ELSEVIER

Available online at www.sciencedirect.com
SCIENCE DIRECT®

European Journal of Pharmacology 461 (2003) 129–137

ejp

www.elsevier.com/locate/ejphar

An endothelium-derived factor modulates purinergic neurotransmission to mesenteric arterial smooth muscle of hamster

Sharada Thapaliya, Hayato Matsuyama, AbuBakr El-Mahmoudy, Yasutake Shimizu, Tadashi Takewaki*

Department of Pathogenetic Veterinary Science, The United Graduate School, Gifu University, Yanagido 1-1, Gifu 501-1193, Japan

Received 18 April 2002; received in revised form 4 January 2003; accepted 7 January 2003

Abstract

The interaction between the endothelium and purinergic perivascular nerves was investigated by measuring the changes in amplitude of excitatory junction potential (EJP) of smooth muscle cells in hamster mesenteric arteries (100–350 μm). Uridin-5′-triphosphate (UTP) (100 μM) applied to endothelium-intact preparations evoked a hyperpolarization of 17.0 ± 0.7 mV ($n = 46$). During this hyperpolarization, the amplitude of electrically evoked EJPs was inhibited to about 50% of that of the control. In endothelium-denuded preparations, UTP (100 μM) neither hyperpolarized the smooth muscle nor inhibited the amplitude of the EJP. Neither a nitric oxide (NO) synthase inhibitor, *N*ω-nitro-L-arginine methyl ester (L-NAME) (100 μM), nor a cyclooxygenase inhibitor, indomethacin (1 μM), had an effect on the UTP-evoked hyperpolarization and inhibition of the electrically evoked EJP. The UTP-evoked membrane hyperpolarization and inhibition of the EJP amplitude was antagonized by the P2Y receptor antagonist, cibacron blue (100 μM). Endothelium-derived hyperpolarizing factor (EDHF)-mediated hyperpolarization was inhibited by either adventitial or intimal application of apamin (0.1 μM) and charybdotoxin (0.1 μM). However, the EJP inhibition was still present. In apamin- and charybdotoxin-treated preparations, focal application of adenosine 5′-triphosphate (ATP) (10 mM) evoked a depolarization of 15.5 ± 1.3 mV ($n = 15$). This postjunctional response was not modified by UTP (15.3 ± 1.7 mV, $n = 4$, $P > 0.05$). These results suggest that exogenously applied UTP activates P2Y receptors of endothelium to release endothelium-derived factors, which in turn inhibit ATP release from purinergic nerves.
© 2003 Elsevier Science B.V. All rights reserved.

Keywords: Purinergic inhibitory neuromodulation; Endothelium-derived factor; Mesenteric artery; (Hamster)

1. Introduction

Adenosine 5′-triphosphate (ATP) is of increasing interest as a neurotransmitter in a number of different blood vessels (Burnstock, 1990), including rabbit saphenous and mesenteric arteries (Burnstock, 1990), guinea-pig mesenteric artery (Onaka et al., 1997), artery of guinea-pig choroid (Hashitani et al., 1998) and hamster mesenteric artery (Thapaliya et al., 1999). ATP acting on P2X ligand-gated cation channels on smooth muscle cells evokes depolarization, as excitatory junction potentials (EJPs), subsequently leading to vasoconstriction. The endothelium, on the other hand, is well recognized as a releaser of several vasoactive substances, whose primary site of action is vascular smooth muscle leading to potent vasodilatation. In addition, we have proposed that ATP released from the perivascular nerves may reach the endothelium and induce an endothelium-hyperpolarizing factor in thin arteries of the hamster (Thapaliya et al., 1999). Beside their potent direct vasodilator actions, endothelial factors may exert an indirect control of vascular tone via modulation of purinergic neurotransmission. However, a literature search yielded no observation on the effect of endothelial-derived factors (EDF) on purinergic neuromodulation.

EDF is released by activation of endothelial P2Y receptors (Malmsjö et al., 1999). We have also reported that hamster mesenteric arterial endothelial cells contain $P2Y_2$-like receptors and that EDF is released upon activation of these receptors (Thapaliya et al., 1999). We have used UTP as a $P2Y_2$-like receptor agonist to stimulate the endothelium, as UTP has been reported to have no direct

* Corresponding author. Tel.: +81-58-293-2997; fax: +81-58-293-2992.
E-mail address: tt@cc.gifu-u.ac.jp (T. Takewaki).

0014-2999/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0014-2999(03)01291-3

effect on uptake and release of neurotransmitter at sympathetic perivascular nerves in rabbit ear artery (Saiag et al., 1998).

Thus, the aim of the present study was to investigate if exogenously applied UTP-released EDF could modulate purinergic neurotransmission as judged from the amplitude of electrically evoked EJPs (Brock and Cunnane, 1999). The effect of exogenously applied nitric oxide (NO) on the amplitude of EJP and of UTP-released EDF on exogenously applied ATP-induced depolarization was also studied. A preliminary account of these data was presented to the Japanese Pharmacological Society (Thapaliya et al., 2000). It was hoped that these experiments would shed some light on the role of endothelium in purinergic neuromodulation.

2. Materials and methods

2.1. Tissue preparation

Male Golden Syrian hamsters weighing 100–30 g, killed by an overdose of diethyl ether, were given administered heparin (100 U) into the left ventricle of the heart and exsanguinated, following a protocol approved by the Gifu University Animal Care and Use Committee in accordance with the guidelines of the Japanese Department of Agriculture. The superior mesenteric artery was carefully dissected from the ileal region and placed in a physiological salt solution (PSS) at room temperature. Connective tissue was removed and the vessels were cannulated at the proximal end with a glass micropipette (200 μm tip diameter) attached to the gravity-driven perfusion apparatus to perfuse the vessel with warmed (35 °C) PSS to remove blood from the vessels. Care was taken to ensure that the endothelium was not damaged during processing of the preparation. When required, the endothelial cells were removed by injecting warmed (35 °C) PSS containing collagenase (1 mg ml^{-1}) into the perfusion route for 15 min. Successful removal of endothelial cells was confirmed by the absence of the typical membrane hyperpolarization in response to acetylcholine. To observe changes in the membrane potential, arteries supplying ileal region of about 100–350 μm (outside diameter) were used.

2.2. Electrophysiological experiments

Pre-cannulated arteries were placed in the partition chamber in which large extracellular silver–silver chloride plates were used to elicit nerve stimulation, as described previously (Bolton et al., 1984). The preparation was superfused at a constant flow rate (3 ml min^{-1}) with warmed (35 °C) PSS. The PSS was also passed through the cannula from a reservoir whose height could be adjusted; this system produced a constant perfusion flow rate of about 0.5 ml min^{-1} and pressure of about 14–16 mm Hg. Membrane potentials were recorded with a conventional microelectrode technique, using glass capillary microelectrodes filled with 3 M KCl with tip resistances ranging from 50 to 10 MΩ. Impalements were made from the adventitial side, within 2 mm of the stimulation electrode. Electrical activity was monitored on an oscilloscope (CS 4026, Kenwood, Tokyo, Japan) and recorded on a thermal-array recorder (RTA-1100 M, Nihon Kohden, Tokyo, Japan) and on a PCM data recorder (RD-111T, TEAC, Tokyo, Japan) to allow replay for further analysis.

2.3. Focal application system

To apply small quantities of ATP to localized regions and at a particular time, drugs were pressure-ejected from a micropipette. A Pneumatic PicoPump was used for this purpose with a pressure of 10 psi and pulse duration of 10 ms. Fiber-filled glass micropipettes (outside diameter = 1 mm, inside diameter = 0.5 mm) were drawn with a microelectrode puller (Narishige, Japan, Type PP-83), yielding an outside diameter of the tip of 20–30 μm. The pipette was then filled with a 10 mM solution of ATP. To avoid possible desensitization due to leakage of drugs, the pipette was kept at a distance from the preparation and positioned next to the electrode only after a stable impalement was obtained; drug application was initiated thereafter. After application of a drug, the pipette was withdrawn.

2.4. Drugs and solutions

The composition of the physiological salt solution was (mM): Na^+ 137, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 134, HCO_3^- 15.4, $H_2PO_4^-$ 1.2 and glucose 11.4. The solution in the supply reservoir was gassed continuously with a 95% O_2:5% CO_2 gas mixture creating a pH of 7.2 and was warmed to 35 °C. The drugs used were as follows: uridin-5′-triphosphate (UTP) trisodium salt (Wako, Osaka, Japan); collagenase, ATP disodium salt, *N*ω-nitro-L-arginine methyl ester (L-NAME), carbenoxolone, charybdotoxin, apamin 4-aminopyridine, glibenclamide, TEA, ouabain (Sigma); 1-amino-4-[[4-[[4-chloro-6-[[3 (or 4)-sulphophenyl]-amino-1,3,5-triazin-2-yl]amino]-3-sulphophenyl]amino-9,10-dihydro-9,10-dioxo-2-anthracenesulfonic acid (cibacron blue F3GA) (Funakoshi, Tokyo, Japan); pinacidil and indomethacin (RBI); BQ-123 and Q-788 (Peninsula Laboratories). 17-Octadecynoic acid was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Pinacidil (10 mM) was dissolved in dimethylsulphoxide (DMSO) (100%). Indomethacin (10 mM) was dissolved in an equimolar concentration of Na_2CO_3. 17-Octadecynoic acid was dissolved in ethanol to a stock solution and then diluted in HEPES to give a final ethanol concentration of ≤ 1% (v/v). All other nine drugs were dissolved in distilled water. The drugs were serially diluted in the PSS solution to the required final concentrations just before the experiments.

2.5. Preparation of nitric oxide solution

A stock solution of NO was prepared as described by Stark et al. (1991). NO gas was injected into PSS, which was previously deoxygenated by gassing with helium for 2 h, to give stock solutions of NO ranging from 0.01% to 1.0% (v/v). The deoxygenated solution had no effect on membrane potential. NO was applied onto the adventitial surface.

2.6. Statistics

Data are shown as means ± S.E.M.; n indicates the number of separate arteries in which electrical events were recorded. Statistical analysis was performed with Student's unpaired t-test and a P-value of < 0.05 was regarded as significant.

3. Results

3.1. Effect of UTP on membrane potential and EJPs evoked by trains of brief stimuli

The smooth muscle cells of hamster mesenteric arteries had resting membrane potentials of -64.7 ± 0.5 mV ($n = 151$) and were electrically quiescent when unstimulated. Perivascular nerve stimulation evoked EJPs. Stimulation at a rate of 0.25 Hz frequency during a train evoked EJPs, which did not facilitate. Facilitation of the EJPs was only observed at frequencies $\geqq 0.5$ Hz (data not shown, $n = 15$). The amplitude of EJPs at 0.25 Hz frequency ranged between 4 and 12 mV (6.9 ± 0.7 mV, $n = 82$). Experiments were conducted to observe the effect of UTP (100 μM) on resting membrane potential and EJP amplitude. Fig. 1A shows the effects of UTP on the membrane potential as well as on the EJP amplitude. The EJP amplitude (control: 6.4–0.2 mV) was not significantly inhibited during about the first 25 s of membrane hyperpolarization (6.3 ± 0.5 mV, $n = 15$), although 80% of maximum hyperpolarization had occurred, but the time constant of decay (τ_{decay}) of the EJP was significantly reduced ($P < 0.05$) from the control 345.0 ± 35.0 to 280.0 ± 28.0 ms. The amplitude of EJP started to decrease only after about 30 s of membrane hyperpolarization by UTP and maximum inhibition occurred (control: 6.4 ± 0.2 mV, $n = 15$; during hyperpolarization: 3.1 ± 0.3 mV, $n = 15$, $P < 0.001$; Fig. 1A–D) after about 1 min. During this period, τ_{decay} was also significantly reduced from 340.0 ± 8.0 to 290 ± 8.0 ms. Upon removal of UTP, the time course of recovery of the EJP amplitude was faster than the membrane repolarization. After about 2 min, even though the membrane potential was only 20% recovered (maximum 19 ± 0.6 mV; after 2 min 15.05 ± 0.6 mV, $n = 15$), the amplitude of EJP had fully returned to the control level (6.3 ± 0.3 mV, $n = 15$, $P > 0.05$). It took about 6 min before the membrane potential fully returned to control values.

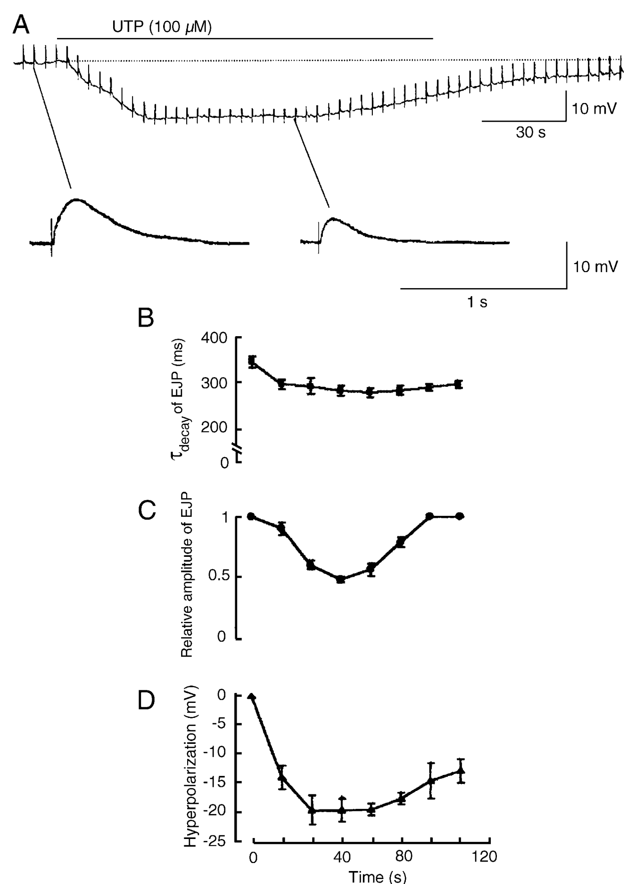

Fig. 1. Effect of UTP on the resting membrane potential and EJPs. The EJP was evoked continuously by perivascular nerve stimulation at a frequency of 0.25 Hz. UTP (100 μM) on adventitial surface application, hyperpolarized the membrane and inhibited the EJP (A). The application time for UTP was about 2 min. (B–D) Summaries of effect of UTP on the membrane potential, the amplitude of EJP and time constant of decay (τ_{decay}) of EJP. The amplitude of each EJP is expressed relative to the amplitude in the control. Absolute values for EJP amplitude ranged between 4 and 8 mV. Each point indicates means ± S.E.M. ($n = 15$–20).

3.2. Involvement of endothelium in UTP-evoked EJP inhibition

The involvement of the endothelium in this UTP-evoked EJP inhibition was studied. Endothelium-denuded smooth muscle cells were electrically quiescent when unstimulated, but the resting membrane potential was significantly depolarized from -64.7 ± 0.5 mV ($n = 151$) to -61.3 ± 1.0 mV ($n = 6$, $P < 0.05$). The amplitude of EJP was not significantly affected by the endothelium denudation (before: 7.3 ± 0.2 mV, $n = 8$; after: 6.9 ± 0.5 mV, $n = 5$, $P > 0.05$). In endothelium-denuded preparations, the intimal application of UTP (100 μM) significantly neither inhibited the amplitude of the EJP (Fig. 2) nor altered the membrane potential (before: 17.5 ± 0.9 mV; after denudation: 0.9 ± 0.3 mV, $n = 5$), but about 4 mV of depolarization was evoked in 2/7 preparations. A hyperpolarizing response to pinacidil (1 μM, 13.2 ± 0.5 mV, $n = 5$) was not affected by the endothelium denudation

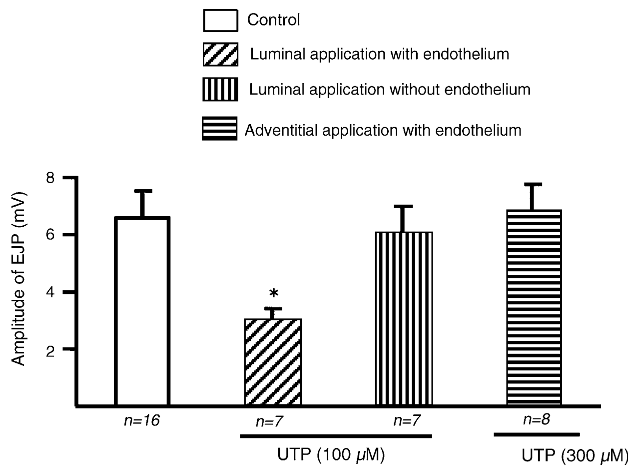

Fig. 2. Involvement of endothelium in UTP-induced EJP inhibition. In endothelium-denuded preparations, luminal application of UTP did not affect the amplitude of the EJP. Adventitial application of UTP also did not modify the EJP amplitude. The application time for UTP was about 2 min. Results are presented as the means ± S.E.M. of the number of experiments, shown below each column. *Significantly different from control ($P<0.001$).

(13.3 ± 1.2 mV, $n=5$, $P>0.05$). The possibility that UTP directly evoked the EJP inhibition was investigated by applying this agonist in the adventitial surface. When UTP (300 μM) was applied onto the adventitial surface, 5 mV depolarization was evoked in 4/11 preparations. However, the amplitude of EJPs was not significantly inhibited (Fig. 2). In other experiments, the endothelin receptor antagonists, BQ-123 and BQ-788, and muscarinic receptor antagonist, atropine, had no effect on the UTP-evoked inhibition of EJP. The amplitudes of the EJPs were 7.1 ± 0.4 and 7.5 ± 0.2 mV before the application of BQ-123 and BQ-788, and 6.9 ± 0.6 and 7.4 ± 0.4 mV, respectively, after the addition of these blockers ($n=5$, $P>0.05$). Atropine had no effect on the EJP amplitude, which was from 7.4 ± 0.3 to 7.2 ± 0.5 mV ($n=5$, $P>0.05$). These results suggest that an UTP-evoked EDF may be responsible for the EJP inhibition as well as the membrane hyperpolarization.

3.3. Effects of prostacyclin, NO and pinacidil on EJPs evoked by trains of stimuli

The involvement of EDRF, NO and prostacyclin in the inhibition of EJP amplitude was investigated. NO (5 μM), when applied adventitially, induced a membrane hyperpolarization of 8.4 ± 0.3 mV ($n=5$), but did not change the amplitude of the EJPs (Fig. 3). However, the time constant of EJP decay was significantly reduced from 346.6 ± 10.4 to 275.0 ± 12.2 ms ($P<0.01$). Prostacyclin also applied adventitially (1 μM) induced a hyperpolarization of 11.1 ± 0.7 mV ($n=8$) and, during this hyperpolarization, the EJP was significantly inhibited (Fig. 3); the τ_{decay} was also significantly reduced from 345.7 ± 17.0 to 285.0 ± 27.0 ms ($P<0.01$). In many instances, hyperpolarization of vascular smooth muscle is due to an increase in K^+ permeability, and this permeability might inhibit the depolarizing responses of the smooth muscle wall. This was assessed using the K^+ channel opener, pinacidil. Luminal application of pinacidil (1 μM) induced a hyperpolarization of 13.2 ± 0.5 mV ($n=8$) and, during this hyperpolarization, the amplitude of EJP was not significantly inhibited (control: 7.4 ± 0.5 mV, $n=5$; pinacidil 7.5 ± 0.9 mV, $n=5$, $P>0.05$; Fig. 3). However, the τ_{decay} was significantly reduced from 345.0 ± 27.0 to 280.0 ± 35.0 ms ($P<0.01$). DMSO (0.01%) at the concentration used to prepare pinacidil (1 μM) had no effect on either the membrane potential or the EJP (data not shown, $n=4$).

3.4. Effects of indomethacin and L-NAME on the EDF-mediated EJPs inhibition

The involvement of prostanoids and NO in the EJP inhibition by the UTP-induced EDF was investigated. Treatment with a cyclooxygenase enzyme inhibitor, indomethacin (5 μM), and nitric oxide synthase inhibitor, L-NAME (100 μM), did not significantly change the resting membrane potential (−65.0 ± 0.2 mV, $n=5$, $P>0.05$). Neither indomethacin nor L-NAME affected the EJP inhibition mediated by UTP-induced EDF (Fig. 4). These results suggest that NO and prostanoid are not involved in this inhibitory modulation of neurotransmission. In the presence of these enzyme inhibitors, the UTP-induced membrane

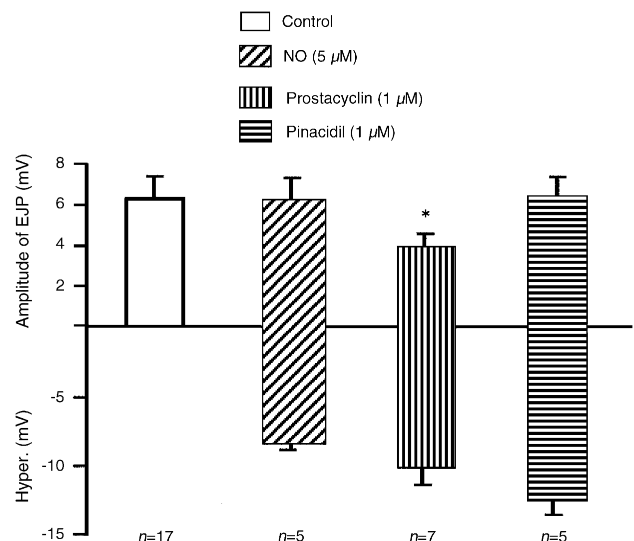

Fig. 3. Effect of NO, pinacidil and prostacyclin on the amplitude of the EJP and the membrane potential. NO and pinacidil did not affect the amplitude of the EJP, though they both produced membrane hyperpolarization. NO, pinacidil and prostacyclin were applied adventitially. The application time for NO, pinacidil and prostacyclin was about 10 s, 2 min and 2 min, respectively. Results are presented as the means ± S.E.M. of the number of experiments, shown below each column. *Significantly different from control ($P<0.01$).

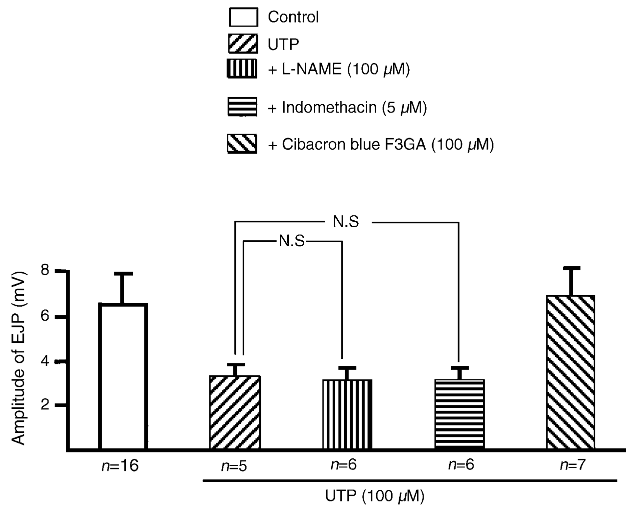

Fig. 4. Effect of L-NAME, indomethacin and cibacron blue F3GA on UTP-evoked inhibition of the EJP. Treatment with L-NAME and indomethacin did not modify the UTP-evoked inhibition of the EJP. In the presence of cibacron blue F3GA, the UTP-evoked EJP inhibition was not seen. Preparations were treated with enzyme inhibitors and antagonist for at least 30 min. After the application of these inhibitors, UTP was applied adventitially for about 2 min. Results are presented as the means ± S.E.M. of the number of experiments, shown below each column. *Significantly different from control ($P<0.001$).

hyperpolarization was also not significantly modified (control: 17.1 ± 0.5 mV, $n=11$; indomethacin: 18.1 ± 0.4 mV, $n=5$; L-NAME: 16.9 ± 0.6 mV, $n=6$). Na_2CO_3 (5 μM), at the concentration used to prepare indomethacin (5 μM), had no effect on either membrane potential or EJP (data not shown, $n=4$). In the rest of the experiments, L-NAME and indomethacin were included in the bath. Under such conditions, UTP-evoked hyperpolarization may be attributed to the production of endothelium-derived hyperpolarizing factor (EDHF).

An experiment was conducted with a P2Y receptor antagonist, cibacrone blue F3GA, to establish the type of endothelial purinoceptor involved in the EDF-mediated EJP inhibition. After treatment with cibacron blue F3GA (100 μM), the resting membrane potential was not significantly changed (− 63.7 ± 0.5 mV, $n=6$, $P>0.05$), but the UTP-induced hyperpolarization was strongly inhibited (control: 16.5 ± 0.5 mV, $n=5$; cibacron blue F3GA: 2.5 ± 0.4 mV, $n=4$, $P<0.001$) and the amplitude of EJPs approached control levels (Fig. 4). In the presence of cibacron blue F3GA (100 μM), the time constant of decay (τ_{decay}) of the EJP before and after the application of UTP (100 μM) was not significantly affected ($P<0.05$) being from 338.6 ±31.5 to 352.5 ± 26.8 ms.

3.5. Effects of apamin, charybdotoxin, 4-aminopyridine, glibenclamide, TEA and ouabain 17-octadecynoic acid on the EDF-mediated EJPs inhibition

Preparations were treated with apamin and charybdotoxin to prevent smooth muscle hyperpolarization, the objective being to rule out the involvement of increased K^+ conductance of the smooth muscle cells in the UTP-evoked EDF-mediated EJP inhibition. Treatment of preparations with apamin (0.1 μM) and charybdotoxin (0.1 μM), by adventitial application, did not change the membrane potential (− 65.3 ± 0.7 mV, $n=7$, $P>0.05$). In the presence of these K^+ channel inhibitors, UTP induced no membrane hyperpolarization, but the EJP was still significantly inhibited, while the τ_{decay} was not significantly changed (control: 343.3 ± 17 ms; during UTP application: 335.0 ±35.0 ms, $P>0.05$) (Fig. 5A and B). In a

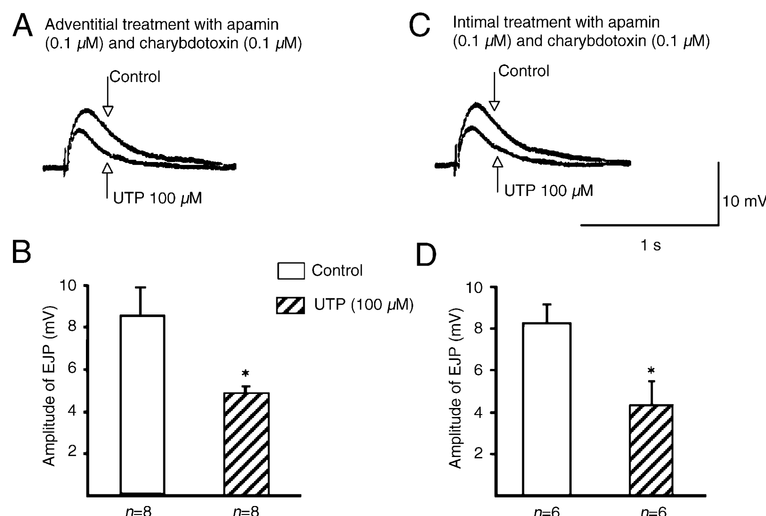

Fig. 5. UTP-evoked EJP inhibition in apamin- and charybdotoxin-treated preparation. Preparations were treated for 30 min with apamin and charybdotoxin by adventitial application (A and B) and by intimal application (C and D). After the application of apamin and charybdotoxin, UTP was applied adventitially for about 2 min. (A and C) Summary of UTP-evoked EJP inhibition in apamin- and charybdotoxin-treated preparations; EJPs were still significantly inhibited. *Significantly different from control ($P<0.001$).

few preparations (2/8), a depolarization of 5 mV was evoked by UTP. Intraluminal application of these toxins did not change the membrane potential (-64.3 ± 1.2 mV, n=6, P>0.05). As with adventitial application, UTP-induced hyperpolarization was inhibited in the presence of these K^+ channel inhibitors; however, the EJP was still significantly inhibited by UTP-induced EDF (Fig. 5C and D).

In other experiments, 1 μM 4-aminopyridine, 1 μM glibenclamide, 1 mM TEA and 10 μM ouabain did not modify the UTP-mediated EJP inhibition. The amplitudes of EJPs before and after the application of 4-aminopyridine, glibenclamide, TEA and ouabain were 5.1 ± 0.8, 4.9 ± 1.0, 5.1 ± 1.3 and 5.2 ± 1.1 mV, and 5.0 ± 0.8, 5.0 ± 0.9, 4.9 ± 0.9 and 5.0 ± 1.1 mV, respectively (n=4, P>0.05).

We also examined the effect of the cytochrome P450 inhibitor, 17-octadecynoic acid, on the UTP-induced EJP inhibition. Treatment of preparations with 17-octadecynoic acid (10 μM), by luminal application, did not change the membrane potential (-64.4 ± 0.6 mV, n=7, P>0.05). 17-Octadecynoic acid had no effect on the amplitude of EJP during the application of UTP from 5.1 ± 0.7 to 5.0 ± 0.8 mV (n=7, P>0.05).

3.6. Effect of UTP-induced EDF on summated EJP

Previously, we have reported that, in prazosin (1 μM)-treated preparations, trains of high-frequency stimuli evoked a summated EJP (Thapaliya et al., 1999). Effect of UTP-induced EDF on the summated EJP was also explored. Under the same experimental condition, 20 Hz and 50 pulse stimuli evoked a summated EJP of 18.7 ± 1.2 mV (n=7). UTP-induced EDF inhibited this summated EJP to 8.7 ± 0.8 mV, P<0.001 (n=6). Treatment of the preparation, either adventitially or intimally, with charybdotoxin (0.1 μM) and apamin (0.1 μM) did not significantly modify the UTP-evoked EDF-mediated EJP inhibition (9.1 ± 1.3 mV, n=8, P>0.05 and 9.7 ± 0.9 mV, n=6, P>0.05, respectively).

The effect of an UTP-evoked EDF on ATP-induced depolarization was investigated in preparations treated with apamin and charybdotoxin adventitially or intimally. In adventitially treated preparations, ATP (10 mM) was pressure-ejected (10 psi, 10 ms duration pulse) in small quantities from a micropipette positioned about 150 μm from the recording electrode. When ATP was applied in this way, membrane depolarization of 15.5 ± 1.3 mV (n=15) was obtained. After 1 min of UTP application, ATP was also applied. The depolarizing response of the mesenteric arterial smooth muscle cells to ATP was not inhibited during this period (15.3 ± 1.7 mV, n=4, P>0.05). In apamin and charybdotoxin intimally treated preparations, the ATP-induced depolarization also was not modified (control: 16.1 ± 0.9 mV, n=8; during UTP application: 15.7 ± 1.4 mV, n=6, P>0.05).

3.7. Effect of gap junction blocker, carbenoxolone, on endothelium-dependent hyperpolarization

The possibility of a passive spread of hyperpolarization from endothelium to smooth muscle was investigated by treating the preparations with the gap junction blocker, carbenoxolone. Incubation of the preparations with carbenoxolone (100 μM) did not significantly change the membrane potential (control: -63.5 ± 0.5 mV; carbenoxolone: -62.9 ± 1.3 mV, n=6, P<0.05) and endothelium-dependent hyperpolarization induced by UTP (100 μM) was unaffected by this gap junction blocker (control: 19.1 ± 0.7 mV; carbenoxolone: 19.7 ± 1.2 mV, n=6, P<0.05).

4. Discussion

In the present experiments, we established that UTP released EDHF, which hyperpolarized the smooth muscle cells of hamster mesenteric artery. This hyperpolarization was not modified by L-NAME and indomethacin, but was inhibited by combined treatment with charybdotoxin and apamin. In the presence of both toxins, EDF still inhibited the EJPs and did not modify the exogenously applied ATP-induced depolarizations. Thus, these results suggested that EDF inhibits purinergic neurotransmission prejunctionally.

Although NO and prostacyclin are reported to be involved in endothelium-dependent hyperpolarization (Tare et al., 1990; Garland and Mcpherson, 1992; Parkington et al., 1993), these factors do not seem to be involved in UTP-mediated hyperpolarization, since the hyperpolarization was not affected by L-NAME and indomethacin. Several hypotheses have been proposed to explain the mechanism involved in the EDHF-mediated hyperpolarization. It is reported that EDHF is the hyperpolarizing current conducted to smooth muscle through the gap junction after hyperpolarization of the endothelium (Yamamoto et al., 1999; Pacicca et al., 1996). However, the existence of humoral EDHF was suggested by results of using sandwiched preparations in guinea-pig coronary artery (Chen et al., 1991), and the release of EDHF from either native or cultured porcine coronary endothelial cells was bioassayed by monitoring the membrane potential in vascular smooth muscle cells located downstream (Popp et al., 1996; Fleming et al., 1999). The hamster mesenteric artery resembles the later type as the UTP-evoked hyperpolarization was not significantly inhibited by the gap junction inhibitor, carbenoxolone.

4.1. Agonist to selectively stimulate the endothelium

To study the neuromodulatory effect of EDF, a specific endothelial agonist should be chosen. Acetylcholine is commonly used as the agonist for studying the different characteristics of endothelium-dependent factors (Rand and Garland, 1992; Corriu et al., 1996; Fukao et al., 1997). However, acetylcholine acts not only on endothelial

muscarinic receptor but also on presynaptic muscarinic receptors, which directly inhibits neurotransmitter release (Komori and Suzuki, 1987). Furthermore, it has been reported that significantly lower concentrations of acetylcholine are sufficient to inhibit neurotransmitter release presynaptically than to release EDHF or endothelial paracrine factors (Kotecha, 1999). Thus, it is necessary to choose an agonist, which does not act presynaptically to modulate transmitter release, and acts specifically on endothelium. In previous studies, EDHF was released upon activation of P2Y receptors in the rat mesenteric artery (Malmsjö et al., 1999) and $P2Y_2$ receptors in the hamster mesenteric artery (Thapaliya et al., 1999). Moreover, UTP has been reported to be unable to modulate the evoked release of noradrenaline from sympathetic nerve in rabbit ear artery (Saiag et al., 1998). In the present study, UTP did not directly inhibit EJP presynaptically, because adventitial application of UTP was without effect. Furthermore, intimal application of UTP was without effect after the endothelium was removed. We, therefore, used UTP to stimulate the endothelium selectively.

4.2. EDF-mediated EJP inhibition

The novel finding of the present study was that intact endothelium is a must for UTP-mediated EJP inhibition. The vascular endothelium is capable of releasing various humoral substances, including NO (Tare et al., 1990; Garland and Mcpherson, 1992; Campbell and Harder, 2001), prostacyclin (Parkington et al., 1993), EDHF (Garland et al., 1995), endothelin (Yanagisawa et al., 1988), ATP, substance P and acetylcholine (Milner et al., 1990). We have devoted all the possible factors released from the endothelium as EDF. In the present study, the exogenously applied NO did not inhibit the amplitude of EJP and treatment of preparations with L-NAME did not modify the UTP-mediated EJP inhibition, suggesting involvement of NO to be less likely. Similar results have been reported from an earlier study (Komori et al., 1988). Exogenously applied prostacyclin inhibited the EJP. However, involvement of protaglandins in UTP-mediated EJP inhibition is unlikely, because indomethacin did not modify the inhibitory effect. Either intraluminal or adventitial treatment of the preparations with apamin and charybdotoxin inhibited the EDHF-mediated hyperpolarization although EJP inhibition was still present. To explain the EDF-mediated EJP inhibition, we offer two hypotheses. An explanation for our finding is that EDHF might be involved in this EJP inhibition. In the present study, apamin and charybdotoxin, inhibitors of small and large conductance Ca^{2+}-activated K^+ channels (K_{Ca}), respectively (Nelson, 1993), inhibited the UTP-evoked hyperpolarization. If these toxins are blocking the target K^+-channels for EDHF on smooth muscle (Bolz et al., 1999; Yamanaka et al., 1998), it seems that the K_{Ca} channels might not be the target K^+ channels for EDHF on nerve endings. In addition to the K_{Ca} channel, three other kinds of K^+ channels have also been reported to mediate the vascular actions of EDHF: (1) K_{ir} channels (Edwards et al., 1998), (2) K_{dr} channels (Petersson et al., 1997), (3) K_{ATP} channels (Liu and Flavahan, 1997). In addition to K^+ channel, Ca^{2+} channels might also be involved in the EJP inhibition. In the present study, the generation of EJP amplitude was dependent on the extracellular calcium concentration and inhibited by ω-conotoxin GVIA (unpublished observations). Previously, Kuriyama and Makita (1982) have reported that the primary prostaglandins inhibit the influx of calcium during transmitter release from nerve terminals. Thus, to identify the K^+ or Ca^{2+} channel involved in mediation of the EJP inhibition by EDHF requires further studies. To date, like EDHF-mediated responses, recording of smooth muscle membrane potential and relaxation has been extensively studied. However, by measuring the modulation of ATP release, our study suggests an additional way of studying the characteristics of EDHF. Alternatively, if according to Edwards et al. (1998) and Doughty et al. (1999), apamin and charybdotoxin act on endothelium to inhibit the production of EDHF, another unidentified short-lived substance may be involved in EDF-mediated EJP inhibition. Release of the unidentified-factor might not have been inhibited by these two toxins, since nitric oxide and cyclooxygenase metabolite release was not inhibited by these two toxins in the rat hepatic artery from the endothelium by acetylcholine and the calcium ionophore A23187 (Zygmunt et al., 1998). Furthermore, it is reported that endothelin reduces the EFS-evoked overflow of ATP (Mutafova-Yambolieva and Westfall, 1998). In addition, acetylcholine is also reported to act presynaptically to inhibit EJP at very low concentrations (Komori and Suzuki, 1987; Kotecha, 1999). However, endothelin and muscarinic receptor antagonists, BQ-123, BQ-788 and atropine, had no effect on the UTP-invoked EJP inhibition. Thus, these substances might not be the possible candidates as unidentified short-lived substance to inhibit the EJP in the hamster mesenteric artery. In endothelium-denuded preparations, the intimal application of UTP did not inhibit the amplitude of the EJP, nor did it significantly alter membrane potential, suggesting that the UTP-invoked EJP inhibition needs endothelial cell and UTP may not directly affect the nerves. We can conclude from these findings that EDF released by UTP may inhibit EJP. However, we cannot exclude the possibility that the inhibitory effect seen with UTP is due to EDHF.

4.3. Increase K^+ conductance and reduced time constant of decay of EJP

Previous observations have suggested that endothelium-dependent hyperpolarization is likely to result from an increase of K^+ conductance in vascular smooth muscle cells (Bolton et al., 1984; Taylor et al., 1988; Chen and Suzuki, 1989; Richards et al., 2001). Such increased K^+ conductance may prevent depolarizing responses of the smooth muscle. However, at the start of EDHF-induced

hyperpolarization, and during NO and pinacidil-induced hyperpolarization and in spite of a reduced time constant of decay of EJP (τ_{decay}), the amplitude of EJP was not reduced. The reduction in τ_{decay} indicates a fall in membrane resistance of the smooth muscle cells (Kotecha and Neild, 1995; Kotecha, 1999) due to increased K^+ permeability. Membrane hyperpolarizations increase the driving forces, which in turn may increase the EJP amplitude. The fact that the EJP amplitude was constant supports the conclusion of a reduced membrane resistance. Therefore, it might be possible that the increase in driving potential is exactly counteracted by the increased membrane conductance. However, additional studies are required to test this hypothesis critically.

4.4. Possible physiological implications

In prazosin-treated preparations, 20 Hz nerve stimulation generates a fast summated EJP, which is inhibited by a P2X receptor antagonist (Thapaliya et al., 1999). Benham and Tsien (1987) have reported that ATP-activated channels provide a distinct mechanism for Ca^{2+} entry into arterial smooth muscle. Furthermore, depolarization could increase Ca^{2+} influx through ATP-activated channels more effectively, leading to contraction (Benham, 1989). In the present study, since the summated EJP was inhibited by EDF, the influx of Ca^{2+} might have been inhibited, resulting in the inhibition of nerve stimulation-induced constriction (Thapaliya et al., 2000, unpublished observation). Therefore, EDF-mediated purinergic neuromodulation may play a very important physiological role in the control of vascular resistance in small arteries.

Acknowledgements

The authors are grateful to Dr. R.J. Lang for helpful comments on the manuscript.

References

Benham, C.D., 1989. ATP-activated channels gate calcium entry in single smooth muscle cells dissociated from rabbit ear artery. J. Physiol. 419, 689–701.
Benham, C.D., Tsien, R.W., 1987. A novel receptor-operated Ca^{2+}-permeable channel activated by ATP in smooth muscle. Nature 328, 275–278.
Bolton, T.B., Lang, R.J., Takewaki, T., 1984. Mechanism of action of noradrenaline and carbachol on smooth muscle of guinea-pig anterior mesenteric artery. J. Physiol. 351, 549–572.
Bolz, S.-S., De Wit, C., Pohl, U., 1999. Endothelium-derived hyperpolarizing factor but not NO reduces smooth muscle Ca^{2+} during acetylcholine-induced dilatation of microvessels. Br. J. Pharmacol. 128, 124–134.
Brock, J.A., Cunnane, T.C., 1999. Effects of Ca^{2+} concentration and Ca^{2+} channel blockers on noradrenaline release and purinergic neuroeffector transmission in rat tail artery. Br. J. Pharmacol. 126, 11–18.
Burnstock, G., 1990. Local mechanisms of blood flow control by perivascular nerves and endothelium. J. Hypertens. 8, S95–S106 (Suppl.).
Campbell, W.B., Harder, D.R., 2001. Prologue: EDHF—what is it? Am. J. Physiol. 280, H2413–H2416.
Chen, G., Suzuki, H., 1989. Some electrical properties of the endothelium-dependent hyperpolarization recorded from rat arterial smooth muscle cells. J. Physiol. 410, 91–106.
Chen, G., Yamamoto, Y., Miwa, K., Suzuki, H., 1991. Hyperpolarization of arterial smooth muscle induced by endothelial humoral substances. Am. J. Physiol. 260, H1888–H1892.
Corriu, C., Fèlètou, M., Canet, E., Vanhoutte, P.M., 1996. Endothelium-derived factors and hyperpolarization of the carotid artery of the guinea-pig. Br. J. Pharmacol. 119, 959–964.
Doughty, J.M., Plane, F., Langton, P.D., 1999. Charybdotoxin and apamin block EDHF in rat mesenteric artery if selectively applied to the endothelium. Am. J. Physiol. 276, H1107–H1112.
Edwards, G., Dora, K.A., Garderner, M.J., Garland, C.J., Weston, A.H., 1998. K^+ is an endothelium-derived hyperpolarizing factor in rat arteries. Nature 396, 269–272.
Fleming, I., Schermer, B., Popp, R., Busse, R., 1999. Inhibition of the production of endothelium-derived hyperpolarizing factor by cannabinoid receptor agonists. Br. J. Pharmacol. 126, 949–960.
Fukao, M., Hattori, Y., Kanno, M., Sakuma, I., Kitabatake, A., 1997. Sources of Ca^{2+} in relation to generation of acetylcholine-induced endothelium-dependent hyperpolarization in rat mesenteric artery. Br. J. Pharmacol. 120, 1328–1334.
Garland, C.J., Mcpherson, G.A., 1992. Evidence that nitric oxide does not mediate the hyperpolarization and relaxation to acetylcholine in the rat small mesenteric artery. Br. J. Pharmacol. 105, 429–435.
Garland, C.J., Plane, F., Kemp, B.K., Cocks, T.M., 1995. Endothelium-dependent hyperpolarization: a role in control of vascular tone. Trends Pharmacol. Sci. 16, 23–30.
Hashitani, H., Windle, A., Suzuki, H., 1998. Neuroeffector transmission in arterioles of the guinea-pig choroid. J. Physiol. 510, 209–223.
Komori, K., Suzuki, S., 1987. Heterogenous distribution of muscarinic receptors in the rabbit saphenous artery. Br. J. Pharmacol. 92, 657–664.
Komori, K., Lorenz, R.R., Vanhoutte, P.M., 1988. Nitric oxide, ACh and electrical and mechanical properties of canine arterial smooth muscle. Am. J. Physiol. 255, H207–H212.
Kotecha, N., 1999. Mechanisms underlying ACh induced modulation of neurogenic and applied ATP constrictions in the submucosal arterioles of the guinea-pig small intestine. Br. J. Pharmacol. 126, 1625–1633.
Kotecha, N., Neild, T.O., 1995. Actions of vasodilator nerves on arteriolar smooth muscle and neurotransmitter release from sympathetic nerves in the guinea-pig small intestine. J. Physiol. 489, 849–855.
Kuriyama, H., Makita, Y., 1982. Modulation of neuromuscular transmission by endogenous and exogenous prostaglandins in the guinea-pig mesenteric artery. J. Physiol. 327, 431–448.
Liu, Q., Flavahan, N.A., 1997. Hypoxic dilatation of porcine small coronary arteries: role of endothelium and KATP-channels. Br. J. Pharmacol. 120, 728–734.
Malmsjö, M., Erlinge, D., Högestätt, F.D., Zygmunt, P.M., 1999. Endothelial P2Y receptors induce hyperpolarization of vascular smooth muscle by release of endothelium-derived hyperpolarizing factor. Eur. J. Pharmacol. 364, 169–173.
Milner, P., Kirkpatrick, K.A., Ralevic, V., Toothill, V., Pearson, J., Burnstock, G., 1990. Endothelial cells cultured from human umbilical vein release ATP, substance P and acetylcholine in response to increased flow. Proc. R. Soc. Lond. B 241, 245–248.
Mutafova-Yambolieva, V.N., Westfall, D.P., 1998. Inhibitory and facilitatory presynaptic effects of endothelin on sympathetic cotransmission in the rat isolated tail artery. Br. J. Pharmacol. 123, 136–142.
Nelson, M.T., 1993. The role of potassium channels in regulation of peripheral resistance. In: Escande, D., Standen, N. (Eds.), K^+ Channels in Cardiovascular Medicine. Springer-Verlag, Paris, pp. 95–106.
Onaka, U., Fujii, K., Abe, I., Fujishima, M., 1997. Enhancement by exogenous and locally generated angiotensin II of purinergic neurotransmis-

sion via angiotensin type 1 receptor in the guinea-pig isolated mesenteric artery. Br. J. Pharmacol. 122, 942–948.

Pacicca, C., Schaad, O., Beny, J.L., 1996. Electronic propagation of kinin-induced, endothelium-dependent hyperpolarizations in pig coronary smooth muscles. J. Vasc. Res. 33, 380–385.

Parkington, H.C., Tare, M., Tonta, M.A., Coleman, H.A., 1993. Stretch released three components in the hyperpolarization of guinea-pig coronary artery in response to acetylcholine. J. Physiol. 465, 459–476.

Petersson, J., Zygmunt, P.D., Högestätt, E.D., 1997. Characterization of potassium channels involved in EDHF-mediated relaxation in cerebral arteries. Br. J. Pharmacol. 120, 1344–1350.

Popp, R., Bauersachs, J., Hecker, M., Fleming, I., Busse, R., 1996. A transferable, β-naphthoflavore-inducible, hyperpolarizing factor is synthesized by native and cultured porcine coronary endothelial cells. J. Physiol. 497, 699–709.

Rand, V.E., Garland, C.J., 1992. Endothelium-dependent relaxation to acetylcholine in the rabbit basilar artery: importance of membrane hyperpolarization. Br. J. Pharmacol. 106, 143–150.

Richards, G.R., Weston, A.H., Burnham, M.P., Feletou, M., Vanhoutte, P.M., Edwards, G., 2001. Suppression of K(+)-induced hyperpolarization by phenylephrine in rat mesenteric artery: relevance to studies of endothelium-derived hyperpolarizing factor. Br. J. Pharmacol. 134, 1–5.

Saiag, B., Shacoori, V., Bodin, P., Catheline, M., Burnstock, G., 1998. Lack of uptake, release and action of UTP at sympathetic perivascular nerve terminals in rabbit ear artery. Eur. J. Pharmacol. 358, 139–145.

Stark, M.E., Bauer, A.J., Szurszewski, J.H., 1991. Effect of nitric oxide on circular muscle of the canine small intestine. J. Physiol. 444, 743–761.

Tare, M., Parkington, H.C., Coleman, H.A., Nield, T.O., Dusting, G.J., 1990. Hyperpolarization and relaxation of arterial smooth muscle caused by nitric oxide derived from the endothelium. Nature 349, 69–71.

Taylor, S.G., Southerton, J.S., Weston, A.H., Baker, J.R.L., 1988. Endothelium-dependent effects of acetylcholine in rat aorta: a comparison with sodium nitroprusside and cromakalim. Br. J. Pharmacol. 94, 853–863.

Thapaliya, S., Matsuyama, H., Takewaki, T., 1999. ATP released from perivascular nerves hyperpolarizes smooth muscle cells by releasing an endothelium-derived factor in hamster mesenteric arteries. J. Physiol. 521, 191–199.

Thapaliya, S., Matsuyama, H., Takewaki, T., 2000. An endothelium-derived factor modulates purineric neurotransmission in mesenteric arterial smooth muscle of hamster. Jpn. J. Pharmacol. (Suppl.) 82 (64 pp.).

Yamamoto, Y., Imaeda, K., Suzuki, H., 1999. Endothelium-dependent hyperpolarization and intercellular electrical coupling in guinea-pig mesenteric arterioles. J. Physiol. 514, 505–513.

Yamanaka, A., Ishikawa, T., Goto, K., 1998. Characterization of endothelium-dependent relaxation independent of NO and prostaglandins in guinea-pig coronary artery. J. Pharmacol. Exp. Ther. 285, 480–489.

Yanagisawa, M., Kurihar, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K., Masaki, T., 1988. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 332, 411–415.

Zygmunt, P.M., Plane, F., Paulsson, M., Garland, C.J., Högestätt, E.D., 1998. Interaction between endothelium-derived relaxing factors in the rat hepatic artery: focus on regulation of EDHF. Br. J. Pharmacol. 124, 992–1000.