

Neurally released ATP mediates endothelium-dependent hyperpolarization in the circular smooth muscle cells of chicken anterior mesenteric artery

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1 The object of the present study was to clarify the neurotransmitter(s) controlling membrane responses to electrical field stimulation (EFS) in the circular smooth muscle cells of first-order branches of chicken anterior mesenteric artery.

2 EFS (five pulses at 20 Hz, 1 ms) evoked a hyperpolarization of amplitude -21.6 ± 1.2 mV, total duration 21.8 ± 1.2 s and latency 641.7 ± 81.9 ms. The response was tetrodotoxin-sensitive and nonadrenergic noncholinergic (NANC) in nature.

3 The NANC response was blocked by the nonspecific purinergic antagonist, suramin, indicating that the response is mediated by the neurotransmitter adenosine 5'-triphosphate (ATP).

4 Either desensitization or blockade of P2Y receptor with its putative agonist 2-methylthioATP ($1 \mu\text{M}$ for 30 min) or with its antagonist cibacron blue F3GA ($10 \mu\text{M}$), respectively, abolished the purinergic hyperpolarization. PPADS at concentrations up to $100 \mu\text{M}$ had no effect on the EFS-induced response, indicating that this response is mediated through P2Y, but not P2X, receptor. In addition, the response was completely abolished by two specific P2Y1 receptor antagonists, namely, MRS 2179 (300 nM) and A3P5PS ($10 \mu\text{M}$).

5 Removal of the endothelium abolished the purinergic hyperpolarization, which was converted, in some preparations, to a small depolarization, indicating that the hyperpolarizing response is endothelium-dependent.

6 The present study suggests that in first-order branches of chicken anterior mesenteric artery, ATP released from perivascular nerves may diffuse to the endothelium-activating P2Y1 receptor to induce release of an inhibitory substance that mediates hyperpolarization in the circular smooth muscle.

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Abbreviations: ACh, acetylcholine; ATP, adenosine 5'-triphosphate; A3P5PS, adenosine-3'-phosphate-5'-phosphosulfate; CBF3GA, cibacron blue F3GA; EDHF, endothelium-derived hyperpolarizing factor; EFS, electrical field stimulation; EJP, excitatory junction potential; IJP, inhibitory junction potential; α, β -MeATP, α, β -methylene ATP; 2-MeSATP, 2-methylthio ATP; MRS 2179, *N*(6)-methyl-2'-deoxyadenosine-3',5'-bisphosphate; NANC, nonadrenergic, noncholinergic; NE, norepinephrine; PPADS, pyridoxalphosphate-6-azophenyl-2',4'-disulfonic; PSS, physiological salt solution; TTX, tetrodotoxin

Introduction

It has been widely recognized that both perivascular nerves and endothelial cells control the tone of the vascular smooth muscles and hence regulate the local blood flow (Furchgott & Zawadzki, 1980; Burnstock, 1990). Adenosine 5'-triphosphate (ATP) has been proven to be a nonadrenergic, noncholinergic (NANC) transmitter, colocalized with norepinephrine (NE) in the sympathetic nerves and being released with NE in variable proportions depending on the tissues and species (Burnstock, 1986). ATP was evidenced to have contributing roles in rabbit saphenous and mesenteric (Burnstock, 1990), guinea-pig mesenteric (Onaka *et al.*, 1997) and hamster mesenteric (Thapaliya *et al.*, 1999) arteries. The receptors mediating

responses to ATP have been characterized as P2 purinoceptors (Burnstock, 1987). P2 purinoceptors have been subdivided into two major classes: P2X and P2Y purinoceptors (Abbracchio & Burnstock, 1994). ATP may evoke a dual response, that is, when it acts on P2X ligand-gated cation channels on smooth muscle cells it evokes transient depolarization termed excitatory junction potential (EJP) (Stjarne, 1986), and when it acts on endothelial G-protein-coupled P2Y receptors, it evokes hyperpolarization (Keef *et al.*, 1992).

On the other hand, endothelial cells were evidenced to release more than one inhibitory factor, including nitric oxide (NO) (Furchgott, 1995), prostanoide (Narumiya *et al.*, 1999) and endothelium-derived hyperpolarizing factor (EDHF) (McGuire *et al.*, 2001). The transmitter (ATP) released from nerves could diffuse and act directly on the endothelial cell,

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releasing one or more of such hyperpolarization substances (Thapaliya *et al.*, 1999) in thin but not thick arteries. The possible presence of such an interaction is also emphasized by Ralevic & Burnstock (1996).

Compared to other species, very little information is known about ATP and endothelial cells as controllers of the circular smooth muscle cells of first-order branches of chicken anterior mesenteric artery. In contrast to mammalian mesenteric artery, Ball *et al.* (1963) have stated that anterior mesenteric artery of chicken contains an additional longitudinal muscle layer in the main branch; however, the side branches contain only the circular muscle layer that is common to all blood vessels. Functionally, Bolton (1969) and Bell (1969) reported that the longitudinal muscle, in contrast to the circular one, is relaxed by adrenergic nerve stimulation acting through β -adrenergic receptors; however, stimulation of cholinergic nerve fibers caused this muscle to contract. On the other hand, parallel to mammals, adrenergic nerve stimulation produces contraction of the circular muscle.

The present study investigates the possible contribution of ATP to the functional activity of the circular smooth muscle cells of first-order branches of chicken anterior mesenteric artery that are devoid of the longitudinal muscle layer using microelectrode and tension recording techniques. The role of the endothelium in modulating neuroeffector transmission and the subtype of P2 purinoceptor involved in this modulating action have been also investigated.

Methods

Tissue preparation

Female white leghorn chickens aged 10–14 weeks old were killed by cervical dislocation. First-order branches of anterior mesenteric artery were carefully dissected from the ileal tissue and placed in a physiological salt solution (PSS; see below) at room temperature. The connective tissue was removed and vessels were cannulated at their proximal ends with glass micropipettes (200 μ m tip diameter) attached to a gravity-driven perfusion apparatus that perfused the vessel with warmed (33°C) PSS to remove the blood in the vessels. Ethics and experimental procedures were approved by the Gifu University Animal Care and Use Committee, and were in accordance with the Japanese Department of Agriculture Guidelines, and all efforts were made to minimize animal suffering and to reduce the number of animals used. Care was taken to ensure that the endothelium was not damaged during processing of the preparation. When required, the endothelium was removed by injecting warmed (33°C) PSS containing collagenase (1 mg ml⁻¹) into the lumen of the vessels for 15 min.

Electrophysiological recording

Arterial preparations were placed in a partition chamber in which large extracellular silver–silver chloride plates were used to elicit nerve stimulation as described previously (Bolton *et al.*, 1984). The preparations were perfused at constant flow rate (3 ml min⁻¹) with prewarmed (33°C) PSS containing the cholinergic blocker atropine (0.5 μ M) and the adrenergic blockers prazosin (5 μ M) and propranolol (1 μ M) to establish

the NANC condition. Tissue preparations were allowed to equilibrate for approximately 1 h before experiments were undertaken. Membrane potentials were recorded with conventional glass capillary microelectrodes, filled with 3 M KCl with tip resistances ranging from 50 to 80 M Ω . The microelectrode insertions were made into the circular muscle cells through the adventitial side within 2 mm of the stimulating plate (Takewaki & Ohashi, 1977). Electrical activity was monitored on an oscilloscope (CS 4026, Kenwood, Japan) and recorded on a thermal-array recorder (RD – 111T, TEAC, Japan). Electrical field stimulation (EFS; 15 V, 1 ms pulse width) with single stimuli and variable numbers of stimuli at 20 Hz was applied with a SEN-3301 stimulator (Nihon Kohden, Japan). In experiments of investigating the effects of exogenous ATP, care was taken that ATP was applied to the adventitial side only by cannulating the arterial segment from both sides. Recordings were carried out from preparations either with or without endothelial cells. Endothelial removal was performed as described above and considered successful when no hyperpolarization was elicited by acetylcholine (ACh; 5 μ M). Integrity of the smooth muscle cells after enzymatic removal of endothelium was confirmed by successful recording of the hyperpolarizing effect of the ATP-sensitive K⁺ channel opener, cromakalim (1 μ M) (data not shown).

Tension recording

Arterial segments of 3–5 mm length were cut and mounted for isometric tension recording in a 5 ml organ bath containing PSS at 33°C that was gassed with 95% O₂:5% CO₂. Two fine, stainless steel pins 100 μ m in diameter were introduced through the lumen of the segment. One pin was fixed to the organ bath floor, while the other was connected to a force transducer (AD instrument MLT 050/D, Australia) for tension recording. An initial resting tension of 1 g was applied to the arterial rings, which were subsequently left to equilibrate for 60–90 min. At the end of this period, the tension on the vessel was taken as the resting tension and no further mechanical adjustment was made during experimentation. Changes in isometric force were recorded using a PowerLab 2/25 data acquisition system (Chart software, version 5.0.2, AD instruments, Australia).

The relaxant effect of exogenously applied ATP was studied by adding ATP after initial precontraction using NE (2 μ M). This experiment was performed using endothelium-intact or -denuded preparations. Endothelium integrity was confirmed by the presence of inhibitory response upon application of ACh (5 μ M) and cromakalim (1 μ M) on the NE-induced precontractions. ATP was used in concentration range (100 nM–1 mM). Relaxing effects were expressed as percentage of papaverine-induced relaxation. After exposure to a drug, the preparation was washed with PSS and left for at least 20 min before further experimentation.

Physiological salt solutions

The physiological solutions used in this study had the following composition in mM: NaCl 118, KCl 4.6, CaCl₂ 2.7, MgCl₂ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, glucose 11. The solution in the supply reservoir was gassed continuously with 95% O₂:5% CO₂ gas mixture, creating a pH of 7.4, and was warmed to 33°C.

Drugs

The following drugs have been used in the present study: 1-amino-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 (CBF3GA)), *N*(6)-methyl-2'-deoxyadenosine-3',5'-bisphosphate (MRS 2179), 2-methylthio ATP (2-MeSATP), α,β -methylene ATP (α,β -MeATP) lithium salt, adenosine-3'-phosphate-5'-phosphorsulfate (A3P5PS), ACh, ATP, atropine sulfate monohydrate, collagenase, cromakalim, guanethidine sulfate, indomethacin, *N* ω -nitro-L-arginine methyl ester (L-NAME), NE, papaverine, prazosin hydrochloride, propranolol hydrochloride, pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic (PPADS) acid, suramin sodium and tetrodotoxin (TTX). All drugs have been purchased from Sigma chemicals (St Louis, MO, U.S.A.).

Indomethacin (10 mM) was dissolved in an equimolar concentration of Na₂CO₃. Prazosin (10 mM) was dissolved in methanol (100%). All other drugs were dissolved in distilled water. Drugs were applied at required concentrations by their addition to the superfusing PSS.

Statistics

Data are expressed as mean $\pm$ s.e.m.; *n* represents the number of chickens from which the tissues were isolated. Statistical analysis was performed with Student's unpaired *t*-test, and *P*-values <0.05 were considered statistically significant.

Results

General observations

The mean resting membrane potential recorded from the circular smooth muscle cells of first-order branches of chicken anterior mesenteric artery was -61.8 ± 0.4 mV (*n* = 77) at a bath temperature of 33°C. At rest, circular smooth muscle cells were electrically quiescent. Single-pulse stimulation evoked a slow hyperpolarization. Multiple (2–20) pulse stimulation at 20 Hz produced hyperpolarization with gradually increasing amplitudes (Figure 1). Usually, a maximal response was

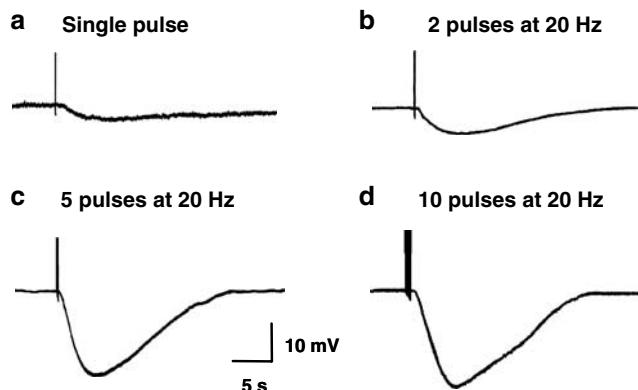


Figure 1 Hyperpolarization produced by single and multiple nerve stimulation at 33°C bath temperature. EFS-evoked hyperpolarization produced by a single pulse (a) or 2 (b), 5 (c) or 10 (d) pulses at 20 Hz. Membrane potential was -62 mV.

achieved when five-pulse stimulation at 20 Hz was applied. EFS using five pulses at 20 Hz was selected for all subsequent experiments because it produced the most constant response. The electrically evoked hyperpolarizations were blocked by both TTX ($0.3 \mu\text{M}$, *n* = 4) and guanethidine ($5 \mu\text{M}$, *n* = 4) (data not shown for both), indicating that they are mediated by activation of the sympathetic nerve axons. Table 1 summarizes the temporal parameters of the hyperpolarizations evoked by a single pulse (1 ms duration) and by five pulses at 20 Hz for the same duration of time.

Effect of endothelium denudation on the EFS-evoked hyperpolarization

In the absence of stimulation, the circular smooth muscle of endothelium-denuded preparations was electrically quiescent. In these tissues, removal of the endothelium with collagenase had no effect on the resting membrane potential and ACh failed to produce hyperpolarization. The EFS-induced hyperpolarization was not observed after endothelium denudation. However, in some other preparations, small depolarizations have been recorded that ranged between 0.5 and 4 mV in amplitude (Figure 2B).

Effects of L-NAME and indomethacin on the EFS-evoked hyperpolarization

Endothelial cells are known to release more than one inhibitory factor including NO (Furchgott, 1995), prostanoid (Narumiya *et al.*, 1999) and EDHF (McGuire *et al.*, 2001).

Table 1 Parameters of slow hyperpolarization evoked by single pulse and by five pulses at 20 Hz

	Amplitude (mV)	Latency (ms)	Time to peak (s)	Total duration (s)
Single pulse (<i>n</i> = 10)	3.5 ± 1.1	615.4 ± 63.2	4.2 ± 0.3	20.8 ± 1.8
Five pulses (20 Hz) (<i>n</i> = 24)	21.6 ± 1.2	641.7 ± 81.9	4.1 ± 0.2	21.8 ± 1.2

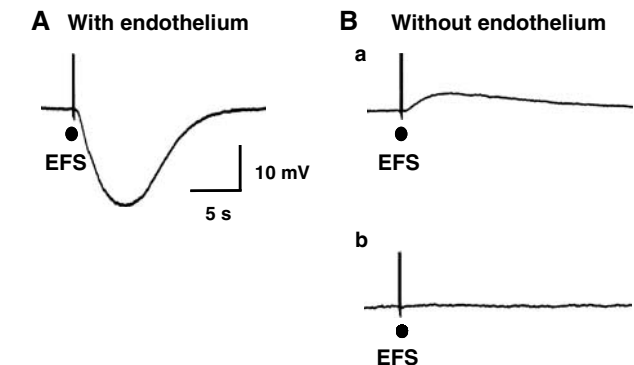


Figure 2 Effect of endothelium denudation on nerve stimulation using a train of five pulses. (A) Typical recordings showing EFS-evoked hyperpolarization in artery with endothelium. (B) Typical recordings showing EFS-evoked hyperpolarization in artery without endothelium. Membrane potential for (A, Ba, Bb) were -63 , -62 and -63 mV, respectively.

In this experiment, involvement of NO and prostanoid in the EFS-induced hyperpolarization was investigated. L-NAME, an NO synthase inhibitor ($100\text{ }\mu\text{M}$), had no effect on EFS-evoked hyperpolarization ($n=5$). Similarly, indomethacin, a cyclooxygenase inhibitor ($10\text{ }\mu\text{M}$), did not affect the hyperpolarizing response ($n=5$).

Effects of suramin, PPADS and CBF3GA on the EFS-evoked hyperpolarization

Application of the nonspecific purinergic antagonist, suramin, did not cause any changes in the resting membrane potential ($n=5$). Suramin exhibited a dose-dependent ($100\text{--}500\text{ }\mu\text{M}$) inhibition of the amplitude of EFS-evoked hyperpolarization, with complete abolition of the response upon application of $500\text{ }\mu\text{M}$ suramin (Figure 3a and b). It is well established that ATP mediates its action through two types of purinergic receptors, namely P2X (mainly excitatory) and P2Y (mainly inhibitory) receptors. Therefore, the following experiments have been performed to elucidate the receptor type through which ATP produces its hyperpolarizing action. The blocker for P2X receptor, PPADS ($100\text{ }\mu\text{M}$), had no effect on the recorded EFS-evoked hyperpolarization ($n=5$, Figure 3c). In contrast, the P2Y receptor-specific blocker, CBF3GA,

exhibited dose-dependent ($50\text{--}100\text{ }\mu\text{M}$) inhibition on amplitude of EFS-evoked hyperpolarization, with complete abolition at $100\text{ }\mu\text{M}$ (Figure 4a and b). CBF3GA did not cause any change in the resting membrane potential ($n=6$).

Effects of P2X and P2Y receptor agonists' desensitization on NANC EFS-evoked hyperpolarization

For further confirmation of P2 receptor type which mediated the NANC EFS-evoked hyperpolarization, the putative P2X and P2Y receptor agonists, α,β -MeATP ($1\text{ }\mu\text{M}$) and 2-MeSATP ($1\text{ }\mu\text{M}$), respectively, have been used. Application of α,β -MeATP did not cause any changes in the membrane potential. However, application of the P2Y receptor agonist 2-MeSATP resulted in hyperpolarization ($-16.8 \pm 3.8\text{ mV}$; $n=4$). The response was slowly developing and long-lasting, where the membrane potential repolarized back to the baseline value after $4.6 \pm 1.0\text{ min}$.

To desensitize a certain class of P2 receptor, specimens were incubated for 30 min with the respective agonist and then the EFS-evoked response was recorded. After P2X receptor desensitization, no effect on the EFS-evoked hyperpolarization was observed. In contrast, desensitization of P2Y receptor with 2-MeSATP completely abolished the EFS-evoked hyperpolarization ($n=6$) or even converted it into a depolarization in some other preparations ($4.0 \pm 1.2\text{ mV}$; $n=3$) (Figure 5B).

P2Y receptor subtyping

The P2Y receptor subtype involved in EFS-evoked hyperpolarization was investigated. A specific P2Y₁ receptor antagonist, MRS 2179 (300 nM), completely abolished the EFS-evoked hyperpolarization (Figure 6a; $n=5$). Another specific P2Y₁ antagonist, A3P5PS, at a concentration of $10\text{ }\mu\text{M}$, similarly abolished the response ($n=7$) or even converted it to a small

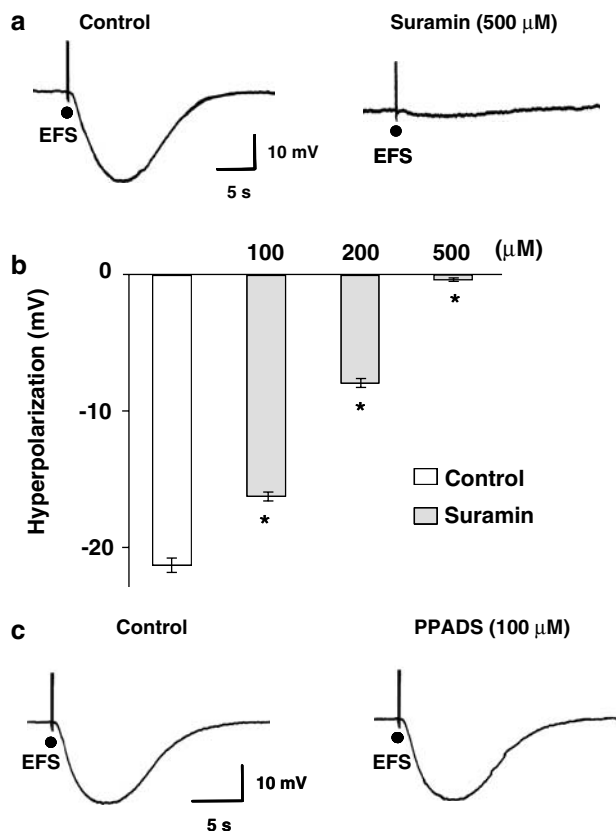


Figure 3 Effects of suramin on the EFS (using a train of five pulses)-evoked hyperpolarization. (a) Typical recording showing the effect of suramin ($500\text{ }\mu\text{M}$; $n=5$) on the EFS-evoked hyperpolarization. (b) Summary graph showing concentration-dependent inhibition of suramin on the amplitude of EFS-evoked hyperpolarization. (c) Typical recording showing the effect of PPADS ($100\text{ }\mu\text{M}$; $n=5$) on the EFS-evoked hyperpolarization. Membrane potentials for (a, c) were -60 and -59 mV , respectively.

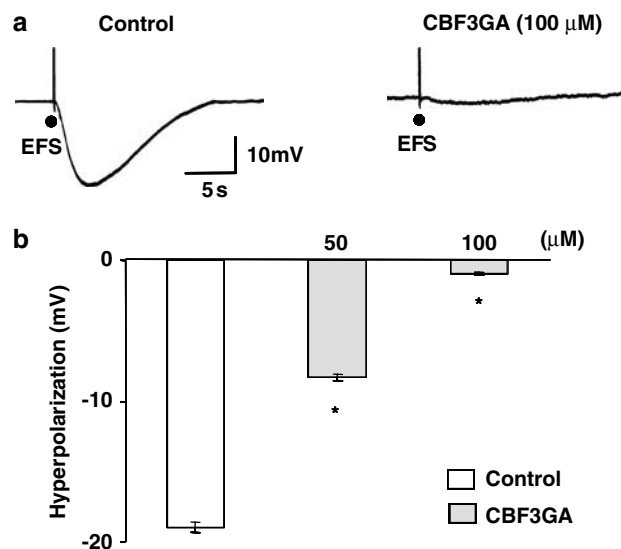


Figure 4 Effects of CBF3GA on the EFS (using a train of five pulses)-evoked hyperpolarization. (a) Typical recording showing the effect of CBF3GA ($100\text{ }\mu\text{M}$; $n=6$) on the EFS-evoked hyperpolarization. (b) Summary graph showing concentration-dependent inhibition of CBF3GA on the amplitude of EFS-evoked hyperpolarization. Membrane potential for (a) was -62 mV .

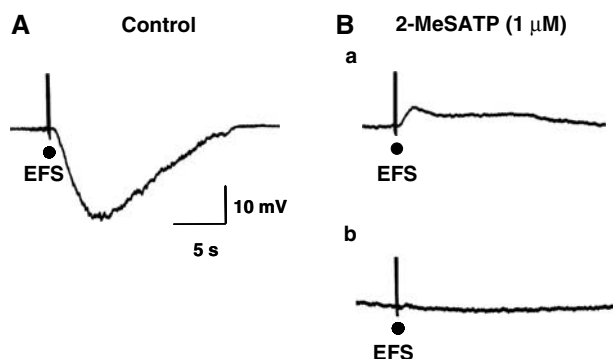


Figure 5 Effect of desensitization of P2Y receptor by its agonist, 2-MeSATP, on the EFS (using a train of five pulses)-evoked hyperpolarization. (A) Typical recordings showing EFS-evoked hyperpolarization. (B) Typical recording showing the effect of P2Y receptor desensitization by its agonist 2-MeSATP that EFS-evoked hyperpolarization was converted to a small depolarization (1 μM; $n=3$). Membrane potential for (A, Ba, Bb) were -62 , -60 and -61 mV, respectively.

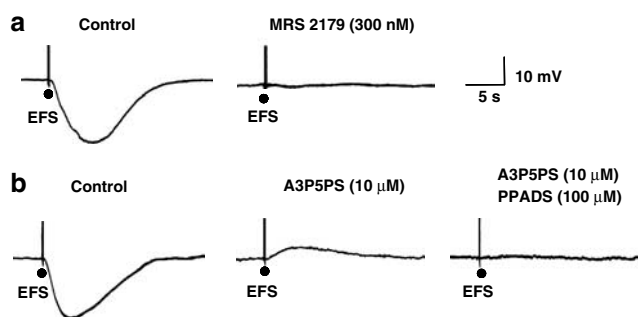


Figure 6 Effects of P2Y1 receptor antagonists on the EFS (using a train of five pulses)-evoked hyperpolarization and effect of P2X receptor antagonist on depolarization. (a) Typical recording showing the effect of MRS 2179 (300 nM; $n=5$) on the EFS-evoked hyperpolarization. (b) Typical recording showing the effects of A3P5PS (10 μM; $n=7$) on the EFS-evoked hyperpolarization, and effect of PPADS (100 μM; $n=3$) on the A3P5PS-induced depolarization. Membrane potentials for (a, b) were -60 and -62 , respectively.

depolarization in some preparations (0.5–3 mV; $n=3$); the resultant depolarizing response was blocked by the P2X receptor antagonist PPADS (100 μM) (Figure 6b).

Exogenous application of ATP

For further confirmation of the contribution of ATP to EFS-evoked hyperpolarization exhibited by the circular smooth muscle cells of chicken anterior mesenteric artery, the effect of exogenously applied ATP was investigated using preparations with or without endothelia. In case of preparations with intact endothelia, exogenous application of ATP produced slowly developing hyperpolarization in a dose-dependent manner. The hyperpolarizing effect was recorded even after using nanomolar concentration of ATP, where 100 nM produced a hyperpolarization ranging in amplitude between -0.7 and -3.2 mV (Figure 7). Hyperpolarizations produced by 100 nM ATP were completely blocked after prior incubation

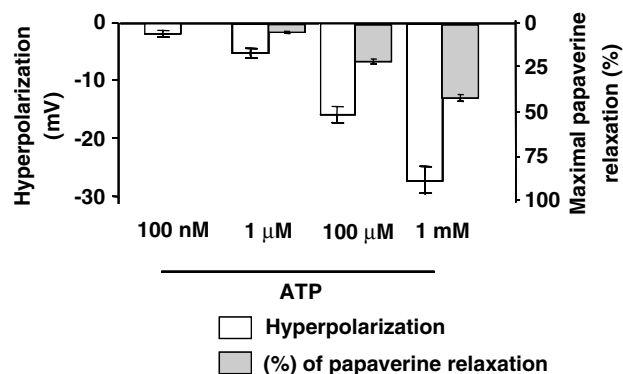


Figure 7 Effect of exogenously applied ATP on membrane potential and muscular tone of chicken mesenteric artery. Summary graph showing concentration-dependent membrane responses of ATP ($n=6$) and summary graph showing concentration-dependent relaxation of ATP ($n=7$).

of the preparations with either suramin (100 μM) or CBF3GA (50 μM).

As shown in Figure 7, experiments of mechanical recording revealed that ATP (100 nM–1 mM) relaxed the NE-precontracted endothelium-intact preparations in a dose-dependent manner. However, such relaxant effect of ATP was not observed in preparations with denuded endothelium. ATP-induced relaxation was evident even at low concentrations (100 nM) parallel to those of ATP-induced hyperpolarization.

Discussion

The primary aim of this study was to investigate the mediators and the neuroeffector mechanisms of the nerve stimulation-induced hyperpolarization in the circular smooth muscle cells of first-order branches of chicken anterior mesenteric artery. The major finding was that ATP released from nerve terminals innervating the first-order branches of chicken anterior mesenteric artery mediates a hyperpolarization by acting at P2Y receptors on the endothelium (an indirect action) and in a few cases EFS-evoked depolarization by ATP acting on the smooth muscle (a direct action). This conclusion is based on the following lines of evidence: (i) sensitivity of the EFS-evoked hyperpolarization to the neuronal blocker TTX, (ii) abolition of the hyperpolarization response by endothelial denudation, (iii) blockade of the response by the nonspecific purinergic antagonist, suramin, and by the P2Y receptor antagonist CBF3GA, (iv) evoking of a slowly developing hyperpolarizing response, similar to that mediated by EFS, by exogenous application of ATP and of the P2Y receptor agonist 2-MeSATP, (v) conversion of the hyperpolarizing response to a small depolarizing one after desensitization of the P2Y receptor by long-time application of 2-MeSATP, (vi) blockade of the hyperpolarizing response by the specific antagonists of P2Y1 receptor, MRS 2179 and A3P5PS.

To our knowledge, this study is the first to investigate the membrane potential properties of the circular smooth muscle cells of first-order branches of chicken anterior mesenteric artery. The resting membrane potential did not differ greatly from that of other animals being -60 mV or lower (Levick, 1995). Unlike mammalian circular arterial smooth muscle cells

in which EFS evokes a fast EJP with short latencies of 10–20 ms and total durations of 0.3–1 s (Thapaliya *et al.*, 1999), EFS of the circular smooth muscle cells of first-order branches of chicken anterior mesenteric artery produced hyperpolarizations that were slowly developing (641.7 ± 81.9 ms latency; 4.1 ± 0.2 s time to peak) and longlasting (21.8 ± 1.2 s total duration).

Inhibitory neurotransmission has been reported in different blood vessels. NO released from nerves has been proposed to mediate neurally evoked relaxation in various mammalian arteries (Toda & Okamura, 1992). Cholinergic nerve stimulation-induced hyperpolarization and/or vasodilatation was also reported in arterioles of guinea-pig submucosal plexus (Kotecha & Neild, 1995) and choroidal arterioles (Hashitani *et al.*, 1998) and rabbit lingual artery (Brayden & Large, 1986). Thapaliya *et al.* (1999) demonstrated that ATP released from the perivascular nerves may diffuse as far as the endothelium and activate P2Y2-like receptors to induce the release of EDHF in thin hamster mesenteric arteries, but not in thick arteries. These findings may be consistent with ours. We suppose that the only way by which ATP transverse to endothelium is diffusion *via* intercellular spaces. That is because application of ATP from the adventitial side produced the response, even when both side ends of the preparation were closed by threads.

In the present experiments, the nerve stimulation-induced hyperpolarization was blocked by blocking the purinoceptors with CBF3GA. These results indicate that the response may be evoked by activation of purinergic neurons and P2Y receptors. The significant inhibition of the transient hyperpolarization by the purinoceptor antagonist is unlikely to be due to a nonspecific action, because this antagonist has been demonstrated to be a selective P2Y receptor antagonist in vascular tissues (Burnstock & Warland, 1987; Hopwood & Burnstock, 1987). It has also been reported that the desensitization of P2Y receptors by 2-MeSATP abolishes electrical and vasodilator responses evoked by purinergic nerve stimulation in various vascular tissues (Hourani *et al.*, 1993; Ziganshin *et al.*, 1994). This may be consistent with our results where EFS-evoked hyperpolarization was abolished by desensitization of P2Y receptors by 2-meSATP, but not by that of P2X receptors using α, β -meATP. Currently, eight mammalian P2Y receptor subtypes have been cloned and functionally characterized, namely, P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 and P2Y14 (Ralevic & Burnstock, 1998; von Kugelgen & Wetter, 2000; Sak & Webb, 2002). Data presented in this study showed that EFS-evoked hyperpolarization was completely blocked by the specific antagonists of P2Y1 receptor, MRS 2179 (Camaioni *et al.*, 1998) and A3P5PS (Boyer *et al.*, 1996). In a few cases the hyperpolarizing response was not only abolished but also converted to a depolarizing response. These data indicate that the subtype involved in the hyperpolarizing response is likely to be a P2Y1 receptor.

Adventitial application of ATP resulted in a slowly developing hyperpolarizing response that was not evident after endothelial denudation. This finding suggests that the ATP-mediated hyperpolarization is endothelium-dependent, and that it is mediated *via* P2Y1 receptor located on the endothelium. This may be further supported by what has been previously reported that P2Y receptors are typically located on the endothelium (O'Connor *et al.*, 1991). The finding that both EFS-evoked and ATP-mediated hyperpolarizations were abolished by endothelium denudation may indicate that the neurally released ATP diffuses to the endothelium and acts on P2Y1 receptor located there, releasing some inhibitory substance. This substance, in turn, exerts its action on the vascular circular muscle, hyperpolarizing it. It has been reported that ATP-stimulated NO release occurred in endothelial cells from the guinea-pig pulmonary artery (Liu *et al.*, 1992). In addition, stimulation of endothelium with ACh is reported to release prostacyclin in guinea-pig coronary artery (Parkington *et al.*, 1993). However, in our experiments, treatment with L-NAME and indomethacin did not modify the nerve stimulation-induced hyperpolarization, suggesting that the involvement of NO and prostacycline is unlikely. There are two hypotheses explaining this result. One is that ATP-induced hyperpolarization might be explained on the basis of release from endothelial cells of EDHF. Similar data have been reported in the small mesenteric arteries of hamster (Thapaliya *et al.*, 1999). The second hypothesis is the movement of ATP-mediated hyperpolarization in the endothelial cells to the neighbor circular smooth muscle cells *via* cell–cell gap junctions (Segal, 1994).

Tension-recording experiments revealed that ATP mediates relaxant responses in a dose-dependent manner. This relaxation was completely absent in endothelium-denuded preparations, indicating that the intact endothelium is a must for mediating relaxation parallel to the membrane potential results.

In conclusion, the present findings demonstrate that ATP released from the perivascular nerves supplying first-order branches of chicken anterior mesenteric artery may diffuse as far as the endothelium, activating P2Y1 receptors to induce hyperpolarization *via* either release of substance other than NO and prostacycline, may be the yet-unknown EDHF, or mediating hyperpolarization in the endothelial cells that is transferred to the smooth muscle cell *via* gap junctions. ATP also may act locally on the smooth muscle cells, activating the less-sensitive P2X receptor to induce depolarization. Thus, we propose that ATP may play an important physiological role in the local regulation of vascular resistance in chicken anterior mesenteric artery.

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