British Journal of Pharmacology

DOI:10.1111/bph.12878
www.brjpharmacol.org

RESEARCH PAPER

Protons modulate perivascular axo-axonal neurotransmission in the rat mesenteric artery

Shingo Takatori[1,2], Kazuhiro Hirai[2], Shuichiro Ozaki[2], Panot Tangsucharit[2], Satoko Fukushima-Miyashita[2], Mitsuhiro Goda[2], Narumi Hashikawa-Hobara[3], Nobufumi Ono[4] and Hiromu Kawasaki[1,2,4]

[1]*Department of Clinical Pharmacy, College of Pharmaceutical Sciences, Matsuyama University, Matsuyama, Ehime, Japan,* [2]*Department of Clinical Pharmaceutical Science, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama, Japan,* [3]*Department of Life Science, Okayama University of Science, Kita-ku, Okayama, Japan, and* [4]*Department of Drug Information, Faculty of Pharmaceutical Sciences, Fukuoka University, Fukuoka, Japan*

Correspondence
Shingo Takatori, Department of Clinical Pharmacy, College of Pharmaceutical Sciences, Matsuyama University, 4-2 Bunkyo-cho, Matsuyama, Ehime 790-8578, Japan. E-mail: stakator@cc.matsuyama-u.ac.jp

Received
7 October 2013
Revised
7 July 2014
Accepted
1 August 2014

BACKGROUND AND PURPOSE

Previous studies have demonstrated that nicotine releases protons from adrenergic nerves via stimulation of nicotinic ACh receptors and activates transient receptor potential vanilloid-1 (TRPV1) receptors located on calcitonin gene-related peptide (CGRP)-containing (CGRPergic) vasodilator nerves, resulting in vasodilatation. The present study investigated whether perivascular nerves release protons, which modulate axon-axonal neurotransmission.

EXPERIMENT APPROACH

Perfusion pressure and pH levels of perfusate in rat-perfused mesenteric vascular beds without endothelium were measured with a pressure transducer and a pH meter respectively.

KEY RESULTS

Periarterial nerve stimulation (PNS) initially induced vasoconstriction, which was followed by long-lasting vasodilatation and decreased pH levels in the perfusate. Cold-storage denervation of the preparation abolished the decreased pH and vascular responses to PNS. The adrenergic neuron blocker guanethidine inhibited PNS-induced vasoconstriction and effects on pH, but not PNS-induced vasodilatation. Capsaicin (CGRP depletor), capsazepine and ruthenium red (TRPV1 inhibitors) attenuated the PNS-induced decrease in pH and vasodilatation. In denuded preparations, ACh caused long-lasting vasodilatation and lowered pH; these effects were inhibited by capsaicin pretreatment and atropine, but not by guanethidine or mecamylamine. Capsaicin injection induced vasodilatation and a reduction in pH, which were abolished by ruthenium red. The use of a fluorescent pH indicator demonstrated that application of nicotine, ACh and capsaicin outside small mesenteric arteries reduced perivascular pH levels and these effects were abolished in a Ca^{2+}-free medium.

CONCLUSION AND IMPLICATION

These results suggest that protons are released from perivascular adrenergic and CGRPergic nerves upon PNS and these protons modulate transmission in CGRPergic nerves.

Abbreviations

CGRP, calcitonin gene-related peptide; CGRPergic, CGRP-containing; LI, like immunoreactivity; mACh receptor, muscarinic ACh receptor; MOPS, 3-(N-morpholino) propanesulfonic acid; nACh receptor, nicotinic ACh receptor; NPY, neuropeptide Y; PNS, periarterial nerve stimulation; TRPV1, transient receptor potential vanilloid-1

Tables of Links

TARGETS
GPCRs
α_1-adrenoceptor
Muscarinic ACh receptor
CGRP receptor
Ligand-gated ion channels
Nicotinic ACh receptor
Ion channels
TRPV1 channel

LIGANDS	
ACh	Mecamylamine
Atropine	Methoxamine
Capsaicin	Neuropeptide Y
Capsazepine	Nicotine
CGRP	Nitric oxide (NO)
Guanethidine	Noradrenaline
	Ruthenium red

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013a,b,c).

Introduction

We have previously demonstrated that rat mesenteric arteries are innervated by NANC nerves, including calcitonin gene-related peptide (CGRP)-containing (CGRPergic) nerves, which release the neurotransmitter CGRP and cause vasodilatation (Kawasaki *et al.*, 1988), and NO-containing nerves involved in the modulation of adrenergic neurotransmission (Hatanaka *et al.*, 2006). Our previous studies also revealed that the elimination of CGRPergic nerve function augmented adrenergic nerve-mediated vasoconstriction, and conversely, adrenergic nerves via presynaptically released noradrenaline inhibited the neurogenic release of CGRP from the nerve, decreasing the function of CGRPergic nerves (Kawasaki *et al.*, 1990a,b). These findings led to the hypothesis that adrenergic nerves interact with CGRPergic nerves. In support of this hypothesis, immunohistochemical studies demonstrated that CGRP-immunopositive nerve fibres accompanied adrenergic TH or neuropeptide Y (NPY)-immunopositive nerve fibres in the rat mesenteric artery (Eguchi *et al.*, 2004), which indicated the reciprocal regulation of tone in the mesenteric resistance artery with CGRPergic vasodilator nerves and sympathetic vasoconstrictor nerves (Kawasaki *et al.*, 2009; 2011).

To prove our proposal of an axo-axonal interaction in rat mesenteric perivascular nerves, we demonstrated that nicotinic ACh receptor (nACh receptor) agonists induced perivascular adrenergic nerve- and CGRPergic nerve-mediated vasodilatation in the rat mesenteric artery, which was blocked by nACh receptor antagonists (hexamethonium and mecamylamine), an adrenergic neuron blocker (guanethidine), transient receptor potential vanilloid-1 (TRPV1) receptor inhibitors (capsazepine and ruthenium red), a CGRP receptor antagonist (CGRP 8-37) and CGRP depletor (capsaicin) (Shiraki *et al.*, 2000; Eguchi *et al.*, 2004; 2007). We explained that the underlying mechanism of nicotinic agonists is firstly to stimulate nACh receptors located on adrenergic nerves to release a transmitter, which activates adjacent CGRPergic nerves through TRPV1 receptors to induce CGRP-mediated vasodilatation. Based on these studies, a recent report showed that nACh receptor agonists release protons (H^+), an endogenous agonist for TRPV1 receptors, from adrenergic nerves (Kawasaki *et al.*, 2009). However, in our previous study we demonstrated that the CGRPergic neurotransmitter, CGRP, induced presynaptic inhibition of CGRPergic neurotransmission through a negative feedback mechanism (Nuki *et al.*, 1994). We hypothesized that CGRPergic nerves release protons, which have a feedback effect on presynaptic TRPV1 receptors. However, the substance transmitting axo-axonal information between perivascular adrenergic nerves and CGRPergic nerves has not been elucidated. Therefore, in the present study we investigated whether perivascular adrenergic and CGRPergic nerves release protons, which modulate axon-axonal neurotransmission by acting as an endogenous agonist of TRPV1 receptors.

Methods

The drug/molecular target nomenclature applied (e.g. receptors, ion channels) conforms to the British Journal of Pharmacology's Concise Guide to Pharmacology (Alexander *et al.*, 2013a,b,c).

Animals

One hundred and fifty-five male Wistar rats weighing 250–350 g (purchased from Shimizu Experimental Animal Co., Ltd., Shizuoka, Japan) were used in the present 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 ± 10% relative humidity and a 12 h light/12 h dark cycle (lights on 8:00 a.m.). This study was carried out 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 studies

involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Perfusion of the mesenteric vascular beds

Rats were anaesthetized with sodium pentobarbital (50 mg·kg^{-1}, i.p.) before the mesenteric vascular beds were isolated and prepared for perfusion as described previously (McGregor, 1965; Kawasaki *et al.*, 1988). Briefly, 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 isolated and perfused with modified Krebs solution (Kawasaki *et al.*, 2009) at a constant flow rate of 5 mL·min^{-1} with a peristaltic pump (model AC-2120; ATTO Co., Tokyo, Japan). 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 the vascular endothelium

The vascular endothelium was removed chemically by perfusing tissues with sodium deoxycholate (1.8 mg·mL^{-1}) for 30 s (Shiraki *et al.*, 2000). Successful removal of the endothelium was assessed by the lack of vasodilatation to 1 nmol ACh in preparations precontracted by perfusion with Krebs solution containing the α_1-adrenoceptor agonist methoxamine (2 μM). ACh was injected directly into the perfusate proximal to the arterial cannula with an infusion pump (model 975; Harvard Apparatus, Holliston, MA, USA); the volume injected was 100 μL over 12 s.

Periarterial nerve stimulation

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

In vitro *treatment with capsaicin*

The depletion of perivascular CGRPergic nerves was achieved by perfusing the endothelium-denuded preparations with Krebs solution containing capsaicin (CGRP depletor) (5 μM) for 20 min (Kawasaki *et al.*, 1988; 1990a). In preparations precontracted with methoxamine (2 μM), successful depletion of CGRPergic nerves was confirmed by the lack of vasodilatation to PNS (2 Hz).

Cold-storage denervation

The isolated mesenteric vascular bed was stored in cold Krebs solution at 4°C for 72 h to achieve cold-storage denervation (Kawasaki *et al.*, 1991). After being perfused with Krebs solution at 37°C for 60 min, the preparation was subjected to the experiment. The endothelium-denuded preparation was contracted by perfusion with Krebs solution containing methoxamine (10 μM), because the cold-storage treatment decreased vasoconstrictor reactivity to methoxamine. To determine the vascular smooth muscle activity, CGRP (50 pmol) was applied to induce vasodilatation. Successful denervation of the periarterial nerves was confirmed by the lack of PNS-induced vasoconstriction (8 and 12 Hz) at resting tone and vasodilatation (2–8 Hz) at active tone.

Measurement of pH levels in the perfusate

The pH levels of perfusate flowing out from the preparation were measured as described previously (Kawasaki *et al.*, 2009). In the preparation perfused with Krebs solution, a pH sensor was placed around the apical portion of each preparation and changes in the pH of the perfusate were continuously monitored with a pH meter (model F-54; HORIBA Co., Kyoto, Japan). Changes in pH are expressed as the difference in pH values before and after the perfusion of Krebs solution containing the agents tested.

Measurement of noradrenaline in the perfusate

The perfusate of the mesenteric vascular bed perfused with Krebs solution was collected before and after PNS (8 Hz for 30 s) for 3 min in the absence (control) or presence of capsazepine (5 μM). Noradrenaline in the perfusate was assayed by HPLC with electrochemical detection (model HTEC-500; Eicom, Kyoto, Japan) after being adsorbed onto alumina and eluted with acetic acid.

Experimental protocols for PNS and pharmacological analysis

In denuded preparations with active tone, the first series of PNS (S1, control) at 2–8 Hz was applied, and thereafter Krebs solution alone or Krebs solution containing methoxamine and guanethidine (an adrenergic neuron blocker) (5 μM), capsazepine (a TRPV1 receptor antagonist) (5 μM), or ruthenium red (a TRPV1 receptor inhibitor) (30 μM) were perfused. After the elevated perfusion pressure had stabilized, the second series of PNS (S2) was applied. In experiments using capsazepine and ruthenium red, the concentration of methoxamine was increased to 5–7 μM because these antagonists caused vasodilatation.

In the experiments in which ACh and capsaicin were applied, in the first series (control) ACh (1–100 nmol) or capsaicin (50–200 pmol) was applied to endothelium-denuded preparations with active tone. Thereafter, Krebs solution containing methoxamine (1.5–2 μM) and guanethidine (5 μM), capsazepine (5 μM), atropine (a mACh receptor antagonist) (10 nM), mecamylamine (a nACh receptor antagonist) (1 μM), atropine plus ruthenium red (10 μM), or atropine plus mecamylamine (1 μM) was perfused. After the elevated perfusion pressure had stabilized, Krebs solution containing methoxamine and ACh or capsaicin was infused into the perfusate at 20 min intervals.

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

Measurement of perivascular pH levels in small mesenteric arteries

A fluorescent pH indicator (LysoSensor Green DND-189; Molecular Probes, Life Technologies, Osaka, Japan) was used

to visualize perivascular pH changes. To remove the endothelium, the isolated mesenteric vascular bed with resting tone was perfused with 3-(N-morpholino) propanesulfonic acid (MOPS) buffer solution (see below) and then with sodium deoxycholate for 30 s as mentioned previously. In some preparations, capsaicin (10 μM) was perfused for 20 min to deplete perivascular CGRPergic nerves. Removal of the endothelium or depletion of CGRPergic nervea was confirmed by the lack of a vasodilator response to ACh (1 nmol) or PNS (2 Hz) in preparations precontracted with methoxamine. Thereafter, the third branch of the mesenteric arteries (50 mm length) was isolated, immersed and placed between two glass slides. MOPS buffer solution containing DND-189 (2.5 μM) and methoxamine (2 μM), and either nicotine (1 mM), ACh (1 mM) or capsaicin (10 μM) was injected between the two slides, and changes in fluorescent over 1 min were observed with fluorescent microscopy (SZX12; Olympus, Tokyo, Japan). The MOPS buffer solution had the following composition (mM): NaCl 118.0; KCl 4.7; $CaCl_2$ 2.4; $MgSO_4$ 1.15; MOPS 10.0; KH_2PO_4 1.1; disodium EDTA 0.027; dextrose 11.1 (pH 7.4 adjusted with NaOH 1 mM). In the series of experiments with a Ca^{2+}-free medium, the MOPS buffer solution without $CaCl_2$ was used. The arteries were incubated with the MOPS buffer solution containing 5 μM guanethidine or 10 μM capsazepine for 10 min, and then a MOPS buffer solution containing DND-189, methoxamine, each agonist (nicotine, ACh or capsaicin), and 5 μM guanethidine or 10 μM capsazepine was applied to the arteries.

To determine the pH-dependent fluorescence, saline containing DND-189 (2.5 μM) at pH 4.5–7.5, which was adjusted with hydrochloric acid (HCl), was injected into the double glass slides in the absence of the artery, and the fluorescence obtained at each pH was measured with a fluorescent microscope. To quantify the changes in fluorescent activity with pH, each image with a green colour was converted to a grey colour and quantified by a computer-assisted image analyser. The grey scale and width of the artery before and 1 min after application of each agent were expressed as a ratio.

Statistical analysis

Experimental results are presented as the mean ± SEM. Statistical analysis was performed using Student's unpaired *t*-test and one-way ANOVA followed by Tukey's test. A $P < 0.05$ was considered significant.

Drugs

Chemicals were from Sigma-Aldrich (Tokyo, Japan) if not stated otherwise: ACh chloride (Daichi Pharmaceutical Co., Tokyo, Japan), guanethidine sulfate (Tokyo Kasei, Tokyo, Japan), noradrenaline hydrochloride (Sankyo-Daiichi, Tokyo, Japan), methoxamine hydrochloride (Nihon Shinyaku Co., Kyoto, Japan), rat CGRP (Peptide Institute, Inc., Osaka, Japan), sodium deoxycholate (Ishizu Seiyaku, Tokyo, Japan). All drugs, except for capsaicin and sodium deoxycholate, were dissolved in pure water and diluted with Krebs solution. Capsaicin was dissolved in 50% ethanol and diluted with Krebs solution (final alcohol concentration, 0.4 mg·mL^{-1}) or MOPS buffer solution. Sodium deoxycholate was dissolved in 0.9% saline.

Results

PNS-induced vascular responses and pH changes

The first series of PNS (S1) at 2–8 Hz caused an initial increase in perfusion pressure due to vasoconstriction, followed by a long-lasting decrease in perfusion pressure due to vasodilatation in the denuded preparation with active tone. Vasoconstrictor and vasodilator responses to PNS were frequency-dependent and reproducible when PNS (S2) was repeated (Figure 1E and F).

PNS (2–8 Hz) caused a decrease in pH levels of the perfusate during vasodilatation in frequency-dependent and reproducible manner, reaching a maximum of 5–10 min after PNS and returning to pre-PNS (except for 8 Hz) levels within 15 min (Figure 1B and C). Basal pH levels in the perfusate before the first and second series of PNS were 7.546 ± 0.064 (n = 6) and 7.668 ± 0.044 (n = 6) respectively.

Effect of cold-storage denervation on PNS-induced vascular responses and pH lowering

PNS (8 and 12 Hz) did not induce vasoconstriction in the denuded preparation with resting tension and cold-storage denervation (Figure 2A). In the same preparation with active tone, PNS (2–8 Hz) caused no vascular response and pH lowering (Figure 2A–D), indicating effective denervation of perivascular nerves. However, a CGRP injection caused vasodilatation (Figure 2A), indicating that the function of vascular smooth muscles was left intact. Basal pH levels in the perfusate before the first series of PNS were 7.741 ± 0.027 (n = 6).

Pharmacological analysis of PNS-induced vascular responses and pH lowering

Effect of guanethidine. The elimination of adrenergic nerves with guanethidine (5 μM) significantly reduced the PNS-induced pH lowering (only at 8 Hz) and adrenergic vasoconstriction (at 4 and 8 Hz), but not CGRPergic vasodilatation (Figure 3A–C). Basal pH levels in the perfusate before the first (control) and second (guanethidine) series of PNS were 7.630 ± 0.030 (n = 7) and 7.583 ± 0.033 (n = 7) respectively.

Effect of capsaicin pretreatment. The pH lowering to PNS (8 Hz) was markedly inhibited in preparations pretreated with capsaicin (5 μM) (Figure 3D), which eliminated CGRPergic vasodilatation. Basal pH levels in the perfusate of control and capsaicin-treated preparation were 7.546 ± 0.064 (n = 6) and 7.599 ± 0.066 (n = 6) respectively. The vasoconstriction to PNS (2–8 Hz) in the capsaicin-pretreated preparation was significantly greater than that in the control; however, PNS-induced vasodilatation was pronouncedly reduced (Figure 3E and F).

Effect of capsazepine. To confirm whether pH lowering and vascular responses to PNS are involved in the activity of TRPV1 receptors, which have been shown to localize in perivascular nerves (Eguchi *et al.*, 2004), we checked the effect of capsazepine. Capsazepine (5 μM) markedly inhibited

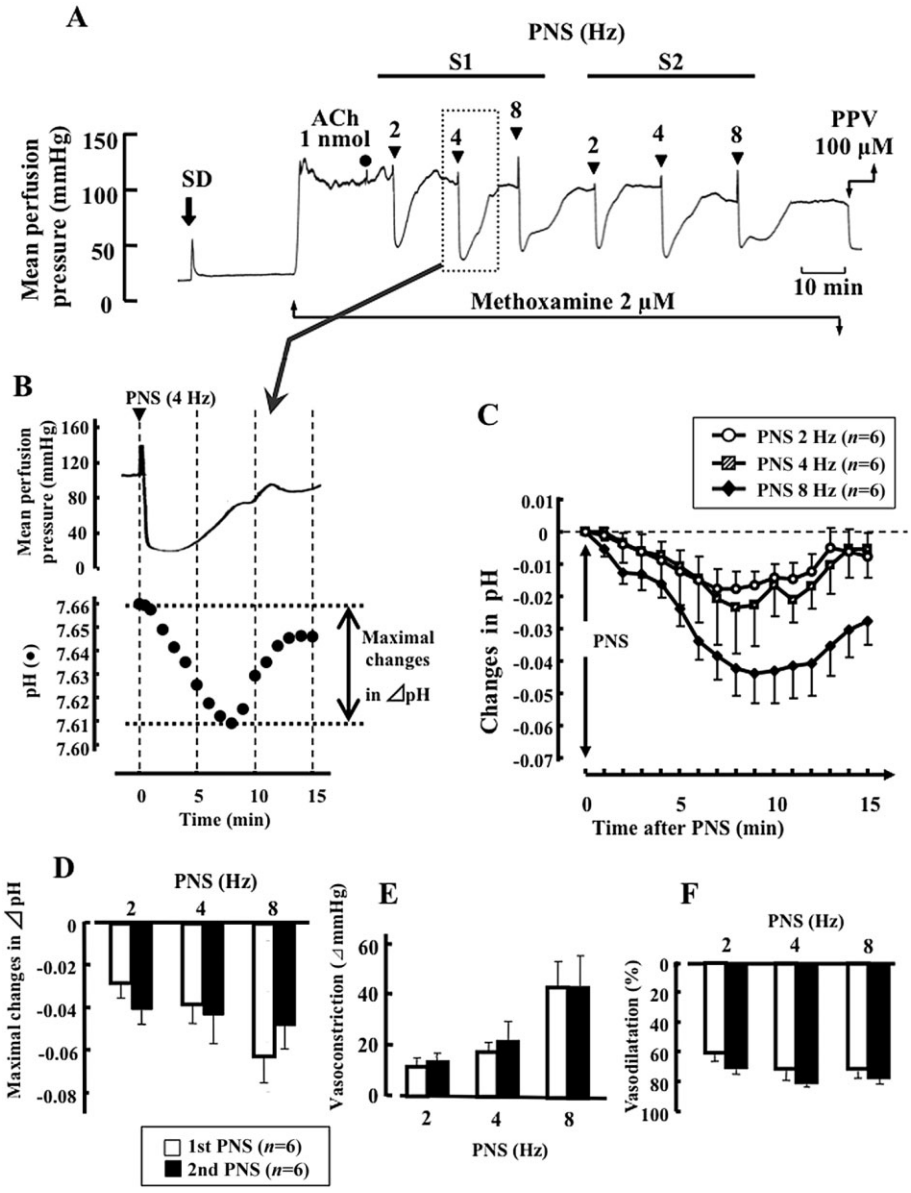

Figure 1

A typical record (A) showing vascular responses (B, E, F) and changes in overflow perfusate pH levels (B, C, D) induced by PNS (closed inverse triangles) in the rat mesenteric vascular bed with active tone and without an endothelium. In (A), PNS (S1 and S2) shows the first and second series respectively. SD, ACh (closed circle) and PPV indicate the perfusion of sodium deoxycholate, a bolus injection of ACh (1 nmol) and papaverine (100 μM) respectively. (B) A typical chart of changes in perfusion pressure and overflow perfusate pH levels (closed circles) after PNS [4 Hz, indicated by a dotted-line square in (A)]. (C) The time-course of changes in overflow perfusate pH levels after PNS at 2, 4 and 8 Hz. Bar graphs in D, E and F show the overflow perfusate pH levels, and the increases (vasoconstriction) and decreases (vasodilatation) in the perfusion pressure induced by the first and second series of PNS respectively.

PNS-induced vascular responses and PNS-induced (4 and 8 Hz) pH lowering (Figure 4A–C). Basal pH levels in the perfusate before the first (control) and second (capsazepine) series of PNS were 7.645 ± 0.048 (n = 6) and 7.665 ± 0.044 (n = 6) respectively.

Effect of ruthenium red. The inhibition of TRPV1 receptors with ruthenium red (30 μM) caused the significant inhibition of PNS-induced (4 and 8 Hz) pH lowering and vascular responses (Figure 4D–F). Basal pH levels in the perfusate before the first (control) and second (ruthenium red) series of PNS were 7.700 ± 0.043 (n = 6) and 7.626 ± 0.040 (n = 6) respectively.

Effect of capsazepine on PNS-induced noradrenaline release

The spontaneous release of noradrenaline was detected in the perfusate of preparations with active tone (40.7 ±

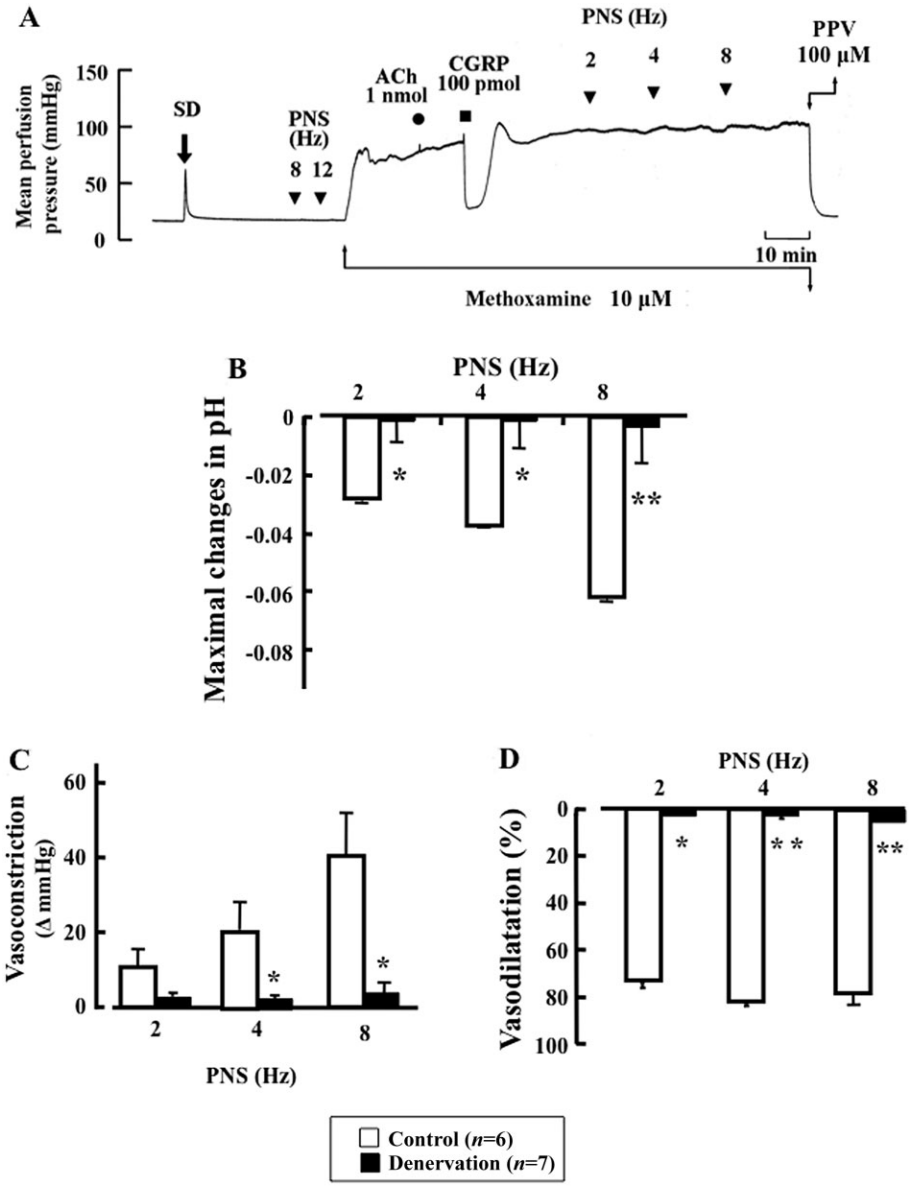

Figure 2

Effect of cold-storage denervation on the decrease in overflow perfusate pH levels and vascular responses induced by PNS (closed inverse triangles) in rat mesenteric vascular beds with active tone and without an endothelium. (A) A typical record of vascular responses in the denervated preparation. In (A), SD, ACh, CGRP and PPV indicate the perfusion of sodium deoxycholate, a bolus injection of ACh (1 nmol), calcitonin gene-related peptide (100 pmol) and the perfusion of papaverine (100 µM) respectively. Bar graphs in (B), (C) and (D) show changes in pH levels, and vasoconstriction and vasodilatation in control and denervated preparations respectively. *$P < 0.05$, **$P < 0.01$ versus control.

15.8 pg·mL^{-1}, $n = 5$). Noradrenaline level after 2 min of PNS at 8 Hz (119.5 ± 4.9 pg·mL^{-1}, $n = 5$; $P < 0.05$) was significantly higher than that at pre-PNS level. Increased noradrenaline levels were observed until 5 min after PNS.

To examine whether TRPV1 receptors may affect the noradrenaline release to PNS, the spontaneous release of noradrenaline was also detected in the perfusate (33.8 ± 8.6 pg·mL^{-1}, $n = 5$) in the presence of capsazepine (5 µM). Capsazepine did not affect the noradrenaline release to PNS (8 Hz) at 2 min after the PNS (145.0 ± 35.1 pg·mL^{-1}, $n = 5$), while noradrenaline release at 5 and 7 min after PNS in capsazepine-treated preparations was significantly smaller than that in the control (Figure 5).

Vasodilatation and pH changes to the ACh injection

Because ACh has been shown to induce an endothelium-independent vasodilatation via direct stimulation of CGRPergic nerves (Takenaga *et al.*, 1995), we examined whether ACh causes pH lowering concomitant with vascular responses. The ACh injection (1 nmol) did not induce vasodilatation in the denuded preparation with active tone (Figure 6A). However,

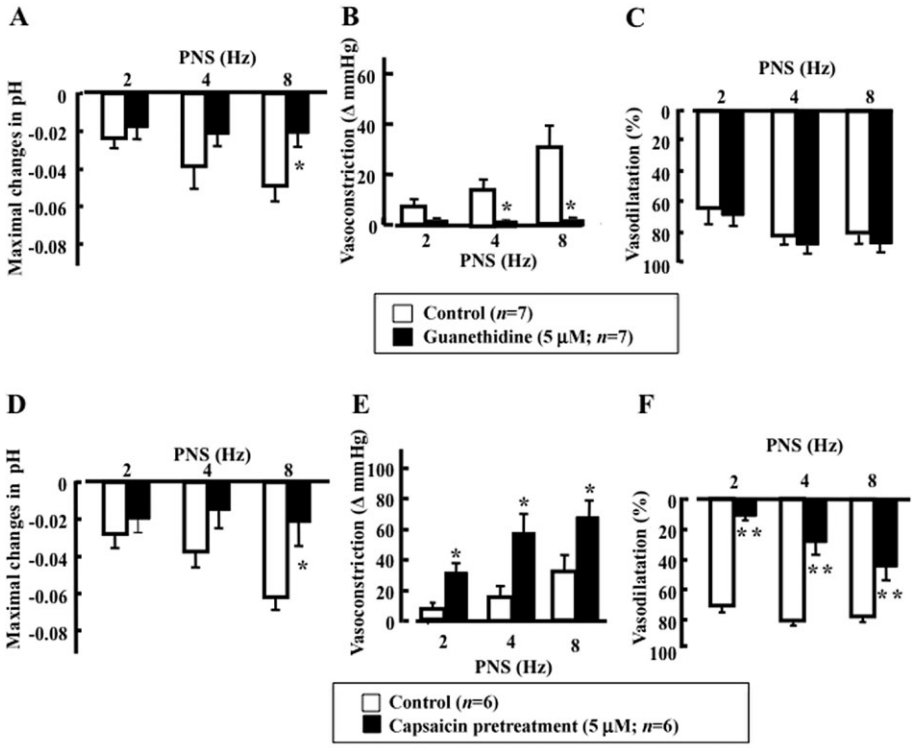

Figure 3

Effects of guanethidine (A, B, C) and capsaicin (D, E, F) on the decrease in overflow perfusate pH levels, vasoconstriction and vasodilatation induced by PNS (2, 4 and 8 Hz) in rat mesenteric vascular beds with active tone and without an endothelium respectively. *$P < 0.05$, **$P < 0.01$ versus control.

ACh (10 and 100 nmol) induced long-lasting vasodilatations with pH lowering in a concentration-dependent manner (Figure 6A and B), indicating that ACh produced an endothelium-independent vasodilatation. The pH changes to the ACh reached a maximum of 7–10 min after the injection and returned to pre-injection pH levels within 15 min. The basal pH level in the perfusate before the injection of ACh was 7.378 ± 0.107 (n = 6).

To determine whether vasodilatation affects pH levels in the perfusate, CGRP was injected. The CGRP injection (50 pmol) induced a long-lasting vasodilatation without a change in pH (Figure 6A and B), indicating that a decrease in perfusion pressure is not responsible for the pH lowering. The basal pH level in the perfusate before an injection of CGRP (50 pmol) was 7.327 ± 0.095 (n = 5).

ACh-induced pH lowering and vasodilatation were abolished by the elimination of CGRPergic nerve function with the capsaicin pretreatment (Figure 6C and D). However, the elimination of adrenergic nerve function with guanethidine (5 μM) had no significant effect on ACh-induced pH lowering and vasodilatation (Figure 6C and D).

The blockade of mACh receptor with atropine, but nACh receptor with mecamylamine, abolished both pH lowering and vasodilatations to the ACh injection at 10 and 100 nmol (Figure 7A and B). However, the combined application of atropine and mecamylamine almost abolished the pH lowering and vasodilator responses to ACh (Figure 7C and D).

The suppression of TRPV1 receptors with ruthenium red did not significantly inhibit pH lowering to the ACh injection, while it caused a significant reduction in ACh-induced vasodilatation (Figure 7A and B). The combined application of atropine and ruthenium red markedly inhibited both the pH lowering and the vasodilator responses to the ACh injection (Figure 7C and D).

Vasodilatation and pH changes to the capsaicin injection

Because capsaicin at low concentrations has been shown to induce an endothelium-independent vasodilatation via activation of CGRPergic nerves through stimulation of TRPV1 receptors, we examined whether capsaicin causes a pH lowering concomitant with vascular responses. Bolus injections of capsaicin (50–200 pmol) produced a concentration-dependent vasodilatation with pH lowering (Figure 8A and B), which reached a maximum of 5–10 min after the injection and returned to pre-injection pH levels within 15 min. The basal pH level of the perfusate before the capsaicin injection was 7.375 ± 0.111 (n = 5).

Capsaicin-induced vasodilatation and pH lowering were almost abolished with ruthenium red (10 μM) treatment (Figure 8C and D), indicating that the responses were mediated by TRPV1 receptors on CGRPergic nerve. The basal pH level in the perfusate before injection of capsaicin in the presence of ruthenium red was 7.375 ± 0.111 (n = 5).

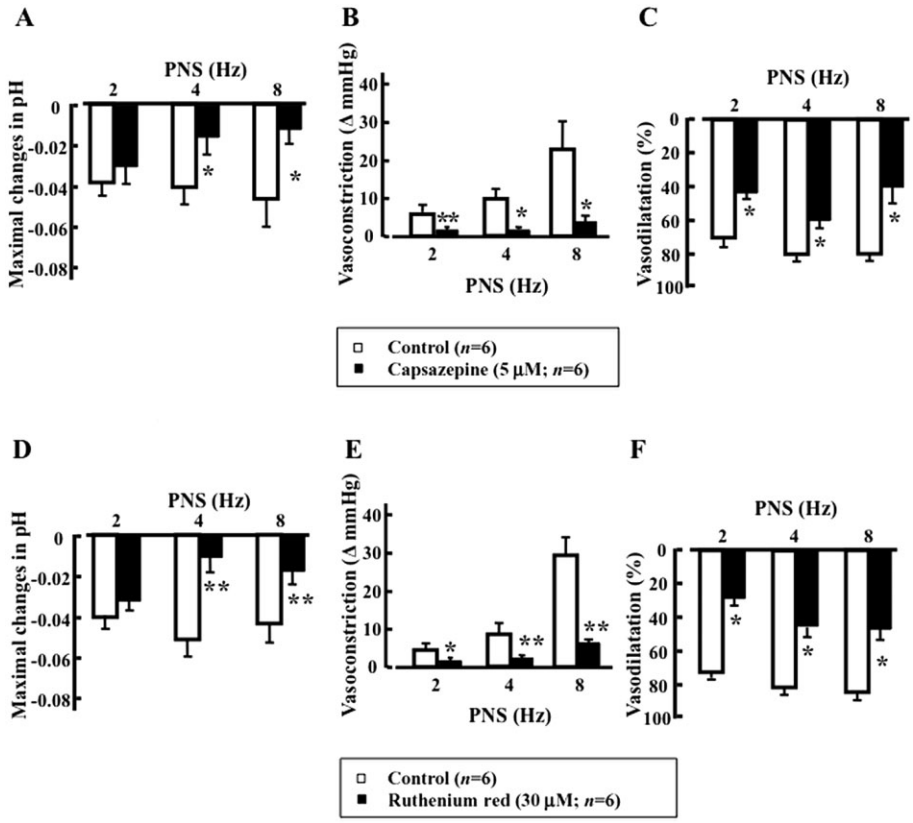

Figure 4

Effects of capsazepine (A, B, C) and ruthenium red (D, E, F) on the decrease in overflow perfusate pH levels, vasoconstriction and vasodilatation induced by PNS (2, 4 and 8 Hz) in rat mesenteric vascular beds with active tone and without an endothelium respectively. *P < 0.05, **P < 0.01 versus control.

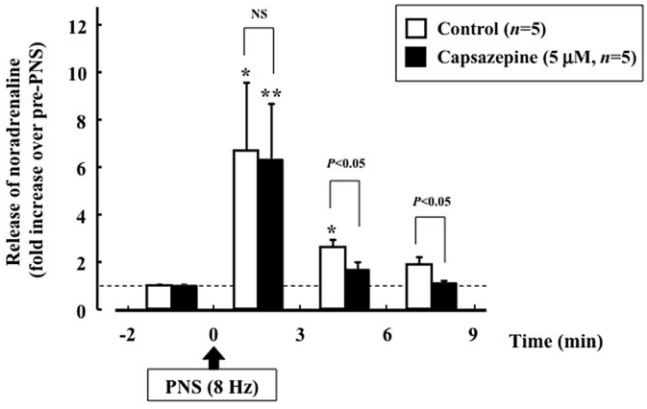

Figure 5

Effect of capsazepine (5 μM) on the release of noradrenaline induced by PNS (8 Hz) in rat mesenteric vascular beds with active tone and without an endothelium. The perfusate was collected every 2 min and PNS (8 Hz) was applied at time 0. *P < 0.05, **P < 0.01 versus pre-PNS values. NS, not significant.

Changes in pH-dependent fluorescence and vasodilatation to nicotine, ACh and capsaicin in small mesenteric arteries

To examine whether protons are released outside the vessel, the pH levels of the vessel were measured using pH-dependent fluorescence. The application of nicotine (nACh receptor agonist) (Figure 9F), ACh (Figure 9G) or capsaicin (Figure 9H), but not saline (Figure 9E), increased the width of arteries due to vasodilatation in denuded mesenteric arteries with active tone. The ratios of vasodilatation to saline (control), nicotine, ACh and capsaicin were 1.057 ± 0.041 (n = 4), 1. 375 ± 0.085 (n = 4; P < 0.05 vs. control), 1.318 ± 0.057 (n = 4; P < 0.05 vs. control) and 1.467 ± 0.118 (n = 4; P < 0.05 vs. control) respectively. Additionally, the application of each agent caused an increase in fluorescence due to lowered pH levels (Figures 9 and 10). However, the blockade of exocytosis in the Ca^{2+}-free medium caused no change in fluorescence of all groups (Figures 9I–K and 10).

Nicotine-induced pH lowering was markedly inhibited by the Ca^{2+}-free medium (82%), guanethidine (80%) and capsaicin (50%) (Figure 10A). Nicotine-induced vasodilatation was also inhibited in all groups (ratio: nicotine alone, 1.376 ± 0.085 (n = 4); nicotine + guanethidine, 1.089 ± 0.045, P < 0.05

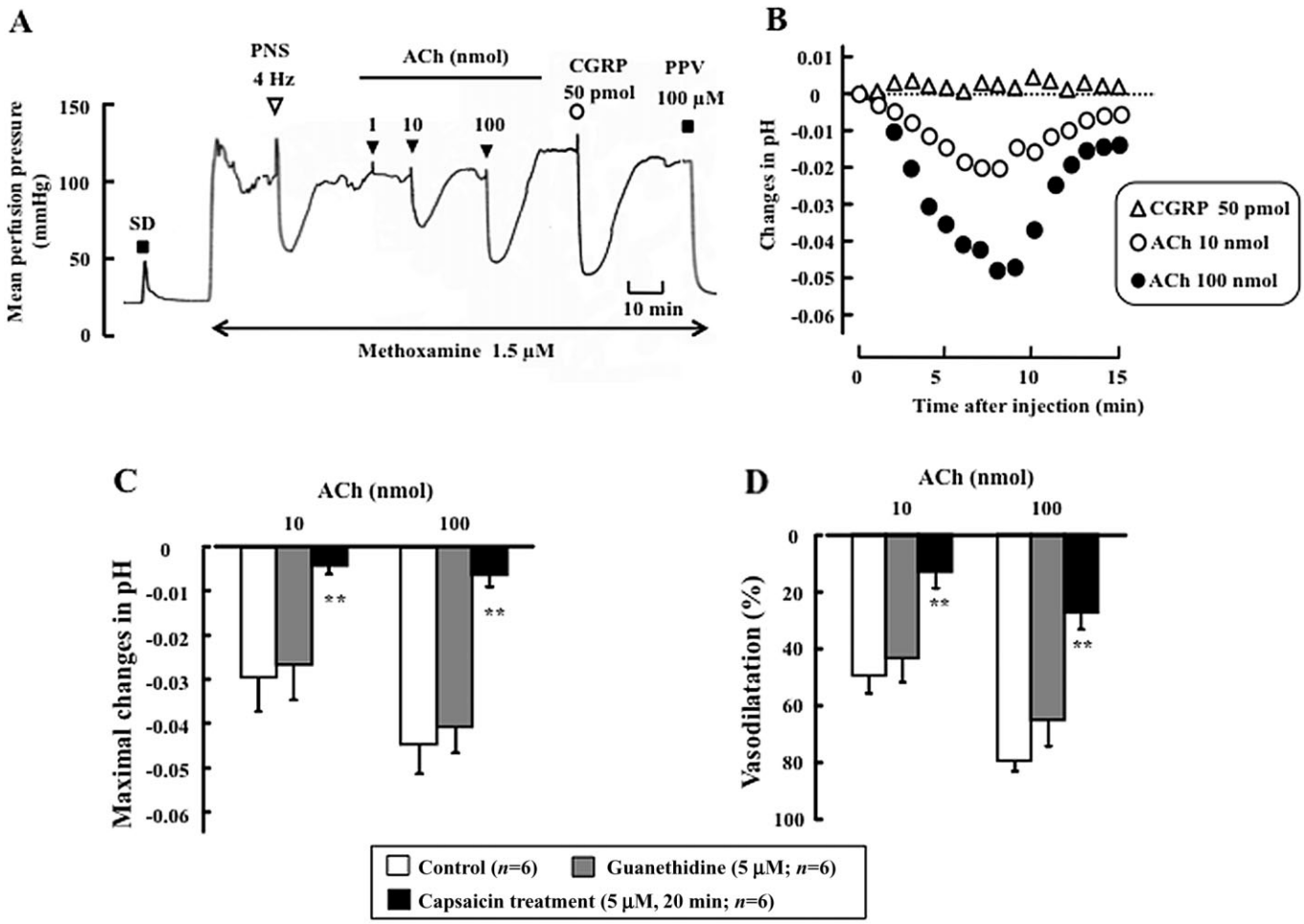

Figure 6

A typical record of vascular responses (A) and time-course changes in pH levels in the perfusate (B) induced by bolus injections of ACh (1, 10 and 100 nmol) and CGRP (50 pmol), and bar graphs (C, D) showing the effects of guanethidine and capsaicin pretreatment on ACh-induced pH lowering and vasodilatation in rat mesenteric vascular beds without an endothelium. In (A), SD, ACh, PNS, CGRP and PPV indicate the perfusion of sodium deoxycholate, PNS (4 Hz), bolus injections of ACh and CGRP, and the perfusion of papaverine (100 μM) respectively. In (C) and (D), each bar indicates the means ± SEM. $^{**}P < 0.01$ versus control.

vs. nicotine alone, $n = 4$; nicotine + capsaicin, 1.130 ± 0.021, $P < 0.05$ vs. nicotine alone, $n = 4$; nicotine + Ca^{2+} free, 1.122 ± 0. 050, $P < 0.01$ vs. nicotine alone, $n = 4$).

ACh-induced pH lowering was almost abolished by the capsaicin pretreatment and Ca^{2+}-free medium; however, the inhibitory effect of guanethidine was weak (20%) (Figure 10B). ACh-induced vasodilatation was also inhibited in all groups (ratio: ACh alone, 1.318 ± 0.048 ($n = 4$); ACh + guanethidine, 1.259 ± 0.022, $P < 0.05$ vs. ACh alone, $n = 4$; ACh + capsaicin, 1.083 ± 0.017, $P < 0.01$ vs. ACh alone, $n = 4$; ACh + Ca^{2+} free, 1.063 ± 0.030, $P < 0.01$ vs. ACh alone, $n = 4$).

Capsaicin application-induced (5 μM) pH lowering mediated by the stimulation of TRPV1 receptors localized in CGRPergic nerve was markedly inhibited by the capsaicin (10 μM) pretreatment (77%), capsazepine (75%) and the Ca^{2+}-free medium (72%). Capsaicin-induced vasodilatation was also inhibited in all groups (ratio: capsaicin alone, 1.467 ± 0.118, $n = 4$; capsaicin + capsazepine, 1.116 ± 0.027, $P < 0.01$ vs. capsaicin alone, $n = 4$; capsaicin + capsaicin (high), 1.103 ± 0.033, $P < 0.01$ vs. capsaicin alone, $n = 4$; capsaicin + Ca^{2+} free, 1.160 ± 0. 017, $P < 0.01$ vs. capsaicin alone, $n = 4$).

Discussion and conclusion

The present study demonstrated that PNS of endothelium-denuded rat mesenteric arteries with active tone produces adrenergic vasoconstriction followed by CGRPergic vasodilatation and a decreased pH in the perfusate. Cold-storage denervation abolished the vascular responses and reduction in pH induced by PNS indicating that these responses are neurogenic in nature. In addition, the perivascular neurons appeared to be the source of protons, which lower the pH levels in the perfusate. The present findings support those found previously that showed adrenergic nerve stimulation via activation of nACh receptors by nicotine releases protons to activate TRPV1 receptors on CGRPergic nerves, leading to vasodilatation (Kawasaki *et al.*, 2009; 2011). Furthermore, in

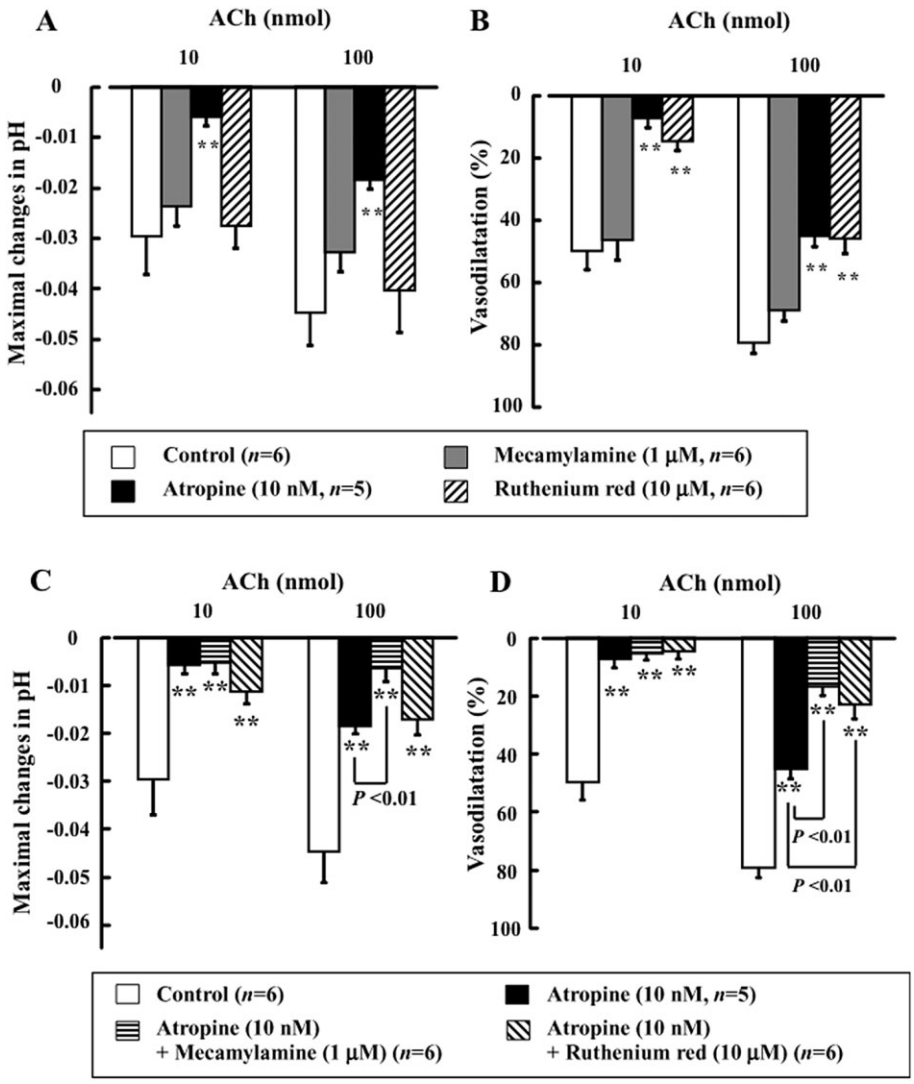

Figure 7

Effects of mecamylamine, atropine, ruthenium red, and the combination of atropine and mecamylamine or ruthenium red on ACh-induced maximal changes in pH levels (A, C) and vasodilatation (B, D) in rat perfused mesenteric vascular beds without an endothelium and with active tone produced by methoxamine. Data indicate the mean ± SEM. $^{**}P < 0.01$ versus control.

the present study it was demonstrated that guanethidine markedly inhibits the ability of PNS to lower pH at 8 Hz, but not at 4 Hz, and blocks adrenergic-mediated vasoconstriction at 4 and 8 Hz without affecting CGRPergic vasodilatation. We previously reported that guanethidine abolishes the reduction in pH induced by adrenergic nerve stimulation via nACh receptor activation by nicotine (Kawasaki *et al.*, 2009). Taken together, these results indicate that it is likely PNS-induced pH lowering in the presence of guanethidine results from protons being released from CGRPergic nerves. Indeed, capsaicin pretreatment abolished CGRPergic vasodilatation and pH lowering to PNS (8 Hz), supporting the hypothesis that CGRPergic nerves release protons and contribute to the reduction in pH induced by PNS.

Capsazepine and ruthenium red significantly inhibited pH lowering (at 4 and 8 Hz) and adrenergic vasoconstriction (at 2–8 Hz). Because capsazepine had no suppressive action on neurogenic noradrenaline release, it is assumed that the inhibitory effects of capsazepine and ruthenium red on vasoconstriction to PNS may result from postsynaptic inhibition due to non-specific actions or TRPV1 receptor antagonism. Additionally, the fact that capsazepine and ruthenium red inhibited CGRPergic vasodilatation and pH lowering (at 4 and 8 Hz) suggests that TRPV1 receptor inhibitors block the actions of protons released from adrenergic nerves at the site of CGRPergic nerves, resulting in inhibition of CGRPergic-mediated vasodilatation and proton release from CGRPergic nerves.

The present and previous studies demonstrated that ACh directly stimulates mACh receptors in CGRPergic nerves to induce endothelium-independent vasodilatation (Takenaga *et al.*, 1995). Interestingly, in the present study it was found that ACh reduced the pH in the perfusate, and this effect was abolished by the mACh receptor antagonist atropine or cap-

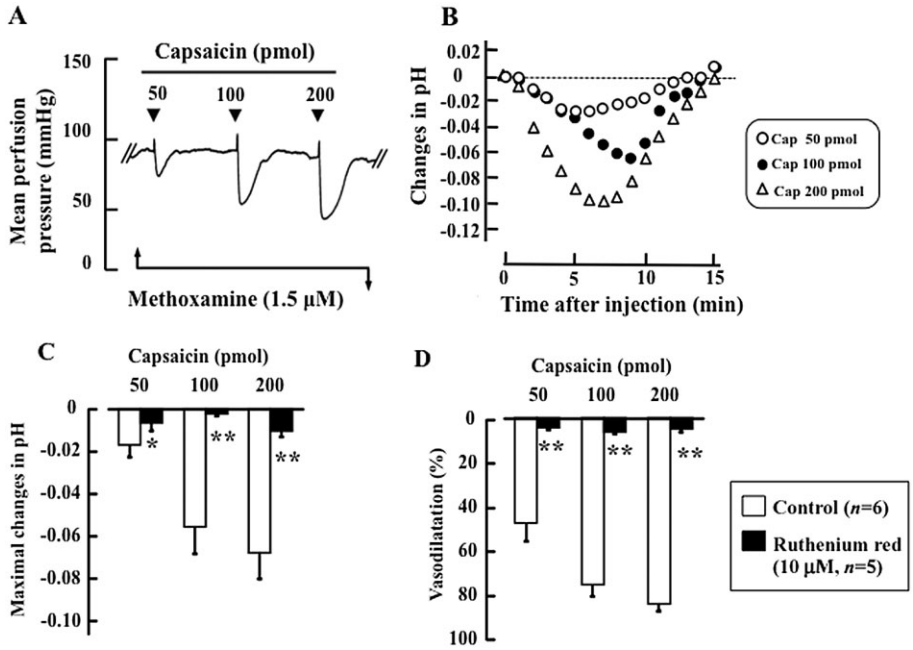

Figure 8

A typical record of vascular responses (A) and time-course changes in pH levels in the perfusate (B) induced by bolus injections of capsaicin (Cap: 50, 100 and 200 pmol), and bar graphs (C, D) showing the effects of ruthenium red on capsaicin-induced pH lowering and vasodilatation in rat mesenteric vascular beds without an endothelium respectively. In (C) and (D), each bar indicates the mean ± SEM. *$P < 0.05$, **$P < 0.01$ versus control.

saicin, which eliminates the function of CGRPergic neurones. Because the exogenous CGRP injection caused vasodilatation, but not pH lowering (Figure 6B), it is very likely that protons and/or acidic substances, which lower pH levels and are stored in synaptic vesicle and/or cytoplasm, might be neurogenic in origin and are released from CGRPergic nerves upon stimulation of mACh receptors. This is also supported by the present results that showed the TRPV1 receptor agonist capsaicin induces pH lowering concomitant with vasodilatation, and these actions were abolished by ruthenium red (Figure 8). Di Giovanni *et al*. (2010) have reported that V-ATPase, which is omnipresent in intracellular membrane compartments, including synaptic vesicles (Stevens and Forgac, 1997), is involved in regulating the acidity of synaptic vesicles and loading with neurotransmitter. However, it is unclear whether V-ATPase is involved in the pH lowering effect of PNS.

ACh has been reported to induce adrenergic nerve-mediated endothelium-independent vasodilatation via the activation of nACh receptora on adrenergic nerves (Shiraki *et al*., 2000). Eguchi *et al*. (2007) reported that nACh receptor agonists initially stimulate $\alpha_3\beta_4$ nACh receptor subtypes, which are located on adrenergic nerves, to release adrenergic neurotransmitter(s) and related substances. These released substances activate TRPV1 receptors located on adjacent CGRP nerves and then release CGRP, causing vasodilatation. We showed that protons, which are an endogenous agonist for TRPV1 receptors (Van der Stelt and Di Marzo, 2004), were released from adrenergic nerves via the activation of nACh receptors (Kawasaki *et al*., 2009). In the present study, atropine partially inhibited the pH lowering induced by a high concentration (100 nmol) of ACh, and the remaining effect of ACh was abolished when the nACh receptor antagonist mecamylamine was combined with atropine (Figure 7C). These findings support the previous reports that protons are released from adrenergic nerves via the activation of adrenergic nACh receptor.

Ruthenium red did not inhibit ACh-induced pH lowering (Figure 7A), but it significantly inhibited ACh-induced vasodilatation (Figure 7B). This is due to the blocking action of ruthenium red to bind protons to TRPV1 receptors at the site of CGRPergic nerves (Kawasaki *et al*., 2011). Therefore, unbound protons in the presence of ruthenium red may overflow and result in no change in pH levels. Additionally, the fact that ruthenium red blunts the vasodilatation mediated by CGRPergic nerves may be due to the blockade of TRPV1 receptor activation by protons.

Ruthenium red almost abolished atropine-sensitive vasodilatation to ACh at 10 nmol (Figure 7B). Because ruthenium red had no inhibitory effect on pH lowering, which differed from atropine, it appears that ruthenium red exhibits no antagonistic effect on mACh receptors. Therefore, it is assumed that the potent inhibitory effect of ruthenium red on ACh-induced vasodilatation may result from the blockade of proton-induced TRPV1 receptor activation. This implies that the proton itself facilitates neurotransmission in CGRPergic nerves via the activation of presynaptic TRPV1 receptors in a positive feedback mechanism.

Protons stimulate TRPV1 receptors to activate primary sensory nerves as an endogenous agonist (Tominaga

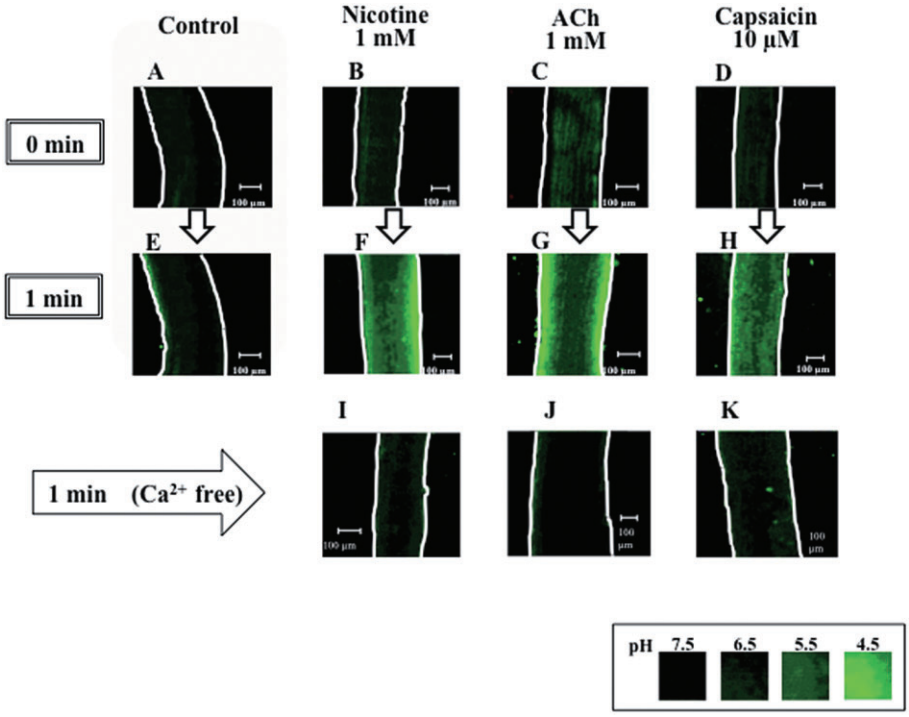

Figure 9

Typical images showing time-course changes in pH-dependent fluorescence before and 1 min after the application of control (saline) (A, E), nicotine (1 mM) (B, F), ACh (1 mM) (C, G) and capsaicin (10 μM) (D, H), and the effect of Ca^{2+}-free medium on the action of nicotine (I), ACh (J) and capsaicin (K) using LysoSensor Green DND-189 in rat mesenteric arteries without an endothelium and with active tone produced by methoxamine. The horizontal bars indicate 100 μm. Lower images show pH-dependent fluorescence changes after application of the HCl-saline solution using LysoSensor Green DND-189.

and Tominaga, 2005). Because exogenous HCl in the denuded preparation produced endothelium-independent and capsaicin-sensitive CGRPergic vasodilatation via activation of TRPV1 receptors (Eguchi *et al.*, 2004; 2007; Kawasaki *et al.*, 2009), protons are likely to mediate PNS-induced vasodilatation and act as a transmitter from adrenergic nerves to CGRPergic nerves. It is also suggested that protons are a neuromodulator that facilitates CGRPergic neurotransmission via a positive feedback mechanism.

Previous immunohistochemical studies demonstrated that TH-, NPY- and CGRP-immunopositive nerve fibres are densely distributed in the adventitia layer of rat mesenteric arteries (Hobara *et al.*, 2004; Eguchi *et al.*, 2007; Kawasaki *et al.*, 2009). Additionally, double immunostainings revealed that both TH- and NPY-LI (like immunoreactivity) and CGRP-LI nerve fibres in rat mesenteric arteries have coalescence sites, suggesting that there are close contact areas, probably at the synapse, between adrenergic and CGRPergic nerves to interact at these areas (Eguchi *et al.*, 2004; Kawasaki *et al.*, 2009). Furthermore, previous studies with double immunostainings of the mesenteric artery showed the dense innervation of CGRP-LI and TRPV1-LI nerves, appearing in the same neuron (Eguchi *et al.*, 2004; 2007). Therefore, these results strongly suggest that CGRPergic nerves innervating mesenteric arteries have TRPV1 receptors.

The pH changes measured in the perfusate were less than 0.05 units. We could not detect the direct pH level around the artery when examining the perfused mesenteric vascular beds, because Krebs solution acted as a buffer to the acid. To overcome this problem and confirm that protons are released from perivascular adrenergic and CGRPergic nerves, we measured the pH changes around the artery using a fluorescent pH indicator, which is sensitive to low pH levels. The results showed that direct stimulation of adrenergic nerves by nicotine and CGRPergic nerves by ACh or capsaicin (low concentration) in the preconstricted artery causes an increase in green fluorescence around the artery, which indicates pH lowering due to the release of protons and vasodilatation. Furthermore, the nicotine-, ACh- and capsaicin-induced fluorescent increase, as well as vasodilatation, did not occur in calcium-free medium. Therefore, it is very likely that adrenergic and CGRPergic nerve stimulation elicits proton release, which is calcium-dependent, from adrenergic nerves and CGRPergic nerves, resulting in pH lowering around the artery. Because the exocytosis process is dependent on calcium, it appears that protons are released into extracellular spaces from perivascular nerves. Furthermore, the pharmacological analysis of pH lowering around the small arteries in response to nicotine, ACh and capsaicin showed that the results were similar to pH lowering in the perfusate in mesenteric vascular beds, confirming that protons are released from perivascular adrenergic and CGRPergic nerves. Additionally, the adrenergic neuron blocker guanethidine abolished the nicotine-induced pH lowering in the artery and

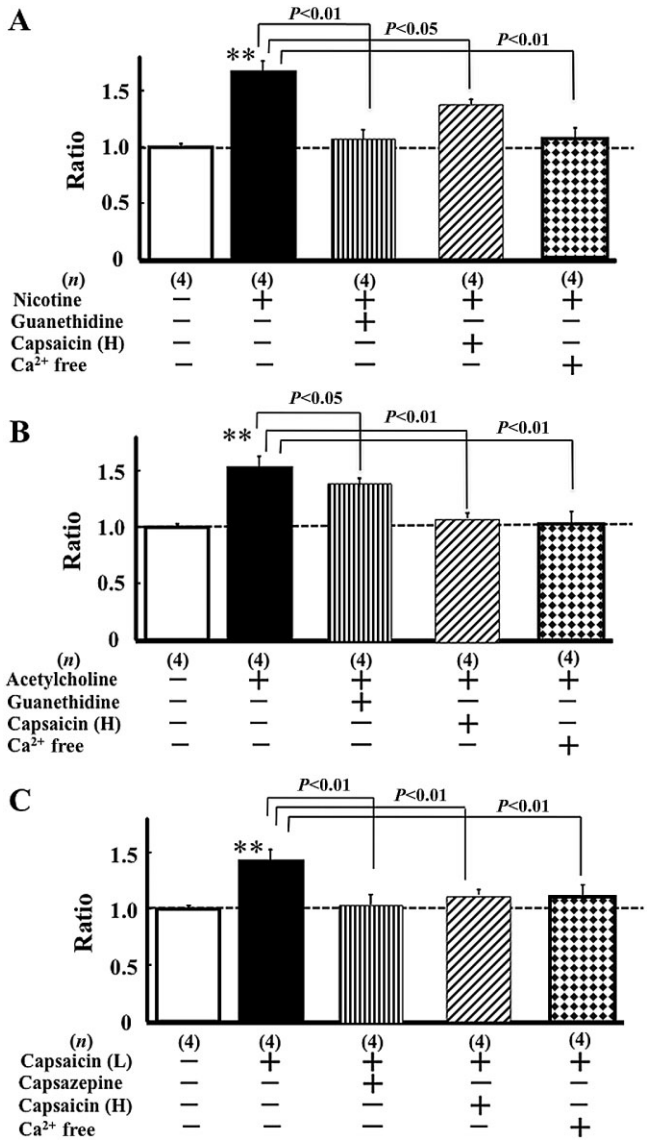

Figure 10

Effects of various inhibitors (guanethidine and capsazepine) and treatments (capsaicin and the Ca^{2+}-free medium) on the pH lowering (an increase in pH-dependent fluorescence) induced by nicotine (A), ACh (B) and capsaicin (C) using LysoSensor Green DND-189 in rat mesenteric arteries without an endothelium with active tone produced by methoxamine. Capsaicin (H) indicates the pretreatment with a high concentration (10 μM). Each bar indicates the mean ± SEM of five experiments. $^{**}P < 0.01$ versus control.

vasodilatation involving CGRPergic nerves. Thus, it appears that the released protons modulate perivascular CGRPergic nerve activity.

Noradrenaline stored in vesicles in the adrenergic nerve terminal is released into the synapse cleft through an exocytosis process in a calcium-dependent manner. These vesicles transport noradrenaline from the cytoplasm using energy provided by the proton gradient, causing protons to be more concentrated inside the vesicle where pH levels are low at 5.5 (Wu *et al.*, 2001). This implies that vesicles in adrenergic nerve terminals contain protons. It is assumed that vesicles in CGRPergic nerve terminals possess similar mechanisms to those in adrenergic nerve terminals. Therefore, it seems likely that protons accompanied by the release of the transmitter noradrenaline or CGRP could be released from adrenergic nerves and CGRPergic nerves when exocytosis occurs. This hypothesis is supported by the results of the present study, which showed that PNS of perfused mesenteric arteries results in a decrease in pH levels in the perfusate concomitant with an initial adrenergic vasoconstriction, followed by CGRPergic vasodilatation, and direct stimulation of adrenergic and CGRPergic nerves induces a calcium-dependent pH lowering around the artery.

In conclusion, the findings of the present study suggest that perivascular adrenergic and CGRPergic nerves innervating rat mesenteric resistance arteries release protons via an exocytosis process in a calcium-dependent manner. It is also proposed that these perivascular nerves have axo-axonal interactions through protons that modulate perivascular nerve function and contribute to the regulation of vascular tone. We hypothesize that the activation of adrenergic nerves could release protons together with the adrenergic neurotransmitter noradrenaline via exocytosis, and the released protons then stimulate TRPV1 receptors on neighbouring CGRPergic nerves, resulting in CGRP-mediated vasodilatation. By this mechanism, efferent adrenergic nerves, which maintain vascular tone through the transmitter noradrenaline, may send their signals via protons to neighbouring CGRPergic nerves. These signal protons may be used as a transmitter for axo-axonal transmission to counteract excess adrenergic vasoconstriction as the efferent function of the afferent sensory nerves, CGRPergic nerves. Additionally, it is also hypothesized that the activation of CGRPergic nerves releases protons together with the CGRPergic nerve transmitter CGRP via exocytosis, and the released protons then stimulate presynaptic TRPV1 receptors on CGRPergic nerves to facilitate neurotransmission in CGRPergic nerves via a positive feedback mechanism, resulting in CGRP-mediated vasodilatation.

Acknowledgements

This work was supported by a grant from the Smoking Research Foundation and in part by a Grant-in-Aid for Scientific Research (KAKENHI) from the Ministry of Education, Science and Technology of Japan (Nos. 13672389 and 16390157).

Author contributions

K. H., S. O. and S. F-M. performed the research. N. O. and H. K. designed the research study. S. T., P. T., M. G. and N. H-H. contributed to discussion and technical guidance or assistance. K. H. and S. O. analysed the data. S. T. and H. K. wrote and reviewed the paper.

Conflict of interest

None.

References

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M *et al.* (2013a). The Concise Guide to PHARMACOLOGY 2013/14: G protein-coupled receptors. Br J Pharmacol 170: 1459–1581.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M *et al.* (2013b). The Concise Guide to PHARMACOLOGY 2013/14: Ligand-gated ion channels. Br J Pharmacol 170: 1582–1606.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M *et al.* (2013c). The Concise Guide to PHARMACOLOGY 2013/14: Ion channels. Br J Pharmacol 170: 1607–1651.

Di Giovanni J, Boudkkazi S, Mochida S, Bialowas A, Samari N, Lévêque C *et al.* (2010). V-ATPase membrane sector associates with synaptobrevin to modulate neurotransmitter release. Neuron 67: 268–279.

Eguchi S, Tezuka S, Hobara N, Akiyama S, Kurosaki Y, Kawasaki H (2004). Vanilloid receptors mediate adrenergic nerve- and CGRP-containing nerve-dependent vasodilation induced by nicotine in rat mesenteric resistance arteries. Br J Pharmacol 142: 1137–1146.

Eguchi S, Miyashita S, Kitamura Y, Kawasaki H (2007). α3β4 nicotinic receptors mediate adrenergic nerve- and CGRPergic nerve-dependent vasodilation induced by nicotine in rat mesenteric arteries. Br J Pharmacol 151: 1216–1223.

Hatanaka Y, Hobara N, Honghua J, Akiyama S, Nawa H, Kobayashi Y *et al.* (2006). Neuronal nitric oxide synthase (nNOS) inhibition facilitates adrenergic neurotransmission in rat mesenteric resistance arteries. J Pharmacol Exp Ther 316: 490–497.

Hobara N, Nakamura A, Ohtsuka A, Narasaki M, Shibata K, Gomita Y *et al.* (2004). Distribution of adrenomedullin in perivascular nerves of the rat mesenteric artery. Peptides 25: 589–599.

Kawasaki H, Takasaki K, Saito A, Goto K (1988). Calcitonin gene-related peptide acts as a novel vasodilator neurotransmitter in mesenteric resistance vessels of the rat. Nature 335: 164–167.

Kawasaki H, Nuki C, Saito A, Takasaki K (1990a). Role of calcitonin gene-related peptide-containing nerves in the vascular adrenergic neurotransmission. J Pharmacol Exp Ther 252: 403–409.

Kawasaki H, Nuki C, Saito A, Takasaki K (1990b). Adrenergic modulation of calcitonin gene-related peptide (CGRP)-containing nerve-mediated vasodilation in the rat mesenteric resistance vessel. Brain Res 506: 287–290.

Kawasaki H, Nuki C, Saito A, Takasaki K (1991). NPY modulates neurotransmission of CGRP-containing vasodilator nerves in rat mesenteric arteries. Am J Physiol 261: H683–H690.

Kawasaki H, Eguchi S, Miyashita S, Chan S, Hirai K, Hobara N *et al.* (2009). Proton acts as a neurotransmitter for nicotine-induced adrenergic and CGRPergic nerve-mediated vasodilation in the rat mesenteric artery. J Pharmacol Exp Ther 330: 745–755.

Kawasaki H, Takatori S, Zamami Y, Koyama T, Goda M, Hirai K *et al.* (2011). Paracrine control of mesenteric perivascular axo-axonal interaction. Acta Physiologic 203: 3–11.

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). Animal research: reporting *in vivo* experiments: the ARRIVE guidelines. Br J Pharmacol 160: 1577–1579.

McGrath J, Drummond G, McLachlan E, Kilkenny C, Wainwright C (2010). Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol 160: 1573–1576.

McGregor DD (1965). The effect of sympathetic nerve stimulation of vasoconstrictor responses in perfused mesenteric blood vessels of the rat. J Physiol 177: 21–30.

Nuki C, Kawasaki H, Takasaki K, Wada A (1994). Pharmacological characterization of presynaptic calcitonin gene-related peptide (CGRP) receptors on CGRP-containing vasodilator nerves in rat mesenteric resistance vessels. J Pharmacol Exp Ther 268: 59–64.

Pawson AJ, Sharman JL, Benson HE, Faccenda E, Alexander SP, Buneman OP *et al.*; NC-IUPHAR (2014). The IUPHAR/BPS Guide to PHARMACOLOGY: an expert-driven knowledgebase of drug targets and their ligands. Nucl Acids Res 42 (Database Issue): D1098–106.

Shiraki H, Kawasaki H, Tezuka S, Nakatsuma A, Kurosaki Y (2000). Endogenous calcitonin gene-related peptide (CGRP) mediates adrenergic-dependent vasodilation induced by nicotine in mesenteric resistance arteries of the rat. Br J Pharmacol 130: 1083–1091.

Stevens TH, Forgac M (1997). Structure, function and regulation of the vacuolar (H^+)-ATPase. Annu Rev Cell Dev Biol 13: 779–808.

Takenaga M, Kawasaki H, Wada A, Eto T (1995). Calcitonin gene-related peptide mediates acetylcholine-induced endothelium-independent vasodilation in mesenteric resistance blood vessels of the rat. Circ Res 76: 935–941.

Tominaga M, Tominaga T (2005). Structure and function of TRPV1. Pflugers Arch 451: 143–150.

Van der Stelt M, Di Marzo V (2004). Endovanilloids: putative endogenous ligands of transient receptor potential vanilloid 1 channels. Eur J Biochem 271: 1827–1834.

Wu MM, Grabe M, Adams S, Tsien RY, Moore H-PH, Machen TE (2001). Mechanisms of pH regulation in the regulated secretory pathway. J Biol Chem 276: 33027–33035.