Regulation of vascular tone by purinoceptor-activation in vascular smooth muscle and endothelial cells

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Ionic membrane currents activated by UTP, a P_{2y}-purinoceptor agonist, and its functional roles to regulate vascular tone were examined in rat aortic smooth muscle (SMCs) and endothelial cells (ETCs). When cells were superfused with K-aspartate rich pipette solution, application of UTP activated transient and following oscillatory inward currents (I_{osci}) at -50 mV in SMCs. The reversal potential of I_{osci} was approximately -35 mV, which was close to the theoretical equibrilium potential of Cl^-. In current-clamp mode, UTP produced depolarising oscillation of membrane potential in SMCs. On the other hand, application of UTP to ETCs elicited membrane currents reversed at -80 mV, and hyperpolarized the cells. When transmembrane potential was recorded from ETCs in an aortic smooth muscle segment (ETC/SMC preparation), UTP elicited only hyperpolarization. Having no effects on dispersed ETCs, phenylephrine (Phe) induced oscillatory depolarization in ETCs in ETC/SMC preparation. While UTP elicited tonic contraction in rat aorta muscle ring without endothelium, the response was significantly small and transient in endothelium-denuded ring. However, an inhibitor of nitric oxide (NO) synthase, effectively reversed UTP-mediated relaxation in aorta with endothelium. Analysis of the expression of P2Y receptor mRNA transcripts revealed that P_{2Y2} and P_{2Y6} receptors were predominantly detected in rat aortic myocytes. These results suggest that UTP itself is a stimulant to both SMCs and ETCs. However, under physiological conditions where intact ETCs were present, UTP predominantly relaxes the muscle via NO-mediating pathway.

Keywords: smooth muscle, endothelial cells, UTP, myo-endothelial conduction

Introduction

The basal tone of vascular smooth muscle is regulated by many factors, such as contracting and relaxant substances mainly coming from nerve endings and endothelium. Stimulation of sympathetic nerves, which releases both noradrenaline and adenosine 5'-triphosphate (ATP), can produce phasic and tonic contractions in vascular smooth muscle via activation of P2 receptors and α-adrenoceptors on cell membrane, respectively ([1],[2],[3]). Pharmacological and electrophysiological studies revealed that several types of P2 receptors are involved in regulation of vascular tone ([2],[4]). P2 receptors (P_{2y2} and P_{2y4}) which have a high affinity to uridine triphosphate (UTP) have been demonstrated in various vascular tissues ([5]). P_{2y2}/P_{2y4} are suggested to be linked to phospholipase C and to increase intracellular Ca^{2+} ($[Ca^{2+}]_i$) by activation of Ca^{2+} release via inositol 1,4,5-trisphosphate mediating pathway ([6]) and their activation elicits contraction in aorta, carotid and mesenteric arteries ([5],[6],[7],[8]). On the other hand, P_{2y2}/P_{2y4} also exist in vascular endothelial cells and mediate the release of vaso-relaxants such as endothelium-dependent relaxing factors (EDRF, [9]). Although UTP affects both vascular smooth muscle and endothelial cells as a stimulant to increase their $[Ca^{2+}]_i$, the physiological output for regulation of vascular tone is clearly opposite.

Recently, we have demonstrated that UTP at concentrations higher than 1 μM activates Ca^{2+}-dependent Cl^- current in rat aortic myocytes and causes oscillatory change

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of membrane potential and corresponding contraction ([8]). Since it has been reported that UTP produces endothelium-dependent relaxation in rat aorta, integral analysis about effects of UTP on smooth and endothelial cells are required to understand the functional role of UTP to regulate vascular tone. Moreover, electrical response elicited in SMCs can conduct to ETCs and vice versa via a myo-endothelial pathway, modulating their cellular functions ([10],[11],[12]). It is therefore, expected that UTP-induced membrane potential oscillation in smooth muscle conducts to and affects neighbouring endothelium. The purpose of the present study is to investigate integral response of SMCs and ETCs to UTP using a vascular preparation where interaction between SMCs and ETCs remains intact.

MATERIALS AND METHODS

Cell-isolation

Male Wistar rats weighing 200-300 g were anesthetized by ether or stunned and killed by exsanguination. All experiments were carried out in accordance with guiding principles for the care and use of laboratory animals (the Science and International Affairs Bureau of the Japanese Ministry of Education, Science, Sports and Culture) and also with the approval of the ethics committee in Aichi Gakuin University. Single aortic cells were dispersed by using collagenase (Amano, Pharm., Co., Nagoya, Japan) as previously described ([8]). For isolation of endothelial cells, the isolated aorta was cleaned of fat and connective tissue, and cut open longitudinally. The tissue was pinned in a 60 mm culture dish filled with Ca^{2+}-Mg^{2+} free phosphate-buffered solution (PBS) containing 0.05% collagenase (Amano, Nagoya, Japan) and 0.05% dispase (Boehringer Mannheim, Tokyo, Japan). The culture dish was kept in an incubator at 37 ℃ for 60 min and then the enzyme solution containing isolated endothelial cells was centrifuged at 1200 rpm for 10 min. Thereafter, the supernatant was removed and the pellet was resuspended in the culture medium. Endothelial cells were allowed to attach to gelatin-coated glass coverslips over 8 hr at 37 ℃ in air with 5% CO2 and used within 48 hr.

Electrophysiological experiments

Membrane currents and transmembrane potentials were recorded with amphotericine B-perforated patch methods. Current and voltage signals were stored on hard disk on an IBM compatible computer by using an analog-digital converter. When transmembrane potential was recorded from endothelial cells in an intact artery, a vascular segment (2 mm×2 mm) was fixed on the luminal side up on the rubber at the bottom of a chamber.

All experiments were carried out at 35±1 ℃ in a physiological salt solution (PSS) containing (mM) NaCl 137, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2.2, glucose 10, and HEPES 10. The pH was adjusted to 7.4 with 10N NaOH. Ca^{2+}, Mg^{2+}-free Hanks' solution for dispersing cells contained (mM) NaCl 137, KCl 5.4, Na_2HPO_4 0.168, KH_2PO_4 0.44, glucose 5.55 and $NaHCO_3$ 4.17. The pipette solution contained (mM) K-aspartate 110, KCl 30, $MgCl_2$ 4, HEPES 10 and amphotericin B 50-100 μg/ml. The pH in these solutions was adjusted to 7.2 with KOH.

Measurement of tension development

After connective tissue and adventia were removed carefully, 1-mm lengths of vessel ring were cut out from the aorta. When endothelial cells were removed, the inner wall of the vessel was rubbed with a cotton pad. The vessel ring was set in a 4 ml organ bath to measure isometric tension with a force-transducer and perfused with Krebs' solution which was maintained at 37±1 ℃ and gassed with 95% O_2 and 5% CO_2. The composition of Krebs' solution was (mM); NaCl 117, $MgCl_2$ 1.2, $CaCl_2$ 2.2, KCl 4.7, KH_2PO_4 1.2, Glucose 14 and $NaHCO_3$ 25.

Reverse Transcription (RT)-PCR amplification

RT-PCR amplification for P2Y receptors expression was performed as described previously ([13]). Total RNAs were extracted from freshly dissociated rat aortic myocytes by the acid guanidium thiocyanate-phenol method following digestion with RNase-free DNase, and RT was performed according to the manufacturer's instructions. Oligonucleotide sequences of primers specific for P_{2Y1}, P_{2Y2}, P_{2Y4}, P_{2Y6}, P_{2Y12}, P_{2Y14} of the rat (sense and antisense, 5' to 3') were GTGTGCTGGTATGGCTCATTGT and GGAGTCGTAGCAGGTGACAGTTT for P_{2Y1}, CCTGGCA GTTTCTGACTCTCTCTAC and GCTTGCAGAGCACTGT GCTAAA for P_{2Y2}, TACCATCCTGTGCCATGACACT and TCAAGAAGGGCAAACCAAAGAG for P_{2Y4}, CCTGTTCA CTGCCCCTACTTATCT and AGGTATCGCTGGAAGCT AATGC for P_{2Y6}, CCAGCCCCAGCAATCTTTT and CCTCCTGTTGGTGAGAATCATGT for P_{2Y12}, and AGCAGATCATCCCCATGTTGTAC and ACTCTTAGAG CTGGGCACGTAAAA for P_{2Y14}. The thermal cycler program used for PCR amplification included a 0.5 min denaturation step at 94℃, a 0.5 min annealing step at 55

℃ and a 0.5 min primer extension step at 72 ℃ for 35 cycles (GeneAmp 2400, Perkin Elmer ABI). Amplified products were separated on 1.5% agarose gels in Tris acetate/EDTA buffer, visualized with 1 μg/ml ethidium bromide, and documented on FAS 1000 (TOYOBO). As a control signal, glycelaldehyde-3-phosphate dehydrogenase (GAPDH) expression was analyzed (sense 5' to 3': CATGGCCTTCCGTGTTCCT, antisense 5' to 3': CCTGCTTCACCACCTTCTTGA).

Drugs

The following drugs were used: uridine triphosphate-2Na (UTP, Yamasa), acetylcholine (ACh, Wako), phenylephrine (Phe, Wako), methoxamine (Metho, Wako), L-nitro-arginine methylester (LNAME, Dojindo, Kumamoto, Japan), amphotericin B (Sigma). Each drug, except LNAME and amphotericin B, was dissolved in distilled water to make 10 mM stock solutions. LNAME and amphotericin B were dissolved in dimethylsulfoxide (DMSO, 100 mM as stock). These solvents (distilled water and DMSO) had no effect on membrane currents and potentials, and tension. All drug concentrations are expressed as the final concentration in the solutions and the pH of the solution was readjusted after addition of drugs. All drugs were applied at a constant flow rate of 0.1 ml s^{-1}. A change of solution could be achieved within 5 s.

Results

In the previous study, we demonstrated that effects of UTP on membrane currents and potential in rat aortic smooth muscle cells (RASMCs, [8]). In Fig.1, these effects were summarized. A cell, which was superfused with K-aspartate rich pipette solution, was exposed to 10 μM UTP under voltage-clamp conditions (Fig.1A). When the cell was alternatively voltage-clamped for 5 s at -50 and -30 mV, inward currents were predominantly elicited at -50 mV by UTP (Fig.1Aa). In the presence of UTP, thereafter, oscillatory (I_{osci}) and the underlying sustained inward currents (I_{sus}) were activated at -50 mV (the lower panel of Fig.1Aa). Application of ramp waveform pulses (90 mV/100 ms) as a voltage command revealed that outwardly rectifying membrane currents were involved in both I_{osci} and I_{sus} (Fig.1Ab), and the subtraction ('2-1', see inset in Fig.1Ab) showed that I_{osci} was reversed at -32 mV, which is close to the theoretical equibrilium potential of Cl^- (-35mV). In addition, as previously described ([8]), I_{osci}

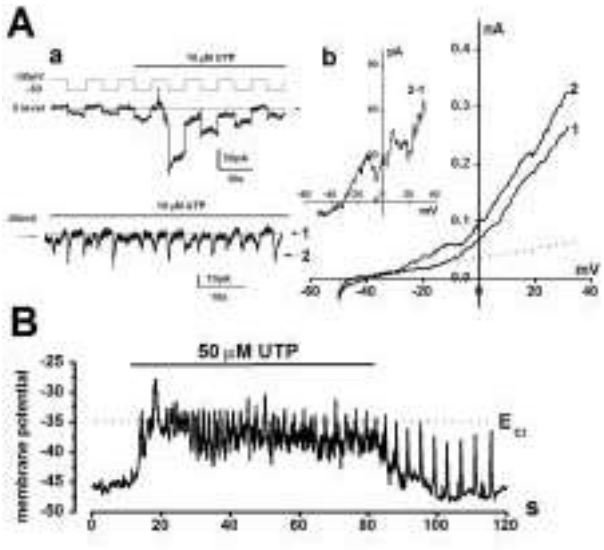

Fig.1 Effects of UTP on membrane currents (A) and potential (B) in single rat aortic smooth muscle cells (RASMCs). **A:** A cell was depolarized for 5 s from a holding potential of -50 mV to -30 at 0.2 Hz. Large transient (upper panel in (a)), and following sustained (I_{sus}) and oscillatory (I_{osci}) inward currents ('1 and 2' in lower panel in (a)) were elicited by application of 10 μM UTP. Dotted lines in (a) indicate zero current. In (b), ramp waveform voltage command pulses were applied at the time indicated by "1 and 2" in lower panel in (a) to obtain the current and voltage relationship of I_{sus} and I_{osci}. The inset figure shows the subtracted current (2-1). **B:** Under the current-clamp conditions, UTP (50 μM) caused oscillatory depolarization. E_{Cl} indicates the theoretical equibrilium potential of Cl^- in the present experimental conditions.

was effectively inhibited by 10 μM niflumic acid, an effective blocker of Ca^{2+}-activated Cl^- current ($I_{Cl\text{-}Ca}$) (not shown), suggesting that activation of $I_{Cl\text{-}Ca}$ is responsible for I_{osci}. Under current-clamp conditions (Fig.1B), application of 50 μM UTP to RASMCs elicited oscillatory depolarization ([8]). Moreover, RASMCs were shortened by 28.7± 2.7% (n=19, not shown) in the presence of 50 μM UTP. Taken together, it is obvious that UTP affects RASMCs as an excitatory agonist and causes contraction in aorta muscle.

In Fig.2, effects of UTP on membrane currents and potential in rat aortic endothelial cells (RAETCs) were examined. At a holding potential of -50 mV, application of 30 μM UTP as well as 10 μM acetylcholine (ACh) elicited outward currents (Fig.2A). The amplitude of the outward currents at -50 mV elicited by UTP (115±25.8 pA, n=6) was not significantly different from that by ACh (80.8± 30.0 pA, n=5). Representative current and voltage (I-V) relationships of UTP- and ACh-activated membrane currents were shown in Fig.2B. These currents were

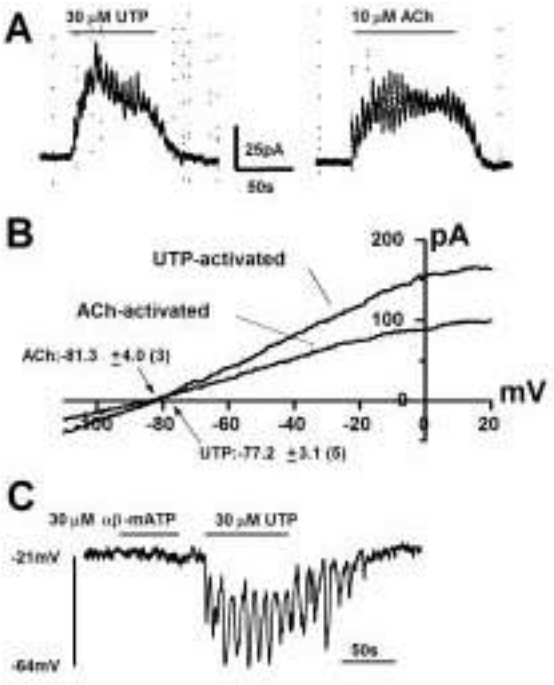

Fig.2 Effects of UTP on membrane currents (A) and potential (C) in single rat aortic endothelial cells (RAETCs). **A:** Under voltage-clamp conditions at -50 mV, 30 μM UTP and 10 μM ACh elicited oscillatory outward currents. **B:** Current and voltage relationships of UTP- and ACh-induced currents were obtained by ramp waveform voltage command and illustrated. Both currents were inwardly rectified at positive potentials and reversed at around -80 mV. **C:** In current-clamp mode, a RAETC had oscillatory hyperpolarization in the presence of UTP, while not in the presence of a P_{2X} receptor agonist, αβ-methylene ATP (αβ-meATP).

obtained by subtracting the current in the presence of a drug from that in the absence. Both membrane currents were reversed at around -80 mV; -77.2±3.1 (n=5) and -81.2±4.0 mV (n=3) for UTP and ACh, respectively, indicating that activation of K^+ currents was responsible for these currents. Under current-clamp conditions, RAETCs were significantly hyperpolarized from -32.4±4.6 to -69.1±1.5 mV ($p<0.01$, n=5) by 30 μM UTP (Fig.2C) and from -30.5±4.2 to -65.2±3.0 mV ($p<0.01$, n=5) by 10 μM ACh (data not shown). In contrast, 30 μM αβ-mATP, a selective P_{2x} agonist, had little effects on membrane potential of RAETCs (Fig.2C, n=4).

It was shown that UTP had a distinct action on RASMCs and RAETCs: depolarization and hyperpolarization, respectively. In Fig.3, therefore, effects of UTP on endothelial cells in intact aorta segment (ETC/SMC preparation) were examined and interaction between SMCs and ETCs was investigated. Resting membrane potential of ETCs recorded in ETC/SMC preparation was -50.0±2.1 mV (n=27). Application of 30 μM UTP to ETCs in ETC/SMC preparation elicited a hyperpolarization (Fig.3Aa). On the other hand, 3 μM phenylephrine (Phe) induced oscillatory depolarization in ETCs in ETC/SMC preparation (Fig.3Aa, n=13, [11],[12]). In contrast, neither 10 μM methoxamine (Metho, n=4, Fig.3Ab) nor 3 μM Phe (not shown, n=3) affected RAETCs, while application of UTP and ACh effectively

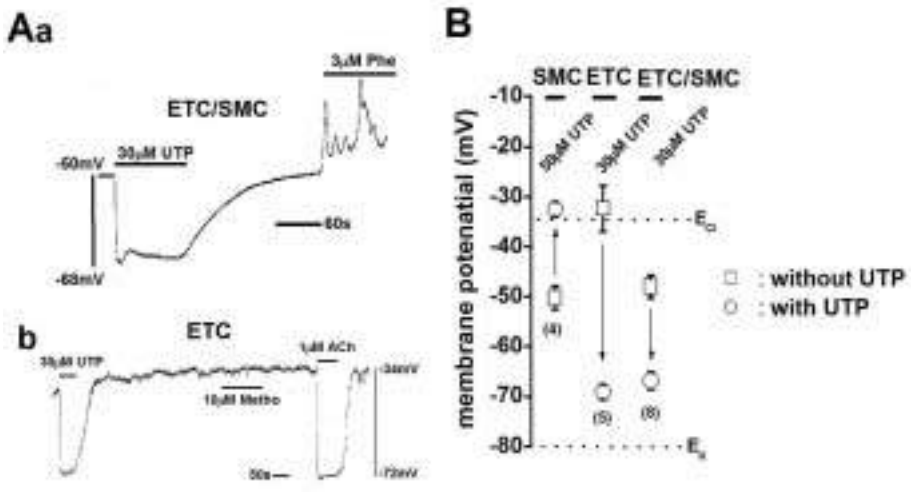

Fig.3 Electrical responses of ETCs in an intact aorta segment (ETC/SMC preparation) to UTP and α-adrenergic agonists. Transmembrane potential was recorded from ETCs in ETC/SMC preparation. **A:** Application of UTP caused a hyperpolarization, whereas 3 μM phenylephrine (Phe) elicited oscillatory depolarization **(Aa)**. **(Ab):** A dispersed ETC did not have any response to 10 μM methoxamine (Metho) and 3 μM Phe (not shown) under the current-clamp conditions. In contrast, both UTP and ACh produced significant hyperpolarizations. **B:** Summary of membrane potential changes elicited by UTP in RASMCs (SMC), RAETCs (ETC) and ETCs in ETC/SMC preparation (ETC/SMC). Each arrow indicates the change direction in membrane potential by UTP. E_{Cl} and E_K are theoretical equilibrium potential of Cl^- and K^+, respectively, under the present experimental conditions.

hyperpolarized the cell (Fig.3Ab, Fig.3B), implying that Phe- but not UTP-induced depolarization in SMCs effectively conducts to ETCs and contributes to endothelial depolarization in ETC/SMC preparation ([11],[12]).

To explain functional roles of UTP in vascular tissue, effects of UTP on vascular tone in rat aorta muscle ring with or without endothelium were examined to measure tension development (Fig.4). Amplitude of the contraction caused by UTP was normalized to that by 1 μM Phe. Application of 30 μM UTP produced a tonic contraction in endothelium-denuded muscle ring (n=5, Fig.4aA). Addition of 30 μM N^G-nitro-L-arginine (LNAME), a nitric oxide (NO) synthase inhibitor, to the muscle ring with tonic contraction, had no effects on the UTP-induced contraction (21.3±2.8 vs. 18.7±2.2%, n=5, P>0.05, Fig.4Aa, B). In contrast, in aorta muscle ring with endothelium, UTP induced small and transient contraction (the amplitude with and without endothelium; 3.2±0.7 and 21.3±2.8 %, P<0.01, n=5, Fig.4Ab, B). Addition of 30 μM LNAME to the muscle ring, however, produced substantial contraction (3.2 ± 0.7 vs. 13.5 ± 1.3%, P<0.01, n=5, Fig.4Ab, B), demonstrating that NO produced by UTP in ETCs effectively prevents muscle tone from being higher.

Next, we evaluated the expression of P_{2Y} receptor mRNA transcripts (P_{2Y1}, P_{2Y2}, P_{2Y4}, P_{2Y6}, P_{2Y12}, P_{2Y14}) in rat aortic myocytes. Total RNA isolated from acutely dispersed aortic myocytes was subjected to RT-PCR. Among these P_{2Y} receptors, transcripts of P_{2Y2} and P_{2Y6} mRNA were predominantly detected (Fig. 5, three separate experiments).

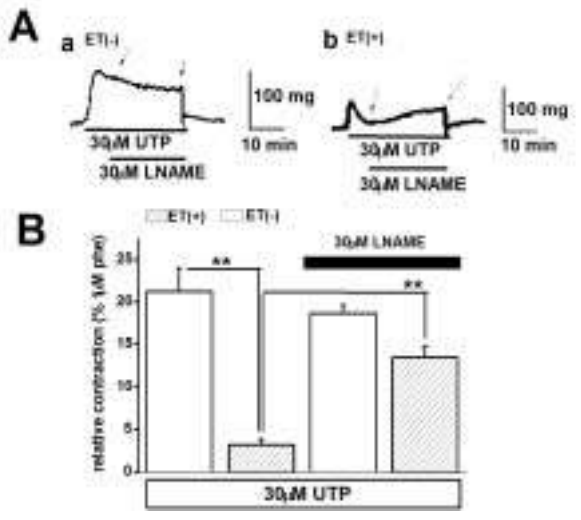

Fig.4 Effects of UTP on tension development in an aortic ring preparation where endothelium was denuded **(Aa, ET(-))** or intact **(Ab, ET(+))**. In the presence of 30 μM UTP, 30 μM L-nitro-arginine-methylester (LNAME), an inhibitor of NO synthase, was applied. B: Summarized data describing effects of UTP and LNAME in endothelium intact- and denuded-aorta. The amplitude of contraction was normalized to that caused by 1 μM Phe. At the time indicated by arrows in **(A)**, the amplitude of contraction was measured.

Discussion

Application of UTP activated Ca^{2+}-dependent Cl^- current and caused oscillatory depolarization in RASMCs. It has been reported that vasoactive substances such as angiotensin II, endothelin, norepinephrine and vasopressin elicit oscillatory change of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) ([3]). The raise of $[Ca^{2+}]_i$ subsequently activates at least two types of Ca^{2+}-dependet currents; $I_{K\text{-}Ca}$ and $I_{Cl\text{-}Ca}$, which make cell-excitability low and high, respectively ([3]). It has been shown that $[Ca^{2+}]_i$ in RASMCs was oscillated in the presence of UTP ([6]). Interestingly, activation of $I_{K\text{-}Ca}$ was transient and disappeared during application of UTP, whereas oscillatory activation of $I_{Cl\text{-}Ca}$ was sustained under the same experimental conditions. Since Ca^{2+}-sensitivity of $I_{Cl\text{-}Ca}$ is higher than that of $I_{K\text{-}Ca}$ ([14],[15]), the raise of $[Ca^{2+}]_i$ in the presence of UTP might not be enough to activate $I_{K\text{-}Ca}$. Alternatively, the activation of $I_{K\text{-}Ca}$ could be inhibited by a certain factor produced by receptor-stimulation, such as a protein Kinase C ([16]) and/or GTP-binding proteins ([17]) even though $[Ca^{2+}]_i$ is enough for the activation. Preferential activation of $I_{Cl\text{-}Ca}$ and corresponding oscillatory depolarization by UTP in RASMCs contribute to a sustained contraction in the presence of UTP ([8], Fig.4Aa in the present study).

When P_2 purinoceptor was activated by UTP, outward currents and hyperpolarization were elicited in RAETCs under the same experimental conditions where $I_{Cl\text{-}Ca}$ and oscillatory depolarization were induced by UTP in RASMCs. The outward currents ($I_{K\text{-}Ca}$) were responsible for activation of small and intermediate conductance Ca^{2+}-dependent K^+

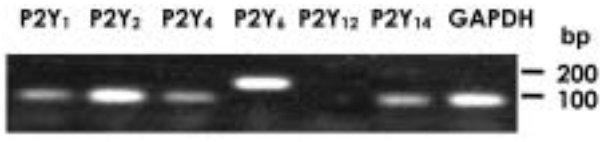

Fig.5 P_{2Y} receptor mRNA transcripts in rat aortic myocytes. A, P_{2Y2} and P_{2Y6} mRNA transcripts were substantially detected in freshly dispersed aortic myocytes using PCR amplification (35 cycles). As a control, GAPDH was amplified.

channels (SK and IK), because application of charybdotoxin and apamin, an inhibitor of SK and IK, respectively, blocked the outward currents ([15]). Similar activation of $I_{K\text{-}Ca}$ by receptor-stimulation has been reported in various vascular endothelial cells ([18]). The negative shift of membrane potential in endothelial cells during stimulation of receptors promotes Ca^{2+}-influx into the cell and subsequently potentiates production of vasoactive substances such as nitric oxide (NO). Consistently, the present result indicates that tension development caused by UTP was significantly larger in aortic ring without endothelium. Moreover, addition of LNAME potentiated the UTP-induced contraction in endothelium-intact muscle, indicating that NO produced by stimulation of P_2 purinoceptor in RAETCs keeps myogenic tone lower. These strongly suggest that UTP has dual and opposite roles in the regulation of vascular tone: a positive and negative contributor in intact vessels via smooth muscle and endothelial cells, respectively. Therefore, when endothelial cells are impaired under pathophysiological conditions such as hypertension, diabetes or arteriosclerosis, ATP- and UTP-induced oscillatory $I_{Cl\text{-}Ca}$ and the attributable change of membrane potential in vascular smooth muscles may be critical for the developement of vascular tone. Even in the presence of 100 μM LNAME, however, UTP-induced contraction had a transient component in aorta with endothelium (data not shown). Since UTP also potentiates release of prostanoids in endothelial cells ([19]), it is possible that the metabolites such as PGI_2 and PGD_2 induces relaxation of aorta muscle after the contraction. Alternatively, it is possible that UTP releases endothelium-derived hyperpolarizing factor from endothelial cells, which is resistant to treatment with LNAME ([20]).

It is well known that P_2 receptors are widely distributed among vascular tissues ([3]). P_{2X} receptors which are selectively stimulated with αβ-meATP are only abundant in vascular smooth muscle and consistently, transient depolarization followed by contraction was observed by application of αβ-meATP to RASMCs ([2,8]). In contrast, in the present study, application of αβ-meATP had no effects on RAETCs, indicating that P_{2X} receptors may not be expressed in aortic endothelial cells. On the other hand, P_{2Y} receptors are widespread in vascular endothelial as well as smooth muscle cells ([7]). A number of studies reports that P_{2Y} receptors in vascular smooth muscle are coupled to contractile responses, whereas activation of P_{2Y} receptor in endothelial cells produces vasorelaxants to dilate vasculatures ([7]). In the present study, we examined expression of P_{2Y} receptor mRNA transcripts in RASMCs and concluded that P_{2Y2} and P_{2Y6} were predominantly detected. Activation of P_{2Y2} as well as P_{2Y6} does increase intracellular Ca^{2+} concentration ([21]), strongly suggesting that both P_{2Y} receptors have an obligatory role in activation of UTP-induced responses which are Ca^{2+}-dependent. In contrast, the type of P_{2Y} receptors in RAETCs were not determined in the present study because collection of pure RAETCs was difficult. Moreover, selective and potent antagonists for all types of P_{2Y} receptors have not been available to pharmacologically identify the subtype involved in UTP-induced responses in RASMCs as well as RAETCs. Nevertheless, it is proposed that P_{2Y2} is involved in UTP-induced response in rat aortic endothelium ([6,5,22]).

Taken together, UTP itself is a stimulant to both SMCs and ETCs. However, under physiological conditions where intact ETCs were present, UTP predominantly relaxes the muscle via NO-mediating pathway. Vascular tone is co-operatively regulated by UTP acting on both smooth muscle and endothelial cells. In pathophysiological conditions where vascular endothelium has dysfunction, UTP possibly causes larger vasocontraction via activation of P_{2Y2} and P_{2Y6}. Therefore, P_{2Y} receptors sensitive to ATP as well as UTP in vascular organs are a potential target to vascular diseases.

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