Full Paper

Dual Signaling Pathways of Arterial Constriction by Extracellular Uridine 5'-Triphosphate in the Rat

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Abstract. We investigated actions of uridine 5'-triphosphate (UTP) in rat aorta, cerebral and mesenteric arteries, and their single myocytes. UTP ($\geq 10 \mu\text{M}$) elicited an inward-rectifying current strongly reminiscent of activation of P2X₁ receptor, and a similar current was also induced by α,β -methylene adenosine 5'-triphosphate (ATP) ($\geq 100 \text{ nM}$). UTP desensitized α,β -methylene ATP-evoked current, and vice versa. The UTP-activated current was insensitive to G-protein modulators, TRPC3 inhibitors, or TRPC3 antibody, but was sensitive to P2-receptor inhibitors or P2X₁-receptor antibody. Both UTP (1 mM) and α,β -methylene ATP (10 μM) elicited similar conductance single channel activities. UTP ($\geq 10 \mu\text{M}$) provoked a dose-dependent contraction of de-endothelialized aortic ring preparation consisting of phasic and tonic components. Removal of extracellular Ca^{2+} or bath-applied 2',3'-O-(2,4,6-trinitrophenyl)-ATP (TNP-ATP) (30 μM) or nifedipine (10 μM) completely inhibited the phasic contraction while only partially reducing the tonic one. The tonic contraction was almost completely abolished by additional application of thapsigargin (2 μM). Similar biphasic rises in $[\text{Ca}^{2+}]_i$ were also evoked by UTP in rat aortic myocytes. In contrast to the low expression of TRPC3, significant expression of P2X₁ receptor was detected in all arteries by RT-PCR and immunoblotting, and its localization was limited to plasma membrane of myocytes as indicated by immunohistochemistry. These results suggest that UTP dually activates P2X₁-like and P2Y receptors, but not TRPC3.

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Keywords: uridine 5'-triphosphate (UTP), P2X receptor, TRPC3, non-selective cation channel

Introduction

It is well documented that adenosine 5'-triphosphate

(ATP) is released as a cotransmitter with noradrenaline simultaneously from the perivascular sympathetic nerve terminals and that released ATP evokes excitatory junction potentials and vasoconstriction (1 – 3). ATP is also released from several other types of cells including endothelial cells, erythrocytes, leucocytes, and platelets, and may function as an extracellular signaling molecule

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in the regulation of the vascular tone (3).

Uridine 5'-triphosphate (UTP) also mediates signaling from the extracellular side to the cell interior. UTP can be released from a variety of cells including platelets, endothelial cells, epithelial cells (e.g., airway and intestinal epithelial cells), and neural cells (e.g., C6 glioma cells and primary astrocytes) (4). In the cardiovascular system, the main source of UTP is endothelial cells from which it is released in response to hypoxia and inflammation (3). UTP is released to the luminal side of blood vessels and thus has a limited access to intact vascular smooth muscle. However, the accessibility may dramatically increase when endothelial integrity is disrupted, for example, arterial ruptures (hemorrhage) or disease state (stroke and atherosclerosis) (5–7).

Nucleotide receptors are classified into two groups, that is, ionotropic P2X receptors and metabotropic P2Y receptors coupled to guanosine 5'-triphosphate binding protein (G-protein) and downstream effectors. UTP has been recognized as a specific agonist for P2Y receptors (but not for P2X receptors) capable of producing vasoconstriction through intracellular Ca^{2+} release from the sarcoplasmic reticulum via activation of the P2Y₂, P2Y₄, and/or P2Y₆ receptor(s) / G_{q/11} / inositol 1,4,5-triphosphate (IP₃) receptor cascade (3, 8). However, several recent studies have shown that UTP can also induce a non-selective cation current, thereby depolarizing the membrane that in turn promotes Ca^{2+} influx through dihydropyridine-sensitive voltage-dependent Ca^{2+} channels (L-type VDCCs) and cause vasoconstriction (9). The induction of this cation current has been proposed to involve the activation of TRPC3 via phospholipase C (PLC)-linked P2Y₂, P2Y₄, and/or P2Y₆ receptor(s) and subsequent generation of IP₃ and diacylglycerol (10). In the same study, the current–voltage (I–V) relationship of UTP-induced cation current has been found to display inward-going rectification. However, this property does not match up with the reported outward-rectification of TRPC3-mediated current (11), but is rather similar to that of the P2X receptor–mediated current (12). This inconsistency prompted us to re-examine the nature of receptors involved in the actions of UTP especially with respect to P2X as well as P2Y receptors. To this end, we employed several functional techniques, that is, patch clamping, intracellular Ca^{2+} imaging, and tension recording techniques, combined with reverse transcriptase–polymerase chain reaction (RT-PCR), Western blotting, and immunostaining of cells and tissues. Our data suggest that UTP exerts dual effects on the contractility of rat arteries via activation of both ionotropic and metabotropic P2 receptors (i.e., P2X₁-like receptor together with P2Y receptors).

Materials and Methods

Tissue and cell preparation

Male Sprague-Dawley rats (10–11-week-old) were anesthetized by inhalation of diethylether and euthanized by decapitation. Arterial tissues (aorta, cerebral, and mesenteric arteries) were removed and placed in cold (4°C) physiological salt solution (PSS). For tension recording, aorta rings were placed in warmed (37°C) Krebs solution equilibrated with 5% CO₂ and 95% O₂. To obtain the cell preparation, arterial segments were enzymatically digested by incubating (37°C) them sequentially in low Ca^{2+} (0.5 mM) PSS containing papain (0.5 mg/mL), followed by 1 mM Ca^{2+} PSS containing type 2 collagenase (2 mg/mL; Worthington, Lakewood, NJ, USA). Digested arterial segments were transferred to PSS containing 1.5 mM Ca^{2+} , stored on ice, and used within 3 h. All animal experiments followed the guideline of the Local Animal Ethics Committee of Kyushu University.

Patch clamp recording

Whole-cell (voltage or current clamp) and single channel recordings were performed by using an Axopatch-1D patch clamp amplifier (Axon Instruments, Foster City, CA, USA) driven by an IBM compatible computer with pClamp v6.04 software (Axon Instruments). In detail, a TL-1 A/D, D/A board was used to generate and apply voltage pulses to clamped cells and to sample corresponding membrane currents. Current signals were low-pass filtered at 1 kHz and digitized at 5 kHz before being stored on a computer hard disk. Data were analyzed by Clampfit v.8.2, (Axon Instruments) after being filtered at 300 Hz with a Gaussian and an 8-pole Bessel filter, for whole cell and single channel recordings, respectively. For continuous recording, data were recorded and analyzed by the PowerLab/4SP system using Chart 5 software. Data analysis and illustration of the results were made by pClamp v8.1, Corel draw v11.0 (Corel Corporation, Ontario, Canada), and Kaleidagraph v3.04 (Synergy Software, Reading, PA, USA). All experiments were performed at room temperature (22°C–25°C).

Tension development

Aortae were cleaned of fat and connective tissues with a fine micro-scissors and forceps and cut into short rings (5–7 mm in length, 1 mm in width). The ring preparation was denuded of endothelium by tenderly rubbing with a wet, thin, twisted strip of tissue paper. Lack of endothelium-dependent relaxation to acetylcholine (10 μM) was confirmed in rings pre-contracted with phenylephrine (1 μM) prior to further experiments. Tension (T) of vessels was normalized by the following formula: $T_N = (T - T_0) / (L \times IC)$, in which T_N is the normalized

force (g), T_0 is the initial tension (g), L is the tissue wall length (mm), and IC is the internal circumference (mm, calculated after applying the initial tension). Data were recorded and analyzed by the PowerLab/8SP system on Chart 5 software and MLT050/D force transducer (AD Instruments Pty., Ltd., Bella Vista, NSW, Australia). The solution was pre-warmed at 37°C and propelled by a Minipuls 3 peristaltic pump (Gilson, Inc., Middleton, WI, USA). The flow rate was 3 mL/min.

Measurement of intracellular calcium

Cells were loaded with 2.5 μ M fura-2/acetoxymethyl-ester on ice for 30 min. Then they were plated on gelatin-coated, glass-bottom, 35-mm dishes for 5 min at room temperature (22°C – 25°C). The 100- μ L external solution was replaced 2 min before the experiment. Concentrated UTP (10 mM, adjusted pH at 7.4) was dropped into the solution directly in the center of dish. The fluorescence intensity of fura-2 was measured at an excitation wavelength of 340 and 380 nm with a video image analysis system (Aquacosmos; Hamamatsu Photonics, Hamamatsu). Emission was collected at 510 nm and the ratio ($R = F_{340}/F_{380}$) of fura-2 fluorescence intensities was used as an index of the intracellular Ca^{2+} concentration (13).

RT-PCR analysis

Total RNA was extracted from whole rat brain and arterial tissues with Trizol reagent (Invitrogen, Carlsbad, CA, USA). After DNase treatment (DNA-free kit; Applied Biosystems, Foster City, CA, USA), total RNA (4 μ g) was subjected to reverse transcription using Super Script III reverse transcriptase (Invitrogen), and a 100-ng sample of the RT products was used for each PCR amplification. The PCR protocol included preheating the sample at 94°C for 1 min followed by 30 cycles consisting of denaturation at 94°C for 10 s, annealing at 64°C for 30 s, and extension at 72°C for 1 min, and final extension at 72°C for 10 min. PCR high fidelity supermix (Invitrogen) enzyme was used for all PCR reactions. The following oligonucleotide primer pairs specific for the respective gene isoforms were used: Rat P2Y₂ (GI: 38197685): forward, TTCCACGTCACCCGCACCCCTC TTATTACT and reverse, CGATTCCCCAACTCACA CATAAAATGATTG (product length: 539 bp); rat P2Y₄ (GI: 13928943): forward, CTTCTCTGCCTGGG TGTTTGGTTGGTAGTA and reverse, TCCCCCGTGA AGAGATAGAGCACTGGA (473 bp); rat P2Y₆ (GI: 48675856): forward, GCCAGTTATGGAGCGGGACA ATGG and reverse, AGGAACAGGATGCTGCCGTG TAGGTTG (357 bp).

Western blot analysis

Tissues (rat brain, cerebral and mesenteric arteries, and aorta) were homogenized in 1 mL ice-cold buffer (50 mM HEPES buffer at pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 50 mM sodium fluoride, 40 mM sodium β -glycerophosphate, 2 mM sodium orthovanadate, 30 mM sodium pyrophosphate, and protease inhibitors cocktail containing 5 μ g/mL pepstatin A, 10 μ M leupeptin, 1.7 μ g/mL aprotinin, and 50 μ M 4-amidinophenylmethanesulfonyl fluoride); then the homogenate was centrifuged at 50,000 $\times g$ for 30 min at 4°C. The supernatants (20 μ g) were separated on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membrane. Immunoblot analysis was performed using antibodies against P2X₁ receptor (anti-rabbit, 1:500; Alomone, Jerusalem, Israel) and TRPC3 (anti-rabbit, 1:500; Alomone). Anti-mouse β -actin antibody (1:1000; Cell Signaling Technology, Inc., Beverly, MA, USA) was used for the positive control, and P2X₁ receptor antibody (1:500) pre-incubated with specific antigen peptide (1:500) was used for the negative control. Blots were developed with horseradish peroxidase-coupled secondary antibodies and visualized using the electrochemiluminescence (ECL) system (GE Healthcare UK Ltd., Buckinghamshire, UK).

Immunohistochemistry

Rat aorta samples were fixed with 4% paraformaldehyde (PFA) in a phosphate-buffered saline (PBS, pH = 7.4) overnight, embedded in paraffin, and sectioned at 7- μ m thickness. Individual sections were deparaffined by treatment with xylene, followed by a stepwise rehydration process with graded ethanol solutions. Samples were incubated in blocking buffer [2.5% bovine serum albumin (BSA) in PBS] for 1 h and then incubated with the first antibodies: anti-rabbit P2X₁ receptor antibody (1:200, Alomone), pre-incubated with antigen peptide (1:200); anti-rabbit TRPC3 antibody (1:100, Alomone); and anti-mouse α -smooth muscle actin antibody (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in 1% BSA-containing blocking buffer overnight at 4°C. The sections were then washed with Tris-buffered saline and incubated with the secondary antibodies (1:1000 Alexa Fluor 594 anti-rabbit antibody and 1:1000 Alexa Fluor 488 anti-mouse antibody; both from Invitrogen) in the blocking buffer for 1 h at room temperature. The slices were mounted with Mountant, PermaFlour (Lab Vision Products; Thermo Fisher Scientific, Fremont, CA, USA).

For cell staining, enzymatically isolated rat aortic smooth muscle cells were dropped on coverslips, bathed in 1 mL low Ca^{2+} (100 μ M) PSS, and incubated at 4°C for 30 min. Then cells were fixed with direct addition of

1 mL of 10% PFA in PBS (pH = 7.4) and incubated at 4°C for 5 min. Cells were rinsed with PBS and fixed again with 4% PFA in PBS for 5 min at room temperature. After fixation, the cells were permeabilized with 50 μ g/mL digitonin for 5 min, subsequently quenched with 50 μ M NH_4Cl for 5 min, and incubated in blocking buffer for 1 h. Samples were incubated with the first antibodies as described above, followed by incubation with the secondary antibody. Finally, samples were mounted with Mountant, PermaFlour (Lab Vision Products, Thermo Fisher Scientific) for visualization. Both tissue and single cell samples were visualized at appropriate wavelengths by a confocal microscope (LSM 510 META; Carl Zeiss Microimaging GmbH, Jena, Germany). The images were processed using Adobe Photoshop 5.5 (Adobe Systems Inc., San Jose, CA, USA).

Solutions and drugs

The internal (pipette) solution for whole-cell and single channel patch-clamp experiment contained 140 mM CsCl, 1.5 mM MgCl_2 , 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 10 mM glucose, 10 mM ethylene glycol-bis (2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) (pH = 7.2, adjusted by Tris), and the external (bath) solution contained 140 mM NaCl, 1.5 mM MgCl_2 , 2 mM CaCl_2 , 10 mM HEPES, and 10 mM glucose (pH = 7.4, Tris). In some experiments, 140 mM CsCl was replaced with 110 mM Cs-aspartate and 30 mM CsCl for the pipette solution, and 140 mM *N*-methyl-D-glucamine (NMDG) substituted for 140 mM Na^+ in the external solution. For the current clamp recording, the internal solution was changed to the following: 140 mM KCl, 1.5 mM MgCl_2 , 10 mM HEPES, 10 mM glucose, and 1 mM EGTA (pH = 7.4, Tris). Chemicals were applied by the 'Y-tube' fast solution change device described of Morita et al (14). Modified Krebs solution, warmed to 37°C for tension recording, contained 121.9 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl_2 , 2.5 mM CaCl_2 , 15.5 mM NaHCO_3 , 1.2 mM KH_2PO_4 , and 11.5 mM glucose (pH = 7.4) continuously aerated with 97% O_2 and 3% CO_2 . All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA), except for uridine 5'-*O*-3'-thiotriphosphate (UTP γ S), which was purchased from Jena Bioscience (Jena, Germany). All solutions containing purines and pyrimidines were used after adjusting the pH to 7.4.

Statistics

All data are expressed as the mean $\pm$ standard error (S.E.M.). A two-tailed unpaired Student's *t*-test and one-way ANOVA were used for statistical evaluation.

Results

UTP activates a non-selective cation current in rat arterial myocytes

When increasing concentrations of UTP (1 – 100 μ M) were applied to single rat aortic myocytes, inward currents were induced in a concentration-dependent manner at the holding potential of –60 mV, near the resting potential of arterial smooth muscle cells (7). UTP activated the current almost immediately after its application, which exhibited, at high concentrations of UTP (≥ 100 μ M), a rapid desensitizing decay (Fig. 1Aa). The time constants for activation and desensitization became progressively shorter as the concentration of UTP was increased, and the current did not recover from the desensitization (Supplementary Fig. 1: available in the online version only). In current clamp mode, UTP (10 μ M – 1 mM) produced concentration-dependent membrane depolarization (up to 0 mV from –60 mV with 140 mM K^+ containing internal solution, Supplementary Fig. 2: available in the online version only), well above the activation threshold for VDCCs (> -40 mV from –70 mV) (15). Furthermore, UTP-induced depolarization was also induced by suppression of ATP-sensitive K^+ and voltage-activated K^+ channels (9, 16), thereby contributing to the generation of sustained membrane depolarization.

The current–voltage relationship of the UTP-induced current showed an inward-going rectification (Fig. 1Ab). The reversal potential of UTP-induced current was 9.1 ± 1.3 mV ($n = 7$) and remained unchanged (9.4 ± 1.9 mV, $n = 5$) when the concentration of Cl^- in the pipette solution was reduced from 143 to 33 mM with aspartate substitution (equilibrium potentials of Cl^- calculated according to Nernst's equation were –1.6 and –39.2 mV at 25.0°C for 143 and 33 mM Cl^- , respectively). Moreover, substitution of Na^+ with NMDG in the bath almost completely abolished the current (in the presence and absence of 2 mM Ca^{2+} : $93.7 \pm 1.8\%$ and 100% inhibition, respectively, $n = 5$). In addition, a potent L-type calcium channel inhibitor, nifedipine (10 μ M), was completely ineffective on the current ($100.0 \pm 2.3\%$, $n = 5$). These results clearly indicate that UTP activates a nonselective cationic current with rapid activating and desensitizing properties. This is strongly reminiscent of P2X receptor as has been reported for rat tail artery smooth muscle cell (17).

Consistent with this idea, similar rapidly activating and desensitizing currents with inward-rectifying property were also induced by a potent P2X₁-receptor agonist, α,β -methylene ATP (right panel in Fig. 1Aa and closed circles in Supplementary Fig. 1A). The concentration–response relationships fitted by the Hill equation indicate that the half maximal effective concentration (EC_{50}) value for α,β -methylene ATP (0.55 μ M) to induce the

Fig. 1. UTP activated inward-rectifying cation current in arterial myocytes. **Aa:** Representative trace of UTP-induced and α,β -methylene ATP (α,β -mATP)-induced current in a single aortic myocyte. **Ab:** The current-voltage relationship of UTP (100 μ M)-induced current. These data were taken from the ramp pulse protocol (-100 to +100 mV, 300 ms) indicated by the asterisk (*) subtracted by averaged three current traces indicated by the double asterisk (**) in panel Aa. **B:** Concentration-response relationships of UTP, UDP, and α,β -mATP. These data were standardized by α,β -mATP (10 μ M)-induced current 5 min after application at each concentration; they were fitted by the Hill equation: $I_{\max} / \{1 + ([\text{drug}] / K_d)^n\}$, where [drug], K_d , n , and $I_{\max}$ denote the drug concentration, its IC_{50} value, cooperativity factor, and maximum current amplitude, respectively. UTP, UDP, and α,β -mATP are indicated by filled circles with a solid line, open circles with a dotted line, and filled triangle with a dotted line, respectively. **C:** Effects of UTP on cerebral and mesenteric artery and aortic myocytes. Data from cerebral artery (CA), mesenteric artery (MA), and aortic (AO) myocytes are indicated by open, filled, and striped squares ($n = 3 - 5$). No significant difference was observed among them by one-way ANOVA. All data are expressed as the mean $\pm$ S.E.M.

inward current is much lower than that for UTP (1.2 mM) (Fig. 1B), suggesting that the former is a more potent agonist.

UTP as well as α,β -methylene ATP could also induce cation currents with fast kinetics in cerebral and mesenteric artery smooth muscle cells (Supplementary Fig. 3A: available in the online version only). Although the maximum current density of the UTP-current was variable for three distinct arterial myocytes (20.1 ± 0.6 , 20.7 ± 3.8 , and 2.4 ± 0.2 pA/pF at -60 mV for aortic, mesenteric, and cerebral artery myocytes, respectively, $n = 3 - 10$). Its concentration-dependence was similar when standardized by the maximum amplitude attained by 10 μ M α,β -methylene ATP (Fig. 1C).

The induction of inward current by α,β -methylene ATP in aortic myocytes was greatly reduced by preceding desensitization with UTP, and vice versa, suggesting that the actions of both agonists desensitized each other (Fig. 2A). Furthermore, cumulative application of UTP at concentrations over 1 mM also showed desensitization of the current (Supplementary Fig. 3B). In contrast, other uridine derivatives such as uridine 5'-diphosphate (UDP),

uridine 5'-monophosphate (UMP), and uridine exhibited only a weak or little efficacy in inducing the inward current; UDP induced an inward cationic current at concentration as high as 1 mM (filled triangles in Fig. 1B), and UMP (1 mM) and uridine (1 mM) were totally ineffective. We also used UTP γ S, a stable UTP analog, to exclude the effect of possible contamination by ATP or other ATP analogs of UTP or UDP (18). UTP γ S (>10 μ M) activated the inward current (data not shown). These results in aggregate support the view that UTP induces the cationic current via activation of P2X receptor(s).

To observe the maximum effect of UTP, a high concentration of UTP (1 mM) was used in following experiments.

UTP activated P2X₁-like receptor in aortic myocytes

To further confirm that induction of rapidly desensitizing cationic currents by UTP is mediated via P2X-receptor activation, we next examined the effects of nonspecific P2-receptor antagonists, suramin and pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) (PPADS), and a P2X receptor-selective antagonist, 2',3'-O-(2,4,6-

Fig. 2. UTP activated P2X receptor in aortic myocytes. **A:** Competitive effect of α,β -mATP and UTP on each agonist-induced current. **B:** Effects of the P2X-receptor specific antagonist TNP-ATP and potent P2-receptor antagonists PPADS and suramin on UTP (1 mM)-induced current. Effects of TNP-ATP, PPADS, and suramin are indicated by closed triangles with dotted line, closed circles with solid line, and open circles with dotted line, respectively. Inset traces show effect of TNP-ATP on UTP-induced current. Data were fitted by the Hill equation: $(1 - I_{\text{rest}}) / \{1 + ([\text{drug}] / K_i)^n\} + I_{\text{rest}}$, where $[\text{drug}]$, K_i , n , and I_{rest} denote the drug concentration, its IC_{50} value, cooperativity factor, and the fraction of unblocked current, respectively.

trinitrophenyl)-ATP (TNP-ATP) (19, 20). As illustrated in Fig. 2B, the half maximal inhibitory concentration (IC_{50}) and the cooperativity coefficient estimated by Hill analysis were, respectively, 0.43 μ M and 1.3 for suramin, 0.59 μ M and 1.1 for PPADS, and 0.95 μ M and 0.9 for TNP-ATP ($n = 3 - 5$). Interestingly, a low concentration of suramin (100 nM) potentiated the UTP-induced current ($141.1 \pm 1.3\%$, $n = 3$) (Fig. 2B), similar to the effect of suramin (300 nM) on α,β -methylene ATP (3 μ M)-induced P2X₁ receptor-like current in rat mesenteric artery myocytes (21). We also observed the effects of gadolinium (Gd^{3+}) and lanthanum (La^{3+}), potent non-selective cation-channel antagonists, on UTP-induced current and found that the IC_{50} values were 51.3 μ M for Gd^{3+} and 21.8 μ M for La^{3+} , respectively ($n = 3 - 5$).

In another series of experiments, we investigated the possible contribution of P2Y receptors to the UTP-induced current by intracellularly applying the activator and inhibitor for trimeric G-protein-mediated signaling, guanosine 5'-O-3'-thiotriphosphate ($\text{GTP}\gamma\text{S}$) and guanosine 5'-O-thiodiphosphate ($\text{GDP}\beta\text{S}$), respectively. Neither $\text{GTP}\gamma\text{S}$ nor $\text{GDP}\beta\text{S}$ (1 mM each) affected the UTP-induced current (Fig. 3A). We also examined the effects of P2X₁-receptor antibody (1:200), which has been shown to functionally inhibit P2X₁ receptor in rabbit portal vein myocytes (22). P2X₁-receptor antibody applied from the patch pipette greatly suppressed the magnitude of cationic currents maximally activated by 1 mM UTP (Fig. 3A, $P < 0.01$ by one-way ANOVA). The inhibitory effect of the antibody was diminished when it

Fig. 3. Insensitivity of UTP-induced current to G-protein modulators and TRPC3 antibody, but sensitivity to P2X₁ receptor antibody in aortic myocyte. **A:** Effects of G-protein modulators and functional P2X₁-receptor antibody on UTP-induced current. UTP (1 mM) was applied in every 5 min. Effects of intracellularly applied GTP γ S, GDP β S, functional P2X₁-receptor antibody (1:200) with antigen peptide (1:200), and functional P2X₁-receptor antibody alone on UTP-induced current are indicated by filled, striped, and crossing pattern squares, respectively ($n = 3 - 5$). The control condition was indicated by open squares ($n = 5$). Only anti-P2X₁ receptor antibody showed a significant difference among them by one-way ANOVA (* $P < 0.05$, ** $P < 0.01$). **B:** Effect of functional TRPC3 antibody (1:200) on UTP-induced current. There was no significant difference (NS) between control and anti-TRPC3 antibody-treated conditions in each stimulus by UTP.

was pre-incubated with the epitopic peptide before use. These results largely exclude the contribution of P2Y receptor / G-protein-mediated pathway, but strongly suggest the involvement of P2X₁ receptor in the generation of cationic currents by UTP.

TRP protein does not contribute to UTP-induced current in aortic myocyte

In previous studies with the anti-sense technique by the reverse permeabilization method, it was reported that in cerebral artery myocytes (10), UTP activates a TRPC3-like channel through P2Y receptors. However, we could not record a TRPC3-like outwardly rectifying, G-protein dependent current (11, 23 - 26) (Figs. 1A, 3A, and Supplementary Fig. 3) from rat isolated aortic, cerebral, and

mesenteric arterial myocytes. Furthermore, the functionally effective antibody for TRPC3 (27, 28) (Fig. 3B) and ethyl-1-(4-(2,3,3-trichloroacrylamide)phenyl)-5-(trifluoromethyl)-1H-pyrazole-4-carboxylate (pyrazole 3), a relatively specific inhibitor of TRPC3 (29), exerted little discernible effects on UTP-induced current (1 μ M; $100.0 \pm 1.5\%$ of the control, $n = 8$). SKF-96365 and 2-aminoethyl diphenylborinate (2-APB), potent TRPC3 blockers, were also ineffective to inhibit the current (for 30 and 100 μ M; $102.8 \pm 4.6\%$ and $100.3 \pm 2.6\%$ of the control, $n = 5$, respectively) (24, 25, 28, 30). These results strongly negate the involvement of TRPC proteins and further corroborate our above conclusion that the molecular entity of UTP-induced current is a P2X (probably P2X₁) receptor-type cation channel.

UTP-induced single channel activity

We further performed single channel recording in outside-out patch membranes. Whereas α,β -methylene ATP (10 μ M) elicited typical P2X₁ receptor-like fast inactivating current, UTP (1 mM) evoked a sustained inward current at -60 mV (Fig. 4A). The unitary conductance estimated by a ramp potential protocol (-160 to $+40$ mV; 1.5 s) was 10.5 ± 0.1 (n = 5) and 10.4 ± 0.1 pS (n = 5) for α,β -methylene ATP and UTP, respectively (Fig. 4B).

The average of 30 ramp current traces disclosed an inward-rectifying current reversing near 0 mV, the properties being similar to those observed for macroscopic currents (Fig. 4C vs. Fig. 1Ab).

Fig. 4. Single channel analysis of UTP-activated current. A: Single channel properties of α,β -mATP (10 μ M) and UTP (1 mM)-induced currents in outside-out mode patch clamp. B: Ramp pulse protocol (-160 to $+40$ mV, 1.5 s) showed almost the same conductance channel (10.5 ± 0.1 and 10.4 ± 0.1 pS) when activated by α,β -mATP (10 μ M) and UTP (1 mM), respectively. C: Assembled data from panel B (n = 30 each).

These results further confirm that α,β -methylene ATP and UTP can activate an identical P2X-type receptor.

UTP contracts rat aortic smooth muscle

To observe the functional effects of UTP on intact arteries, we performed a tension recording with the rat aortic ring preparation denuded of endothelium to exclude the endothelium-dependent effects via P2 receptors (3). UTP elicited a biphasic contraction consisting of an initial fast twitch-like or “phasic” component and a subsequent sustained or “tonic” component in Krebs solution containing 2 mM Ca^{2+} and tetrodotoxin (1 μ M). The phasic contraction was first evident at a concentration as low as 10 μ M and its magnitude was constant over a wide range of concentrations examined. It was similar to the purinergic component of the neurogenic contraction in rat mesenteric artery (31). In contrast, the magnitude of the tonic contraction was increased concentration-dependently (1 μ M – 3 mM, Fig. 5A: a and b). However, when we applied UTP cumulatively, the phasic contraction disappeared at 1 mM due presumably to desensitization (Fig. 5Aa). A similar result was also observed in the UTP-induced currents by whole cell patch-clamp experiment (Supplementary Fig. 3B). These results suggest that UTP-induced contraction and current are activated by the same receptor.

In Ca^{2+} -free solution, the phasic contraction was completely abolished, while the tonic contraction was only partially inhibited. Re-addition of external Ca^{2+} (2 mM) restored both contractions (Fig. 5B). Similar abolition of the phasic contraction and partial inhibition of the tonic contraction was observed with pre-applied nifedipine (10 μ M), a specific blocker for L-type voltage-dependent calcium channels, or TNP-ATP (30 μ M), the P2X-receptor antagonist (Fig. 5: C and D; the effect of TNP-ATP on the phasic contraction is not shown). The effects of pre and post-applied nifedipine (10 μ M) on tonic contraction was statistically different ($35.8 \pm 5.8\%$ and $11.7 \pm 1.7\%$ inhibition, respectively; n = 6, $P < 0.005$) (Fig. 5C). Therefore, we used pre-applied and high concentration of nifedipine (10 μ M) and TNP-ATP (30 μ M) for observing the maximum effect on contraction (see Figs. 2B and 5C). Inhibitory effects of pre-applied 0 mM Ca^{2+} Krebs + 0.5 mM EGTA, nifedipine (10 μ M), and TNP-ATP (30 μ M) on tonic contraction were statistically different from control condition (2 mM Ca^{2+} Krebs; $P < 0.01$). However, there were no significant differences among their inhibitory effects (Fig. 5D). The remaining component of the UTP-induced tonic contraction in the absence of Ca^{2+} (0 mM Ca^{2+} + 0.5 mM EGTA) was almost completely inhibited by 5-min pretreatment with thapsigargin (TG) that depletes internal Ca^{2+} stores (Fig. 5: B and D, $P < 0.001$).

Fig. 5. UTP-induced contraction of endothelial layer-denuded rat aorta ring. **Aa:** Representative trace of UTP (1 μ M – 1 mM)-induced contraction in rat aorta ring preparation. **Ab:** Summary of UTP-induced phasic and tonic contractions at each concentration. **B:** UTP (1 mM)-induced contraction in normal Krebs solution (containing 2 mM Ca²⁺), 0.5 mM EGTA containing Ca²⁺-free Krebs, and 0.5 mM EGTA containing Ca²⁺-free Krebs with thapsigargin (TG, 2 μ M) pretreatment and washed-out with normal (2 mM Ca²⁺) Krebs solution. **C:** Effects of post- or pre-treatment with nifedipine (10 μ M) on UTP-induced contraction. **D:** Summary of pre-applied TNP-ATP (30 μ M), nifedipine (10 μ M), 0.5 mM EGTA containing Ca²⁺-free Krebs, and 0.5 mM EGTA containing Ca²⁺-free Krebs with thapsigargin (TG, 2 μ M) on UTP-induced tonic contraction (n = 6). Data were statistically analyzed by Student's *t*-test against each control condition: ***P* < 0.01 and ****P* < 0.001, respectively. Among preparations pretreated with TNP-ATP (30 μ M), nifedipine (10 μ M), and 0.5 mM EGTA containing Ca²⁺-free Krebs, There were no significant differences (NS) by one-way ANOVA.

Similar to UTP, unique patterns of both phasic and tonic contraction were also observed in response to α,β -methylene ATP (Supplementary Fig. 4: A, B: available in the online version only). However, in contrast to the partial inhibition of the UTP-induced contractions, the entire contractile response was almost completely suppressed by 0 mM Ca²⁺ PSS + 0.5 mM EGTA, nifedipine (10 μ M), and TNP-ATP (30 μ M) (Supplementary Fig. 4: A, C; *P* < 0.001, compared to the control).

These results indicate that the phasic component of UTP-induced contraction of aortic muscle exclusively reflects Ca²⁺ influx through L-type Ca²⁺ channels that are presumably activated by membrane depolarization resulting from P2X₁-like receptor activation. In contrast, the tonic component appears to depend on both voltage-dependent Ca²⁺ influx and intracellular Ca²⁺ release, the latter being independent of P2X receptor and mediated

presumably via activation of P2Y receptors similar to previous reports (32, 33) (also see below).

Intracellular Ca²⁺ imaging

In a final series of functional experiments, we investigated the impact of UTP on intracellular Ca²⁺ dynamics in rat aortic myocytes by using digital Ca²⁺-imaging with fura-2. Application of UTP (1 mM) in 2 mM Ca²⁺-containing PSS evoked a transient rise or oscillatory changes in intracellular Ca²⁺ concentration ([Ca²⁺]_i) (143 or 43 in 186 cells, respectively; Fig. 6Aa), which were accompanied by contraction of the myocyte (Supplementary movie 1: available in the online version only). UTP (1 mM) in Ca²⁺-free PSS also evoked oscillatory or transient changes in [Ca²⁺]_i (54 or 39 in 93 cells, respectively), but their magnitude rapidly diminished with time (Fig. 6Ab, Supplementary movie 2: available in the online version

Fig. 6. $[Ca^{2+}]_i$ measurement of isolated aortic smooth muscle cells. UTP-induced intracellular Ca^{2+} concentration was measured with fura-2 in normal PSS (2 mM Ca^{2+}) (Aa) and 0.5 mM EGTA containing Ca^{2+} -free PSS (Ab). Ac: Comparison of starting time of $[Ca^{2+}]_i$ elevation from time of application of UTP (1 mM) in normal and 0.5 mM EGTA containing Ca^{2+} -free PSS. Mean values are indicated by solid lines with double arrow heads. B: Assembled data in conditions of normal PSS (2 mM Ca^{2+}) (Aa), 0.5 mM EGTA containing Ca^{2+} -free PSS (Ab), and 0.5 mM EGTA-containing Ca^{2+} -free PSS with thapsigargin (TG, 2 μ M) ($n = 11 - 18$).

only). The latency for the first $[Ca^{2+}]_i$ rise varied among the myocytes and became significantly longer in the absence of Ca^{2+} ; the mean value of the latency was 3.3 ± 0.1 ($n = 186$) and 13.5 ± 0.7 ($n = 93$) s for normal (2 mM Ca^{2+} -containing) and Ca^{2+} -free PSS, respectively (Fig. 6Ab). These phenomena were also observed for UTP and ATP in pig aorta, rat pulmonary artery, and human umbilical cord smooth muscle cells (34–36). Averaging individual traces clearly indicated that the initial Ca^{2+} transient was significantly inhibited by removal of extracellular Ca^{2+} . Importantly, the time course of changes in $[Ca^{2+}]_i$ in each condition accords well with that of contractile responses monitored with tension recording produced by UTP and α, β -methylene ATP (Figs. 5B, 6B, and Supplementary Fig. 4: A and D). However, there was no difference in the sustained increase in $[Ca^{2+}]_i$ (sus-

tained Ca^{2+} transient) in normal (2 mM Ca^{2+} -containing) and Ca^{2+} -free EGTA containing PSS. In addition, the waveform of α, β -methylene ATP-induced $[Ca^{2+}]_i$ rise in each single myocyte showed the transient, but not oscillatory pattern (data not shown) (36). This implies that the oscillatory change caused by P2Y mediated intracellular Ca^{2+} release. These results suggest that UTP contracts intact aortic muscle through coincident activation of the P2X₁ receptor / L-type VDCC and P2Y receptor / G-protein / PLC pathways, which lead to Ca^{2+} influx and intracellular Ca^{2+} release, respectively.

Distribution of P2 receptor subtypes in arteries

Finally, we surveyed the expression of P2X₁ receptor and TRPC3 proteins in rat cerebral and mesenteric arteries and aorta by using Western blotting. From all these

tissues, abundant P2X₁-receptor proteins were detected. In contrast, a faint or no expression (each 3 cases) of TRPC3 protein was observed in all arteries compared with rat brain extract (Fig. 7A). Messenger RNA transcripts of both P2X₁ receptor and P2Y₂, P2Y₄, and P2Y₆ receptors were successfully amplified in cerebral and mesenteric arteries and aorta by the RT-PCR technique (Fig. 7B, Supplementary Fig. 5: available in the online version only). Moreover, immunocytochemistry showed the localization of P2X₁ receptor on the plasma membrane of aortic smooth muscle cells, and the intensity was high enough compared to α -smooth muscle actin as a positive control, and some immunofluorescence appeared co-localize at the plasma membrane (Fig. 8: Aa, B) (37). The immunofluorescence by P2X₁-receptor antibody had almost disappeared on pre-incubation with its antigen peptide (Fig. 8Ab). On the contrary, immunofluorescence of TRPC3 was very low compared to P2X₁ receptors and the localization of TRPC3 was diffuse in the cytosol (Fig. 8Ac).

For aorta slice staining, high expression of P2X₁-receptor protein was observed in both the smooth muscle layer (arterial media: middle layer in Fig. 9A) and the endothelial layer (intima: lower layer in Fig. 9A). Pre-incubation of the antibody with the antigen peptide weakened the fluorescence observed in the cell layer; instead, a striped pattern (elastic lamina) between the smooth muscle layers, which are probably non-specific staining, became evident for unknown reason(s) (compare Fig. 9: A and B), indicating the specific staining. An α -smooth muscle actin antibody with Alexa Fluor 488 specifically stained only smooth muscle in arterial media, but not intimal and adventitial layers. Under these conditions, immunofluorescent tissue staining for TRPC3 was low, similar to the result from immunocytochemistry described above (Figs. 8Ac, 9C). Summarizing the above data, P2X₁ receptors were dominantly expressed in aortic smooth muscle cells.

Fig. 7. Molecular existence of P2X₁ receptor and TRPC3 in cerebral and mesenteric arteries, and aorta. A: Western blot analysis of P2X₁ receptor (1:500) (Aa) and TRPC3 (1:500) (Ab). β -Actin (1:1000) was used for the positive control. The immunoreactivity to P2X₁ receptor completely disappeared on treatment of the antibody with the specific antigen peptide (1:500). B: RT-PCR to P2Y₂, P2Y₄, and P2Y₆ receptors in rat brain, cerebral artery (CA), mesenteric artery (MA), and aorta (AO).

Fig. 8. Immunocytochemistry of P2X₁ receptor and TRPC3 in aortic myocytes. **A:** a) The immunofluorescence by P2X₁-receptor antibody shows the localization of P2X₁ receptor (P2X₁) on the plasma membrane, b) when the antibody was pre-incubated, in advance, with the specific antigen peptide (P2X₁-Ag), the immunofluorescence of P2X₁ receptor almost disappeared, and c) shows the immunofluorescence of TRPC3 and α -smooth muscle actin (α -SM actin) in rat aortic myocytes. Antibodies used were as follows: anti-rabbit P2X₁ receptor (1:400), anti-rabbit TRPC3 (1:100), and anti-mouse α -SM actin (1:200) as the first antibodies, and Alexa Fluor 594 anti-rabbit (1:1000, red) and Alexa Fluor 488 anti-mouse (1:1000, green) as the secondary antibodies. Hoechst (1:2000) was used for nuclei staining (blue). As a negative control, anti-rabbit P2X₁ receptor antibody (1:400) with its antigen peptide (1:400) was used as a primary antibody. **B:** Localization of P2X₁ receptor and α -SM actin in aortic myocytes. Immunofluorescent intensities of Alexa Fluor 594 (red) and Alexa Fluor 488 (green) were measured at the red arrow line (a) and illustrated with distance (μm) from the left to the right end of the red arrow line (b).

Discussion

The key findings of the present study can be recapitulated as follows: 1) UTP elicited a rapidly activating and desensitizing inward-rectifying cation current with pharmacological profiles consistent with those of P2X-receptor cation channel to the agonist and antagonists including α,β -methylene ATP, TNP-ATP, suramin, and PPADS; 2) the activation of the current by UTP was counteracted by a functional P2X₁-receptor antibody, but not affected by G-protein modulators (GDP β S and GTP γ S), specific TRPC3 inhibitors such as pyrazole 3, SKF-96365, 2-APB, or a functional TRPC3 antibody; 3) UTP and α,β -methylene ATP activated single channel activities

showing indistinguishable unitary conductances (10.4 ± 0.1 and 10.5 ± 0.1 pS, respectively) with clear inward rectifying property and without significant ion selectivity; 4) UTP evoked biphasic contractions of aortic smooth muscle via elevation in $[Ca^{2+}]_i$ through dual cellular signaling involving extracellular Ca^{2+} -dependent, and -independent pathways; 5) robust expressions of P2X₁-receptor transcript and protein were detected in rat aorta, cerebral, and mesenteric arteries by RT-PCR, Western blotting, and immunohistochemical analyses.

Upon repetitive applications of UTP, evoked currents but not the contraction showed quick run-down phenomena (Fig. 5B, Supplementary Fig. 1). This might be due to preparations and methods used to record the currents

Fig. 9. Distribution of P2X₁ receptor and TRPC3 in aorta thin section. **A:** Immunohistochemistry of rat aorta by anti-rabbit P2X₁ receptor antibody (1:200) shows high expression of P2X₁ receptor protein in arterial media and intima. **B:** Preincubation of the antibody with the specific antigen peptide (P2X₁-Ag, 1:200) weakened the fluorescence observed in the cell layer. **C:** Immunofluorescent tissue staining for TRPC3 by anti-rabbit TRPC3 (1:100) was low. Alexa Fluor 594 anti-rabbit antibody (1:1000) was used for the secondary antibody. α -Smooth muscle actin (α -SM actin) (1:200) and Alexa Fluor 488 anti-mouse antibody (1:1000) were used as the positive control for the smooth muscle layer.

(single myocytes) and tension developments (multi cellular arterial tissues). In the present study, we used the conventional whole-cell patch clamp method rather than the perforated patch clamp method, and it is well documented that the desensitization of P2X receptor and the sensitization (the run-down phenomenon) of L-type VDCC are controlled by cytosolic components including calmodulin, intracellular ATP, and unknown components (21, 38). Therefore, it is reasonable to assume that the observed run-down of the UTP-induced currents in contrast to the tension development would be mainly due to the method (whole-cell patch clamp technique) and preparations.

In addition, Ca²⁺-free EGTA containing PSS evoked small but statistically significant decrease in the amplitude of the sustained component of the UTP-induced contraction without affecting the sustained increase in [Ca²⁺]_i (sustained Ca²⁺-transient). The precise reason for this discrepancy is unknown at present. However, this

might be also due to the different preparations used (single myocytes and multi cellular arterial tissues). Furthermore, it should be mentioned that some myocytes showed an oscillatory Ca²⁺ transient but others showed only a phasic one, and therefore the direct comparison between the sustained contraction and Ca²⁺ transient might not be suitable.

It has long been believed that UTP is a specific P2Y receptor (P2Y₂, P2Y₄, and P2Y₆) agonist (3) and UTP depolarizes by activating the P2Y receptor–TRPC3 cascade (10). Nevertheless, our present results strongly argue that in certain types of arteries, UTP can act as a P2X₁-like receptor agonist to elicit biphasic contraction by opening nifedipine-sensitive VDCC in addition to eliciting intracellular calcium release via P2Y receptor (P2Y₂, P2Y₄, or P2Y₆) stimulation. Supportive evidence can be found in a previous study by McLaren et al. (32, 39), in which UTP activated a P2X₁-like receptor in rat tail artery smooth muscle cell and contracted via P2X₁-like recep-

tors and P2Y receptor(s) activation (32, 39). Moreover, in mesenteric arterial smooth muscle, it was reported that activation of P2X₁ receptors by field stimulation of sympathetic nerves resulted in local, non-propagating junctional Ca²⁺ transients (jCaTs) and concomitant phasic and tonic contractions (31). These jCaTs and accompanying the decrease in vascular diameter completely disappeared by genomic knockout of P2X₁ receptor (40). Furthermore, consistent with our data, TRPC3 proteins are rarely expressed in rat mesenteric artery myocytes (41). In addition, the pharmacological profile of UTP-induced current is clearly different from that reported for expressed TRPC3 or its homologue TRPC6; IC₅₀ values for Gd³⁺ and La³⁺ to block UTP-induced current were similar to P2X₁ receptor expressed in *Xenopus* oocyte, but much higher than those reported for TRPC3 and TRPC6 (42–46).

Moreover, unitary conductance of UTP and α,β -methylene ATP-induced currents were both around 10 pS. These values were a little different to those from recombinant P2X₁ receptor (approximately 18 pS) (47), but quite different to those from TRPC3 (60–66 pS), which showed non-inactivating activities evoked by ATP (100 μ M) or carbachol (100 μ M) (23, 24). It is possible that the difference between the native P2X-like receptor evoked by UTP (about 10 pS) and recombinant P2X₁ receptor (about 18 pS) was due to contamination of the subconductance state, underestimation of channel conductances, or existence of hetero-oligomeric P2X receptors because of their diversity in arterial tissue (Supplementary Fig. 5) (21, 48). These results also suggest that UTP activates the P2X receptor, but not TRPC3.

It has been reported that the P2X receptor-induced responses were lost by cell culture (49). As a possible interpretation, tissue culture for permeabilization of the TRPC3 anti-sense DNA probe would abolish not only the expression of TRPC3 protein, but also the P2X-receptor response (10). On the other hand, we previously reported that ATP can activate P2Y receptors (P2Y₁ and P2Y₁₁-like) in small mesenteric arteries of guinea pigs, thereby regulating nifedipine-insensitive VDCCs positively and negatively via activation of G_s/protein kinase A (PKA) and G_{q/11}/protein kinase C (PKC) pathways, respectively (50, 51). Thus, it is reasonable to assume that dual activation of P2X (P2X₁) and P2Y receptors by ATP or UTP is a common mechanism to regulate the vascular tone, although the P2-receptor subtypes involved may vary with species and region of the vasculature.

In pathophysiological conditions such as hypoxia and inflammation, it is known that UTP and ATP are released from endothelial and smooth muscle cells (3, 52). During the inflammation, cell swelling or tissue congestion may increase the cell membrane tension and/or interstitial

pressure around the cells. This may also facilitate the release of UTP and mechanically sensitize the nearby P2X₁ and P2Y receptors. This would be more prominent during vascular injury where higher amounts of UTP and ATP released from damaged cells may affect local vascular tone via activation of P2X₁ and P2Y receptors. It has been estimated that the resting level of ATP in hypoxic cells would be maintained at more than 3 mM, which is about 20%–30% of normal conditions (53). Simple calculation indicates that at least 300 μ M of UTP (i.e., about 10% of ATP) could be released from damaged cells (52), which would be sufficient to activate P2X₁ and P2Y receptors (Figs. 1, 2, and 5).

UTP shows the dual responses of contraction and relaxation of vasculature as a direct effect on smooth muscle cells and an indirect effect via endothelial cells, respectively (18, 54). During ischemia, vasculature shows acute relaxation, but this is chronically converted to the contraction phase in cerebral and pulmonary artery (55, 56). The chronic effect is more important for vasospasm, which usually develops 4 and 12 days after subarachnoid hemorrhage (57). Intriguingly, the rabbit middle cerebral artery injected with blood as a model of subarachnoid hemorrhage showed an unusually enhanced sensitivity to UTP; contraction to low concentrations of UTP (1–3 μ M) was evidently increased at 24 h (not 10

Fig. 10. Schematic representation of regulation by UTP of arterial smooth muscle contraction. P2X₁: P2X₁ receptor; P2Y_n: P2Y₂, P2Y₄, and/or P2Y₆; IP₃R: IP₃ receptor; SR: sarcoplasmic reticulum; ΔV: membrane depolarization.

min) after the blood injection (58). This chronic vascular hyper-reactivity has been implicated in the pathogenesis of cerebral vasospasm, which usually develops with subsequent elevation of intravascular pressure after subarachnoid hemorrhage (58). These observations, in conjunction with the present results, suggest to us that UTP-mediated mechanisms have an abnormally enhanced vascular reactivity under pathophysiological conditions.

In conclusion, we found that extracellular UTP activates both P2X₁ and P2Y receptors, thereby eliciting biphasic vasoconstriction by promoting Ca²⁺ influx via nifedipine-sensitive Ca²⁺ channels and intracellular Ca²⁺ release from sarcoplasmic reticulum (Fig. 10). These findings may indicate a major pathophysiological role of UTP for abnormal vascular reactivity in ischemia, stroke, and subarachnoid hemorrhage, and thus the receptors for UTP in arteries (P2X₁ and P2Y) may be the targets of hypoxic and inflammatory vascular disorders and vascular injury.

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