M3 expressions were higher than that of M2, suggesting that both M1 and M3 have a critical role in rat mesenteric arteries.

Stimulation of M1 and M3 has been reported to induce endothelium-dependent vasodilatation in various blood vessels from different species (9,19,27), implying that M1 and M3 distribute the endothelium. Hendriks et al. (28) demonstrated that M3 was responsible for an endothelium-dependent vasodilatation in response to methacholine in the perfused rat mesenteric vascular bed. Therefore, to estimate possible distribution site of these subtypes, endothelium removal with sodium deoxycholate resulted in significant decrease in the expression levels of M2 and M3, whereas significant reduction of M1 expression levels was not observed, suggesting that M2 and M3 could partly distribute in the endothelium. It is also suggested that muscarinic AChR might be highly distributed in rat mesenteric smooth muscle cells. Therefore, high concentration of ACh (10 and 100 nmol)-induced vasodilations seems to be partially mediated by the stimulation of muscarinic AChR in vascular smooth muscles. However, our previous study showed that exogenously applied ACh did not cause vascular responses when functions of endothelium and CGRPergic nerves were removed (29). It seems likely that muscarinic AChRs in the vascular smooth muscle have no or less function.

Takenaga et al. (20) reported that the stimulation of muscarinic AChR induced CGRPergic nerve-mediated vasodilatation, which was abolished by cold-storage denervation, indicating that muscarinic AChR was distributed in CGRPergic nerves. In the present study, expression levels of M1 and M2, but not M3, were slightly, but not significantly, decreased by cold-storage

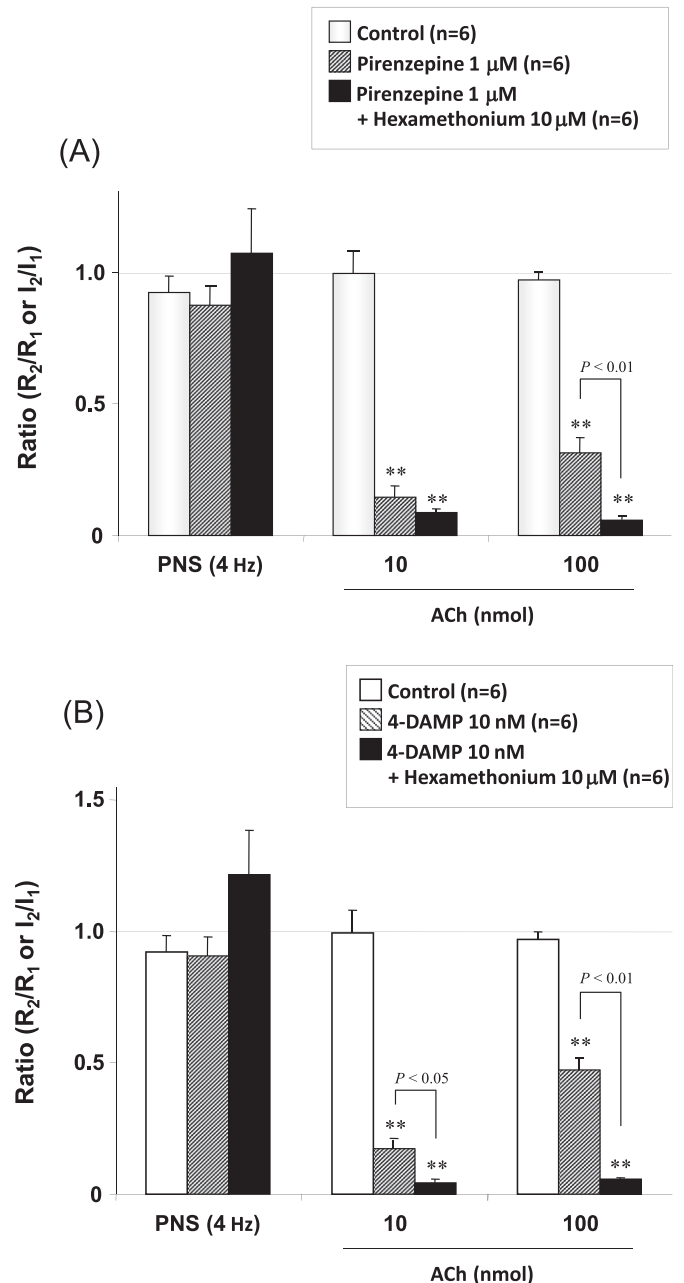


Fig. 6. Effects of pirenzepine (1 μ M) (A) or 4-DAMP (10 nM) (B) and combination with hexamethonium (10 μ M) on vasodilator responses to periarterial nerve stimulation (PNS; 4 Hz) and injections of acetylcholine (10 and 100 nmol) in perfused mesenteric vascular beds without an endothelium. Bars represent vascular responses in the ratio of R2/R1 and I2/I1. Each column and bar represents the mean \pm S.E.M. of six experiments. * $P < 0.05$, ** $P < 0.01$, compared with Control.

denervation, which induced mainly the functional loss of perivascular nerves. It is also inferred that the cold-storage denervation with short-time periods at 72 h could not destroy the structure of nervous system including receptor proteins of M1 and M2. Therefore, it seems likely that the perivascular denervation might cause a slight decrease in M1 and M2 expression levels, since tissue volumes of smooth muscle and endothelium in rat mesenteric arteries were relatively greater than that of perivascular nerves. The present results suggest that muscarinic M1, M2, and M3 are distributed in rat mesenteric arteries, which include endothelium and perivascular nerves, and contribute to induce endothelium- and perivascular nerve-mediated vascular responses.

Interestingly, the present study showed that exogenous application of ACh in the mesenteric artery without an endothelium induced endothelium-independent vasodilatations, which was significantly abolished by elimination of CGRPergic nerve function with the treatment of capsaicin. These findings are good accordance with the previous report by Takenaga et al. (20) that exogenously applied ACh activates presynaptic muscarinic AChR located on CGRP-containing neurons to release CGRP, which then acts at postsynaptic CGRP receptors on vascular smooth muscles to cause the endothelium-independent vasodilatation. However, it has not been reported on the characterization of muscarinic AChR subtypes involving ACh-induced endothelium-independent vasodilatation. To clarify the uncertain facts, the present study demonstrated that pirenzepine (M1 antagonist) and 4-DAMP (M1/M3 antagonist) pronouncedly abolished the vasodilator response to ACh at 10 and 100 nmol in preparations without an intact endothelium and with active tone. However, methoctramine (M2 antagonist) had no effect on vasodilatation in response to ACh at any concentrations. Therefore, it seems likely that M1 and M3 located on perivascular nerves, but not vascular smooth muscle, are responsible for ACh-induced endothelium-independent and CGRPergic nerve-mediated vasodilatations.

In the present study, pirenzepine and 4-DAMP caused 68% and 53% inhibition of vasodilator response to 100 nmol ACh (Fig. 4). In the presence of hexamethonium, pirenzepine or 4-DAMP strongly abolished the vasodilator response to ACh at 100 nmol compared with the administration of pirenzepine or 4-DAMP (Fig. 6A and B). These findings are in line with the findings by Shiraki et al. (21), who reported that high concentration of ACh produced atropine-resistant endothelium-independent vasodilatation in rat mesenteric artery without an endothelium, which was abolished by additional application of hexamethonium, implying that nicotinic AChR is in part involved in the ACh-induced endothelium-independent vasodilatation. Furthermore, additional studies revealed evidence that nicotinic AChR agonist including nicotine and ACh stimulates presynaptic nicotinic AChR on adrenergic nerves to release protons, which then act on transient receptor potential vanilloid 1 (TRPV1) located in CGRPergic nerves to release endogenous CGRP from the nerves (23,30,31). These studies and the present results strongly suggest that a high concentration of ACh induces adrenergic and CGRPergic nerve-mediated vasodilatation via nicotinic and muscarinic M1 and M3, mainly M1.

In conclusion, the present study suggests that muscarinic AChRs, mainly M1 subtypes, distribute rat mesenteric arteries, and that the activation of M1 and M3, but not M2, which may be located on CGRPergic nerves releases CGRP, thereby causing an endothelium-independent vasodilatation.

Conflicts of interest

No competing financial interests exist.

Acknowledgments

This work was supported by Grants from Okayama University, Japan and Faculty of Medicine, Khon Kaen University, Thailand.

References

- (1) Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*. 1980;288:373–376.
- (2) Faraci FM, Sigmund CD. Vascular biology in genetically altered mice: smaller vessels, bigger insight. *Circ Res*. 1999;85:1214–1225.
- (3) Leung HS, Leung FP, Yao X, Ko WH, Chen Z-Y, Vanhoutte PM, et al. Endothelial mediators of the acetylcholine-induced relaxation of the rat femoral artery. *Vasc Pharmacol*. 2006;44:299–308.
- (4) Eglén RM, Whiting RL. Heterogeneity of vascular muscarinic receptors. *J Auton Pharmacol*. 1990;10:233–245.
- (5) Caulfield MP, Birdsall NJ. International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol Rev*. 1998;50:279–290.
- (6) Hynes MR, Banner Jr W, Yamamura HI, Duckles SP. Characterization of muscarinic receptors of the rabbit ear artery smooth muscle and endothelium. *J Pharmacol Exp Ther*. 1986;238:100–105.
- (7) Martínez AC, Hernández M, Rivera L, Recio P, García-Sacristán A, Benedito S. Muscarinic receptor subtypes mediate vasorelaxation in isolated horse deep dorsal penile vein. *Urology*. 2003;62:357–361.
- (8) Norel X, Walch L, Costantino M, Labat C, Gorenne I, Dulmet E, et al. M1 and M3 muscarinic receptors in human pulmonary arteries. *Br J Pharmacol*. 1996;119:149–157.
- (9) Chiba S, Tsukada M. Possible involvement of muscarinic M1 and M3 receptor subtypes mediating vasodilation in isolated, perfused canine lingual arteries. *Clin Exp Pharmacol Physiol*. 1996;23:839–843.
- (10) Bruning TA, Hendriks MG, Chang PC, Kuypers EA, van Zwieten PA. In vivo characterization of vasodilating muscarinic-receptor subtypes in humans. *Circ Res*. 1994;74:912–919.
- (11) Elhusseiny A, Hamel E. Muscarinic—but not nicotinic—acetylcholine receptors mediate a nitric oxide-dependent dilation in brain cortical arterioles: a possible role for the M5 receptor subtype. *J Cereb Blood Flow Metab*. 2000;20:298–305.
- (12) Lamping KG, Wess J, Cui Y, Nuno DW, Faraci FM. Muscarinic (M) receptors in coronary circulation: gene-targeted mice define the role of M2 and M3 receptors in response to acetylcholine. *Arterioscler Thromb Vasc Biol*. 2004;24:1253–1258.
- (13) Pešić S, Jovanović A, Grbović L. Muscarinic receptor subtypes mediating vasorelaxation of the perforating branch of the human internal mammary artery. *Pharmacology*. 2001;63:185–190.
- (14) Phillips JK, Vidovic M, Hill CE. Variation in mRNA expression of alpha-adrenergic, neurokinin and muscarinic receptors amongst four arteries of the rat. *J Auton Nerv Syst*. 1997;62:85–93.
- (15) Yamada M, Lamping KG, Duttaroy A, Zhang W, Cui Y, Bymaster FP, et al. Cholinergic dilation of cerebral blood vessels is abolished in M(5) muscarinic acetylcholine receptor knockout mice. *Proc Natl Acad Sci U S A*. 2001;98:14096–14101.
- (16) Ren LM, Nakane T, Chiba S. Muscarinic receptor subtypes mediating vasodilation and vasoconstriction in isolated, perfused simian coronary arteries. *J Cardiovasc Pharmacol*. 1993;22:841–846.
- (17) Attnä TM, Oliver JJ, Malatino LS, Webb DJ. Contribution of the M3 muscarinic receptors to the vasodilator response to acetylcholine in the human forearm vascular bed. *Br J Clin Pharmacol*. 2008;66:300–303.
- (18) Gericke A, Sniatecki JJ, Goloborodko E, Steege A, Zavaritskaya O, Vetter JM, et al. Identification of the muscarinic acetylcholine receptor subtype mediating cholinergic vasodilation in murine retinal arterioles. *Invest Ophthalmol Vis Sci*. 2011;52:7479–7484.
- (19) Gericke A, Sniatecki JJ, Mayer VGA, Goloborodko E, Patzak A, Wess J, et al. Role of M1, M3, and M5 muscarinic acetylcholine receptors in cholinergic dilation of small arteries studied with gene-targeted mice. *Am J Physiol Heart Circ Physiol*. 2011;300:H1602–H1608.
- (20) Takenaga M, Kawasaki H, Wada A, Eto T. Calcitonin gene-related peptide mediates acetylcholine-induced endothelium-independent vasodilation in mesenteric resistance blood vessels of the rat. *Circ Res*. 1995;76:935–941.
- (21) Shiraki H, Kawasaki H, Tezuka S, Nakatsuma A, Nawa H, Araki H, et al. Adrenergic nerves mediate acetylcholine-induced endothelium-independent vasodilation in the rat mesenteric resistance artery. *Eur J Pharmacol*. 2001;419:231–242.
- (22) Kawasaki H, Takasaki K, Saito A, Goto K. Calcitonin gene-related peptide acts as a novel vasodilator neurotransmitter in mesenteric resistance vessels of the rat. *Nature*. 1988;335:164–167.
- (23) Shiraki H, Kawasaki H, Tezuka S, Nakatsuma A, Kurosaki Y. Endogenous calcitonin gene-related peptide (CGRP) mediates adrenergic-dependent vasodilation induced by nicotine in mesenteric resistance arteries of the rat. *Br J Pharmacol*. 2000;130:1083–1091.
- (24) Kawasaki H, Nuki C, Saito A, Takasaki K. NPY modulates neurotransmission of CGRP-containing vasodilator nerves in rat mesenteric arteries. *Am J Physiol*. 1991;261:H683–H690.
- (25) Kawasaki H, Nuki C, Saito A, Takasaki K. Role of calcitonin gene-related peptide-containing nerves in the vascular adrenergic neurotransmission. *J Pharmacol Exp Ther*. 1990;252:403–409.
- (26) Preiksaitis HG, Krysiak PS, Chrones T, Rajgopal V, Laurier LG. Pharmacological and molecular characterization of muscarinic receptor subtypes in human esophageal smooth muscle. *J Pharmacol Exp Ther*. 2000;295:879–888.
- (27) Bény J-L, Nguyen MN, Marino M, Matsui M. Muscarinic receptor knockout mice confirm involvement of M3 receptor in endothelium-dependent vasodilation in mouse arteries. *J Cardiovasc Pharmacol*. 2008;51:505–512.
- (28) Hendriks MG, Pfaffendorf M, van Zwieten PA. Characterization of the muscarinic receptors in the mesenteric vascular bed of spontaneously hypertensive rats. *J Hypertens*. 1993;11:1329–1335.

- (29) Tangsucharit P, Takatori S, Sun P, Zamami Y, Goda M, Pakdeechote P, et al. Do cholinergic nerves innervating rat mesenteric arteries regulate vascular tone? *Am J Physiol Regul Integr Comp Physiol.* 2012;303:R1147–R1156.
- (30) Eguchi S, Miyashita S, Kitamura Y, Kawasaki H. Alpha3beta4-nicotinic receptors mediate adrenergic nerve- and peptidergic (CGRP) nerve-dependent vasodilation induced by nicotine in rat mesenteric arteries. *Br J Pharmacol.* 2007;151:1216–1223.
- (31) Kawasaki H, Eguchi S, Miyashita S, Chan S, Hirai K, Hobara N, et al. Proton acts as a neurotransmitter for nicotine-induced adrenergic and calcitonin gene-related peptide-containing nerve-mediated vasodilation in the rat mesenteric artery. *J Pharmacol Exp Ther.* 2009;330:745–755.