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α,β-MeATP augments the UTP contraction of rabbit basilar artery

Yasushi Miyagi*, Hitoshi Kimura, Robin C. Carpenter, Andrew D. Parent, John Zhang

Department of Neurosurgery, University of Mississippi Medical Center, Jackson, MS, USA

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Abstract

The mechanism underlying the interaction between α,β-methyleneadenosine 5′-triphosphate (α,β-MeATP) and uridine 5′-triphosphate (UTP) was investigated using the basilar artery of a rabbit. UTP induced a concentration-dependent contraction, whereas P2X receptor agonists, such as α,β-MeATP and 2-methylthioadenosine 5′-triphosphate (2-MeSATP), did not induce any contraction up to 100 μM. α,β-MeATP augmented the UTP contraction two-fold, immediately and reversibly. This effect was observed with ectonucleotidase inhibition with 1 mM Ni^{2+}, the removal of extracellular Ca^{2+} or Evans blue. The contractile response to adenosine 5′-*O*-(3-triphosphate) (ATPγS), a selective agonist for $P2Y_4$, was augmented by pretreatment with α,β-MeATP also. ATPγS had no additional effect on the UTP contraction fully activated with α,β-MeATP. UTP (100 μM) did not induce an increase in cytosolic Ca^{2+} in a rabbit basilar arterial strip; however, in the presence of 1 mM α,β-MeATP, UTP induced a significant increase in cytosolic Ca^{2+}. These results suggest that α,β-MeATP facilitates the activation by UTP of the P2Y receptor ($P2Y_4$) of the rabbit basilar artery through mechanisms other than nucleotidase inhibition, and that it does not do so via a P2X receptor.

Keywords: Uridine 5′-triphosphate; α,β-Methyleneadenosine 5′-triphosphate; $P2Y_4$ receptor; Basilar artery; Ectonucleotidase; Smooth muscle

1. Introduction

According to the current receptor classification, which is based on structure and signal transduction, the P2X family is characterized as ligand-gated cation channels whereas the P2Y family is characterized as G protein-coupled receptors with a seven-transmembrane structure (Ralevic and Burnstock, 1998). In the vascular system, the P2Y families, previously characterized as P2Y ($P2Y_1$) and P2U ($P2Y_2$ or $P2Y_4$), exist on endothelial cells and play an important role in vasorelaxation through the endothelial production of nitric oxide or prostacyclins (Wilkinson et al., 1994; Miyagi et al., 1996a,b). In addition, vascular smooth muscles also have P2X and P2Y families (Ralevic and Burnstock, 1998), both of which assist in the regulation of vascular tone and/or cell proliferation.

Prior to the current classification, the agonist potency order was part of the criteria for the P2 receptor subtypes (Burnstock and Kennedy, 1985; Burnstock, 1996). Since there were no selective antagonists for each subtype, most of the receptor identification depended on cross-desensitization studies or agonist potency order to characterize the P2 receptor subtypes. While desensitization studies among representative agonists have contributed to the evidence of functional P2 receptor subtypes, Von Kügelgen et al. (1987) first reported that α,β-methyleneadenosine 5′-triphosphate (α,β-MeATP) enhanced the vasoconstriction in response to low-concentration uridine 5′-triphosphate (UTP) instead of desensitizing it in rabbit ear artery. Recently, similar interactions among various nucleotides were extensively investigated using rat aorta (Lopez et al., 1998), rat tail artery (McLaren et al., 1998), rat stomach (Otsuguro et al., 1996) and piglet pulmonary artery (McMillan et al., 1999). Although this phenomenon is considered to be important when injury to the tissue results in the release of multiple cytosolic nucleotides, the underlying mechanism of the interactions among different nucleotides remains unclear. The purpose of the present study was to investigate the effect of α,β-MeATP on the UTP-induced contraction in the basilar artery of a rabbit and its underlying mechanism.

The detailed subtypes of the P2 receptor in the rabbit basilar artery have not yet been identified at the molecular

* Corresponding author. Department of Stereotactic and Functional Neurosurgery, Kaizuka Hospital, 7-7-27 Hakozaki, Fukuoka 812-0053, Japan. Tel.: +81-92-632-3333; fax: +81-92-632-2230.
E-mail address: suitecello2003@yahoo.co.jp (Y. Miyagi).

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level; however, the present study outlined a functional pyrimidine ($P2Y_2$ or $P2Y_4$) receptor population. We also report the absence of a functional $P2X_1$ receptor, a finding that is consistent with results of a study by Von Kügelgen et al. (1990). Therefore, the rabbit basilar artery is considered to be an appropriate model for study of the pharmacology of P2U receptors without the possible compromising effect of the $P2X_1$ receptor.

2. Materials and methods

2.1. Tissue preparation and force measurement

New Zealand white rabbits (2.5–3 kg) were killed by an i.v. injection of thiopental (20 mg/kg) and exsanguination. The basilar arteries and ear arteries were excised, and the arachnoid membrane around the basilar arteries and the connective tissues around the ear artery were carefully removed with micro-scissors in physiological saline solution under a microscope. Then, the artery was cut into rings (0.8 mm) with a razor. The luminal surface of each ring was removed with a gentle rubbing motion with a stainless steel needle under the microscope. The rings were mounted vertically in a water-jacketed organ bath (Radnoti Glass, CA, USA) while being connected to a force-transducer. The solution used in the experiment was kept saturated with a mixture of 95% O_2 and 5% CO_2 at 37 °C. During a 1-h equilibration period, the rings were stimulated with 118 mM K^+ solution every 15 min, and the resting tension was adjusted to 100 mg (Chen et al., 1997). The contractile response was expressed as a percentage of the contraction (%force) in response to 118 mM K^+ solution (K^+-contraction). The protocol for this study complies with the *Guide for the Care and Use of Laboratory Animals* published by the National Institute of Laboratory Animal Resources (Commission on Life Science, National Research Council) and was approved by the Animal Care and Use Committee at the University of Mississippi Medical Center.

2.2. $[Ca^{2+}]_i$ measurement of arterial strip

The changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of the rabbit basilar arteries were assessed with fura-2, as described previously (Chen et al., 1997). Briefly, the arterial strips were loaded with fura-2 by incubation in Dulbecco's modified Eagle's medium containing 20 μM fura-2/AM (an acetoxymethyl ester form) and 5% fetal bovine serum for 4 h at 37 °C, under aeration with a mixture of 95% O_2 and 5% CO_2. The fura-2-loaded strips were mounted in a chamber made of SYLGARD silicon (Dow Corning, CA, USA) with insect pins and washed with physiological saline solution to remove the dye in the extracellular space. They were further incubated in physiological saline solution for 30 min. The fluorescence intensities of the fura-2-Ca^{2+} complex were monitored, using Metafluor software (Biorad, CA, USA), specifically designed for fura-2 fluorometry, and the ratio of fluorescence intensities (500 nm emission) at 340 nm excitation to those at 380 nm excitation was monitored. Before starting each measurement, a K^+-contraction was recorded for the purpose of normalization. The fluorescence ratios were expressed as percentages (%ratio), assuming the values in 5.9 and 118 mM K^+ solution, to be 0% and 100%, respectively. Because the calibration of the absolute values of $[Ca^{2+}]_i$ at the end of the experiments was unreliable, statistical analysis of $[Ca^{2+}]_i$ signals was performed using the %ratio, which is stable over an hour of measurements (Chen et al., 1997).

2.3. Drugs and solutions

Physiological saline solution (5.9 mM K^+) was composed as follows (in mM): NaCl 123, KCl 4.7, $NaHCO_3$ 15.5, KH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.25 and D-glucose 11.5. A Ca^{2+}-free version of saline solution (Ca^{2+}-free solution) contained 2 mM ethyleneglycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) instead of 1.25 mM $CaCl_2$. K^+ (118 mM) solution was identical to physiological saline solution, except that 112.1 mM KCl was substituted for 112.1 mM NaCl. All solutions were aerated with a mixture of 5% CO_2 and 95% O_2 (pH 7.4, 37 °C). EGTA and fura-2/AM were purchased from Molecular Probe (CA, USA). Fura-2/AM was dissolved in dimethyl sulfoxide (DMSO) and diluted in a medium just before loading the dye. The following drugs were used: UTP, ATP, α,β-MeATP, adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS), Evans blue (Sigma, MO, USA) and 2-methylthio adenosine 5′triphosphate (2-MeSATP, Research Biochemicals, MA, USA).

2.4. Data analysis

All values were expressed as the means ± S.E.M. Student's *t*-test was used to determine statistical significance of differences between time-matched experiments. To detect the additive effect of cumulated agonists on the sustained contraction or $[Ca^{2+}]_i$ of the artery, the values before and after the addition of the agents were compared using a paired *t*-test. *P*-values less than 0.05 were considered to be significant.

3. Results

3.1. Concentration–response curves of nucleotides in rabbit basilar and ear arteries

Fig. 1 shows the concentration–response curves of nucleotides used in this study as obtained with rabbit basilar and ear arteries. In rabbit basilar artery, α,β-MeATP and 2-MeSATP (P2X receptor agonist) did not induce any signif-

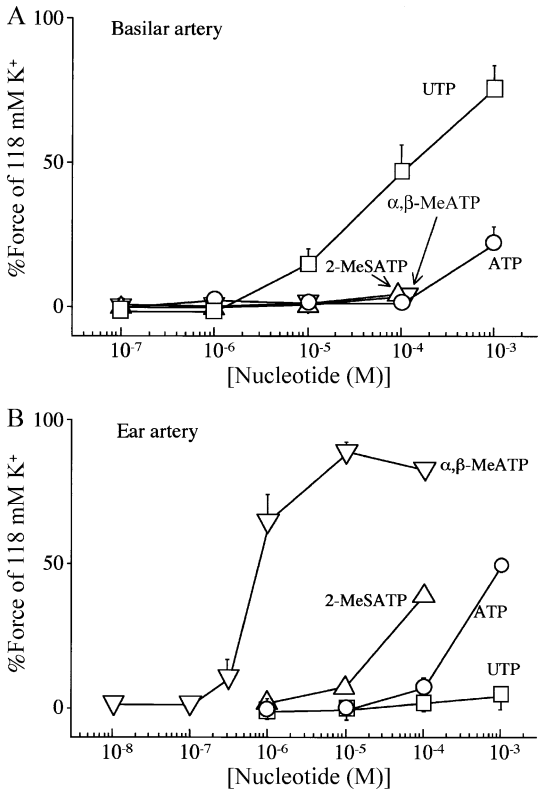

Fig. 1. Concentration–response curves for nucleotides in rabbit basilar (A) and ear (B) arteries. At the beginning of the experiment, the control responses to 118 mM K^+ solution were recorded and taken as 100%. After the arterial force was returned to the basal level (0 %) by washing out 118 mM K^+ solution, a single concentration of each UTP (□), ATP (○), 2-MeSATP (△) or α,β-MeATP (▽) was applied. One concentration was added per arterial ring. The values are expressed as percentages of K^+-contraction and presented as means ± S.E.M. ($n=4$).

icant contraction at up to 100 μM. In rabbit ear artery, however, α,β-MeATP induced a concentration-dependent contraction with a maximal contraction corresponding to 89.3 ± 3.3% of the K^+-contraction at 10 μM ($EC_{50}=0.65$ μM). The maximal contractile effect of 2-MeSATP was much less than that of α,β-MeATP (38.6 ± 2.9% vs. 82.5 ± 1.1%, respectively, of K^+-contraction at 100 μM, $n=4$) in the ear artery. UTP induced a concentration-dependent contraction of the basilar artery with the largest contraction at 1 mM (corresponding to 75.9 ± 7.9% of K^+-contraction, $n=4$). Because the maximum response was not obtained, even at the maximal concentration of nucleotides used in this study, except for α,β-MeATP in the ear artery, most of the EC_{50} values of nucleotides could not be calculated. Unlike its effect on the basilar artery, UTP had almost no effect on the rabbit ear artery even at 1 mM. ATP, a non-specific P2 receptor agonist, induced a small contraction of both the basilar and ear arteries: 22.3 ± 5.6% and 49.4 ± 2.8% of K^+-contraction at 1 mM, respectively ($n=4$, Fig. 1). These results suggested that there is a functional expression of P2X receptors on the rabbit ear artery, but not on the basilar artery.

3.2. α,β-MeATP augments the UTP contraction

The effect of α,β-MeATP on the UTP contraction was investigated using a rabbit basilar artery that had no functional expression of P2X receptors. A single stimulation with 100 μM UTP induced a tonic contraction (up to 78.3 ± 9.1% of K^+-contraction, $n=4$) with a gradual decline (Fig. 2A). α,β-MeATP induced a large contraction additive with the UTP contraction (145.0 ± 4.1% of K^+-contraction, $P<0.05$, $n=4$, Fig. 2), although α,β-MeATP alone does not have any contractile effect on rabbit basilar artery. Thus, the simultaneous stimulation with α,β-MeATP augmented the UTP contraction up to 135–145% of K^+-contraction immediately and reversibly (Fig. 2A).

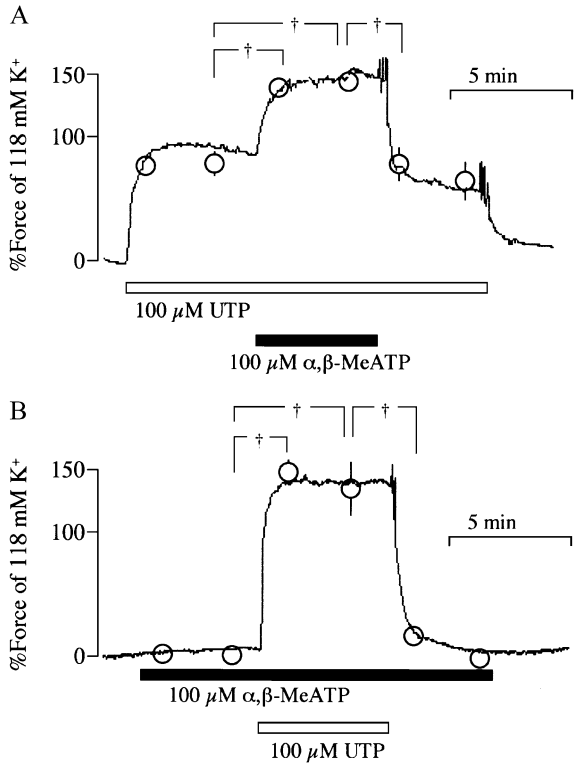

Fig. 2. Representative recordings and summary of the data showing the reversible effect of α,β-MeATP to augment the UTP-induced contraction in the rabbit basilar arterial strips. (A) After the control response to 118 mM K^+ solution, 1 mM UTP induced a tonic contraction. The application of α,β-MeATP (100 μM) induced an additional, rapid and tonic contraction on the steady-state UTP contraction. The augmenting effect of α,β-MeATP was promptly cancelled by washing with normal saline solution containing 1 mM UTP. (B) After the control response to 118 mM K^+ solution, the basilar arterial strips were pretreated with 100 μM α,β-MeATP and 1 mM UTP was applied topically in the presence of α,β-MeATP. α,β-MeATP alone never induced a contraction of the rabbit basilar artery; however, UTP contraction was significantly augmented as compared with the peak and steady-state values of UTP contraction, which were recorded for 5 min. Only UTP was washed out by physiological saline solution containing 100 μM α,β-MeATP. Data were obtained at 1 and 5 min after the application or removal of chemicals and presented as means ± S.E.M. ($n=4$). A paired t-test was used to detect any significant change from the prior value. †$P<0.01$.

3.3. Effect of ectonucleotidase inhibition

It is reported that α,β-MeATP has an inhibitory effect on the ectonucleotidase of the cellular membrane that breaks down the nucleotide triphosphate into nucleotide diphosphate (Chen and Lin, 1997). To distinguish the effect of ectonucleotidase inhibition, Evans blue, another potent inhibitor of ectonucleotidase (Bültmann et al., 1995), was used. Evans blue (100 μM) did not augment the UTP contraction statistically significantly; however, the additive effect of α,β-MeATP on UTP contraction rather increased in the presence of Evans blue ($P<0.01$, $n=4$; Fig. 3A and C). The effect of ectonucleotidase inhibition was also confirmed by treatment with extracellular Ni^{2+}. The augmenting effect of Evans blue was not observed in the presence of 1 mM Ni^{2+}, which suggests that ectonucleotidase activity was completely blocked by 1 mM Ni^{2+} and that the further disinhibition of the enzyme was not achieved by Evans blue. However, the addition of α,β-MeATP apparently augmented the UTP contraction even when ectonucleotidase activity was blocked by both extracellular Ni^{2+} and Evans blue ($P<0.01$, $n=4$; Fig. 3B).

3.4. α,β-MeATP effect on the ATPγS contraction

ATPγS is also one of the selective agonists for P2U subtypes ($P2Y_2$ and $P2Y_4$, but not $P2Y_6$, Chang et al., 1995), which is resistant to nucleotide hydrolysis by ectonucleotidase (Crack et al., 1995). In rabbit basilar artery, ATPγS did not induce significant contraction at up to 30 μM; however, at 100 μM, ATPγS abruptly induced a potent tonic contraction ($101.6 \pm 3.2\%$ of K^+-contraction, $n=4$; Fig. 4A). The maximal response was not determined because the concentration over 100 μM was not available; therefore, the EC_{50} of ATPγS could not be calculated. Pretreatment with α,β-MeATP significantly augmented the ATPγS contraction at 30 μM from $1.8 \pm 0.8\%$ to $38.7 \pm 7.0\%$ ($P<0.001$). At a concentration of 100 μM, ATPγS induced a contraction of 101.6% of K^+-contraction; however, it was significantly augmented both by pretreatment with α,β-MeATP ($137.1 \pm 12.5\%$, $P<0.05$, $n=4$; Fig. 4B) and the addition of α,β-MeATP on ATPγS contraction ($145.4 \pm 20.3\%$, $P<0.05$, $n=4$; Fig. 4A). However, ATPγS (100 μM) could not induce a contraction additive to the UTP contraction after α,β-MeATP pretreatment where the P2Y receptor should be maximally activated (Fig. 4C).

3.5. Role of extracellular Ca^{2+}

The role of extracellular Ca^{2+} in the augmenting effect of α,β-MeATP on UTP contraction in the basilar artery was investigated. In the presence of extracellular Ca^{2+}, 100 μM UTP induced a sustained contraction, and further addition of 100 μM UTP (a total of 200 μM) induced a slight augmentation of UTP contraction although there was no statistical significance ($P=0.52$). Under the same conditions, the addition of α,β-MeATP produced a significant additive contraction ($P<0.05$, $n=4$; Fig. 5A). With Ca^{2+} influx blocked by the removal of extracellular Ca^{2+}, 100 μM UTP induced a significant contraction with a transient peak and subsequent lower sustained phases. Further addition of 100 μM UTP (a total of 200 μM) induced no transient peak but the slight elevation of a sustained phase; however, the addition of α,β-MeATP induced an additive contraction ($P<0.001$, $n=7$; Fig. 5B) that showed the transient feature as well as UTP contraction. Removal of

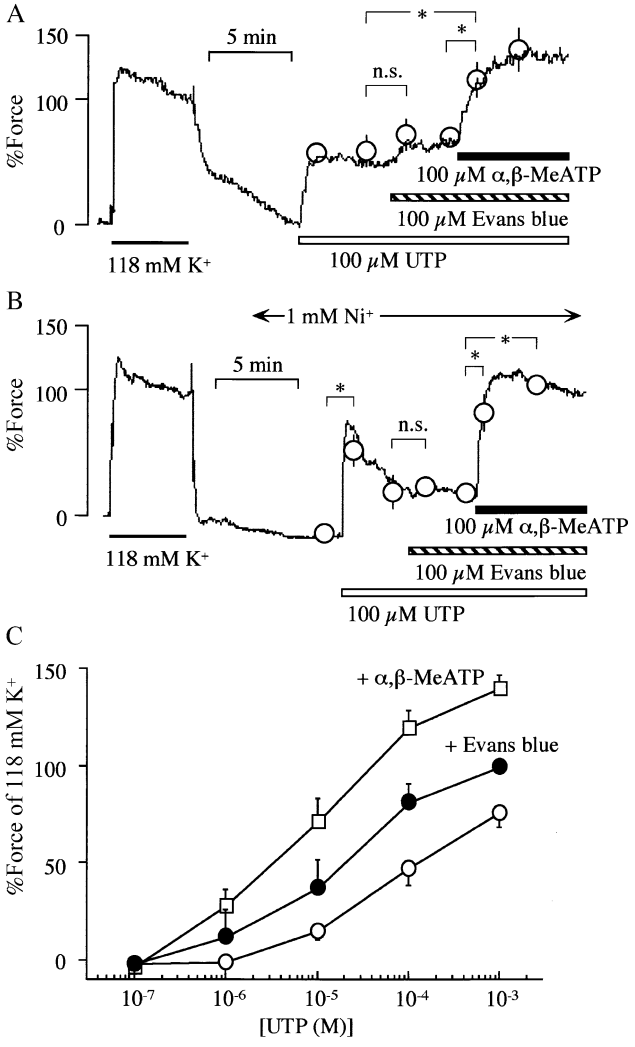

Fig. 3. Effect of Evans blue, an inhibitor of ectonucleotidase, on the α,β-MeATP augmentation of UTP contraction. (A) After the control response to 118 mM K^+ solution, 100 μM UTP induced a tonic contraction. Evans blue (100 μM) did not augment UTP contraction significantly. The further application of α,β-MeATP (100 μM) induced an additional, rapid and tonic contraction on the steady-state UTP contraction. (B) The same protocol was carried out in the presence of 1 mM Ni^+, another inhibitor of ectonucleotidase. Data were obtained at 1 and 5 min after the application or removal of chemicals and a paired t-test was used to detect any significant change from the prior value. $^*P<0.05$, $^\dagger P<0.01$. (C) The effect of Evans blue and α,β-MeATP on the concentration–response curve for UTP. Data were obtained from cumulative application of UTP under no pretreatment (○), in the presence of 100 μM Evans blue (●) or 100 μM α,β-MeATP (□) and are presented as means ± S.E.M. ($n=4$).

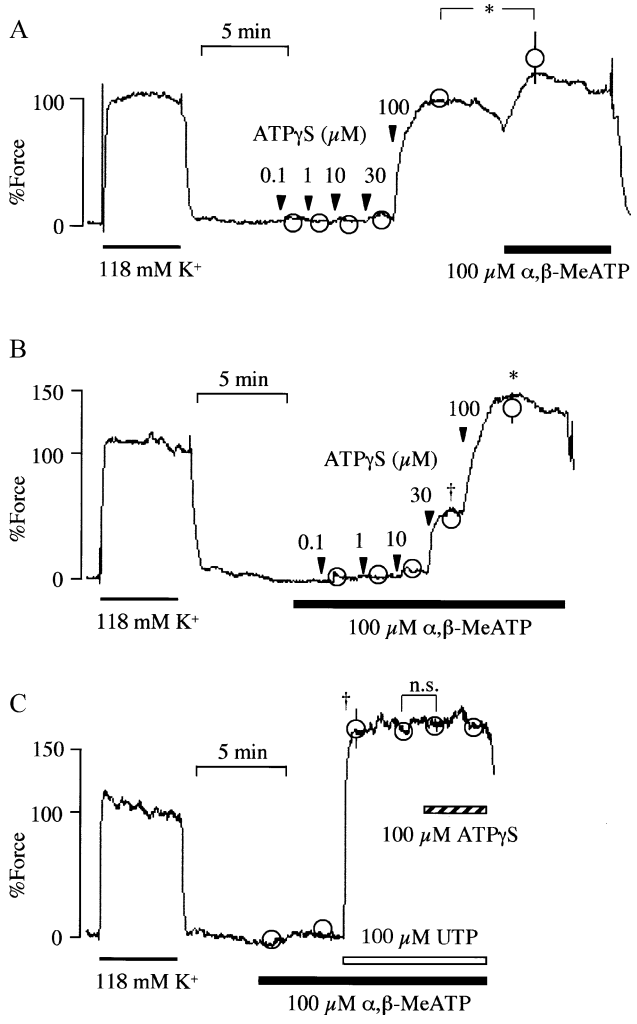

Fig. 4. Effect of α,β-MeATP on ATPγS contraction. Contractile response of basilar artery was induced by cumulative application of ATPγS (0.1–100 μM) in the absence (A) and presence (B) of α,β-MeATP. A Student's *t*-test was used to detect the effect of α,β-MeATP at a given concentration of ATPγS. $^{*}P<0.05$, $^{\dagger}P<0.01$. (C) ATPγS did not elicit an additional contraction on the α,β-MeATP-augmented UTP contraction. Data are presented as means ± S.E.M. ($n=4$).

EGTA and repletion of extracellular Ca^{2+} (1.25 mM) restored the Ca^{2+}-dependent component of UTP contraction as augmented with α,β-MeATP.

3.6. Effect of α,β-MeATP on the UTP-induced $[Ca^{2+}]_i$ transient

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) plays an important role in smooth muscle contraction. Depletion of extracellular Ca^{2+} significantly reduced the UTP contraction, and the repletion of extracellular Ca^{2+} restored the large contraction, as seen in the presence of extracellular Ca^{2+} (Fig. 5B), suggesting the significance of Ca^{2+} influx in the sustained phase of UTP contraction. The finding that α,β-MeATP induced the additional contraction phase in the absence of extracellular Ca^{2+} suggests that the effect of α,β-MeATP on the UTP contraction may include the further release of stored Ca^{2+} in sarcoplasmic reticulum. The augmenting effect of α,β-MeATP was much greater than the UTP contraction in the absence of Ca^{2+}. To observe the augmenting effect of α,β-MeATP on the UTP-induced $[Ca^{2+}]_i$ transient, %ratio was measured using fura-2 fluorometry of the rabbit basilar arterial strip.

In our fura-2 fluorometry, UTP (100 μM) alone did not induce any rise in %ratio. In the presence of UTP, 100 μM α,β-MeATP induced a small increase in %ratio (14.5 ± 2.9%, $P=0.01$; Fig. 6A). α,β-MeATP (100 μM) alone did not induce a rise in %ratio. In the presence of α,β-MeATP, however, 100 μM UTP induced a significant increase in %ratio (19.0 ± 2.7%, $P<0.01$; Fig. 6B). At a higher concentration (1 mM), UTP alone induced a potent but biphasic increase in %ratio; specifically, the first peak of %ratio reached to 88.7 ± 13.2% and then subsequently declined to -23.0 ± 13.4%, which was under the resting level (Fig. 6C). The second addition of 1 mM UTP (a total of 2 mM) induced no significant additive increase in %ratio; however, 100 μM α,β-MeATP induced a significant transient rise to 15.1 ± 7.5%. The same experimental protocol was repeated in the absence of extracellular Ca^{2+} (Fig. 6D). The resting level of the %ratio was significantly decreased by the removal of extracellular Ca^{2+} (−24.5 ± 3.8%), and 1 mM UTP induced a slight increase in %ratio (−18.6 ± 1.4%, $P<0.05$). Furthermore, the second application of

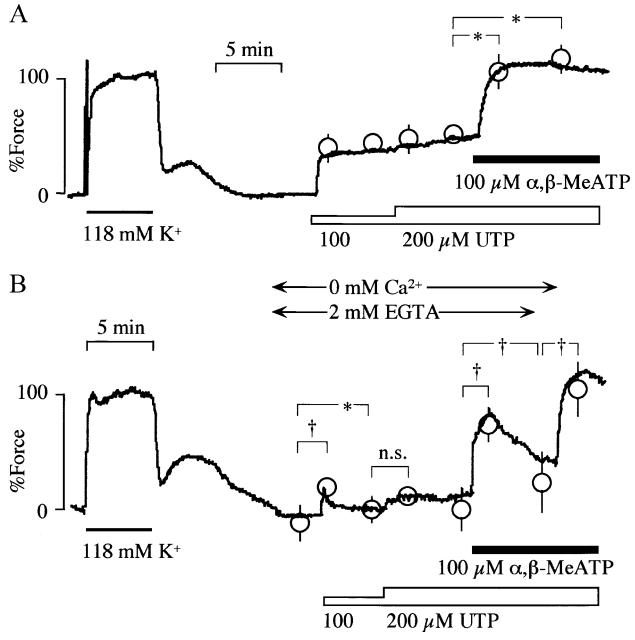

Fig. 5. Effect of extracellular Ca^{2+} depletion on the α,β-MeATP augentation of UTP contraction. Contractile response of basilar artery to UTP (100–200 μM) was augmented by 100 μM α,β-MeATP in the presence (A) and absence (B) of extracellular Ca^{2+}. A paired *t*-test was used to detect any significant change from the prior value. $^{*}P<0.05$, $^{\dagger}P<0.01$. Data were obtained at 1 and 5 min after the application or removal of chemicals and are presented as means ± S.E.M. ($n=4$).

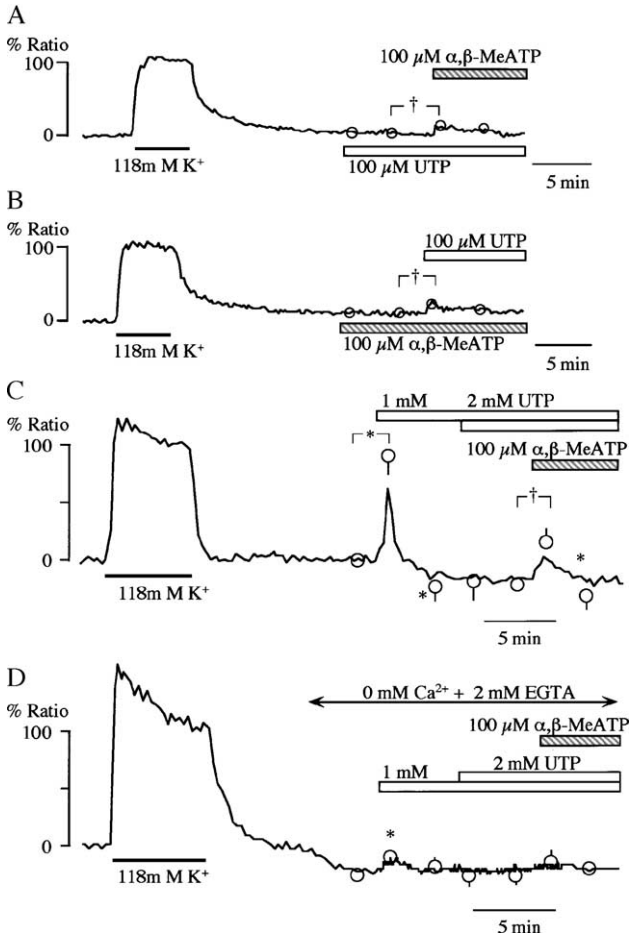

Fig. 6. Representative recordings and summary of the data showing the effect of α,β-MeATP to augment the UTP-induced $[Ca^{2+}]_i$ elevation in the rabbit basilar arterial strips. (A) After the control response to 118 mM K^+ solution, 1 mM UTP did not induce a significant $[Ca^{2+}]_i$ elevation. The application of α,β-MeATP (100 μM) induced a rapid $[Ca^{2+}]_i$ elevation in the presence of UTP. (B) After the control response to 118 mM K^+ solution, the basilar arterial strips were pretreated with 100 μM α,β-MeATP and 100 μM UTP was applied topically in the presence of α,β-MeATP. α,β-MeATP alone never induced a contraction of rabbit basilar artery; however, UTP induced a significant $[Ca^{2+}]_i$ elevation. (C) $[Ca^{2+}]_i$ elevation induced by a higher concentration (1 mM) of UTP. Cumulative application of UTP did not induce any additional $[Ca^{2+}]_i$ elevation; however, 100 μM α,β-MeATP induced an additional $[Ca^{2+}]_i$ elevation. The peak and steady-state values of $[Ca^{2+}]_i$ elevation were recorded for 5 min. (D) The same protocol as (C) was carried out in the absence of extracellular Ca^{2+}. Data were obtained at 1 and 5 min after the application or removal of chemicals and are presented as means ± S.E.M. ($n = 4$). Only significant changes from the prior value by a paired t-test are shown. $^*P < 0.05$, $^\dagger P < 0.01$.

UTP (a total of 2 mM) and α,β-MeATP did not induce any significant increase in %ratio.

4. Discussion

In this study, we investigated the mechanisms of the augmenting effect of α,β-MeATP on UTP contraction. The augmenting effect of α,β-MeATP does not depend on the extracellular Ca^{2+}, which indicates that $P2Y_4$ activation was stimulated by α,β-MeATP but not that P2X activation (ligand-gated cation channel) was stimulated by UTP. It may be partly due to the inhibition of ectonucleotidase activity in smooth muscle. However, this effect cannot be explained solely by ectonucleotidase regulation, because none of the various pharmacological methods of ectonucleotidase inhibition could completely abolish the augmenting effect of α,β-MeATP on UTP contraction.

4.1. P2 receptor subtypes in rabbit basilar artery

The distribution of P2 receptor subtypes is often studied as the expression of mRNA and/or protein levels; however, the results do not always imply a functional coupling between receptor subtypes and biological responses. $P2X_1$ is the main P2X receptor subtype expressed in vascular smooth muscle, whereas $P2X_2$ and $P2X_3$ subtypes are the main receptor subtypes in peripheral sensory ganglia (Buell et al., 1996). $P2X_2$, $P2X_4$ and $P2X_6$ are predominant in brain. $P2X_1$ and $P2X_3$ have a high affinity for ATP and α,β-MeATP but are rapidly desensitized by α,β-MeATP (North and Surprenant, 2000). Among the members of the P2Y family, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ are detected by amplification from mRNA of vascular smooth muscle (Erlinge et al., 1998; Harper et al., 1998; Carpenter et al., 2001). However, $P2Y_1$, $P2Y_2$ and $P2Y_6$ expressed at the mRNA level are not expressed as functional receptors effectively coupled to phospholipase C (Harper et al., 1998). $P2Y_1$ is activated by 2-MeSATP, but insensitive to UTP. $P2Y_2$ (P2U in the earlier classification) has equal affinity to ATP and UTP, whereas $P2Y_3$, $P2Y_4$ and $P2Y_6$ are selectively activated by pyrimidines. Above all, $P2Y_4$ is UTP-selective, while $P2Y_3$ and $P2Y_6$ are UDP-selective (Nicholas et al., 1996). The difficulties of identifying (or characterizing) functional receptor subtypes are due to the mixed population of multiple subtypes in the same tissue and the lack of specific antagonists for each subtype.

Although the molecular biological method showed evidence of $P2X_1$, $P2X_2$ and $P2X_4$ receptors expressed in the vascular smooth muscle of other organs (Nori et al., 1998), the present study revealed that P2X receptor agonists α,β-MeATP and 2-MeSATP did not induce any significant contraction at a concentration up to 1 mM. On the other hand, UTP induced a concentration-dependent tonic contraction in rabbit basilar artery. These results agree with previous reports by Von Kügelgen et al. (1987, 1990) showing the organ-specific distribution of contractile P2X and P2Y families. Therefore, the rabbit basilar artery seems to be an appropriate model for pharmacological study of the functional P2Y family that is free from interference from the functional P2X receptor. The agonist potency order for the contractile $P2Y_2$ subtype is UTP = ATP > α,β-MeATP and the agonist potency order for the $P2Y_6$ subtype shows UTP > 2-MeSATP > ATP > ATPγS (Chang et al., 1995). The functional P2Y receptor involved in UTP contraction as

determined by the present pharmacological study shows the potency order ATPγS ≥ UTP ≫ ATP, α,β-MeATP, 2-MeSATP, which agrees with the agonist potency order of $P2Y_4$. However, the involvement of other subtypes cannot be totally excluded because the contractile response may represent the summation of multiple receptor activation in vascular smooth muscle.

Using the rabbit basilar artery, the present study demonstrated that α,β-MeATP itself did not elicit any contraction, while UTP contraction was augmented by α,β-MeATP. This augmenting effect was immediate and easily ended by the removal of extracellular α,β-MeATP (Fig. 2), suggesting that α,β-MeATP affects the UTP-$P2Y_4$ receptor interaction (not the non-functioning P2X-receptor alone) because α,β-MeATP neither penetrates the plasma membrane nor affects intracellular signal transduction of the P2Y receptor.

4.2. The possible role of ectonucleotidase in the UTP contraction

Ectonucleotidase that exists on the plasma membrane breaks down the nucleotide triphosphate (Kreutzberg et al., 1978; Welford et al., 1987). Since α,β-MeATP is known to inhibit the activity of ectonucleotidase (Chen and Lin, 1997), the augmenting effect of α,β-MeATP on UTP contraction may depend on the activity of ectonucleotidase. A role of endothelial ectonucleotidase activity was suggested (McMillan et al., 1999); however, the endothelium of rabbit basilar artery in this study was mechanically denuded. Although there are no specific inhibitors of ectonucleotidase, several pharmacological methods are available to inhibit this enzyme: (1) deplete the extracellular Ca^{2+}; (2) add divalent cations, such as Ni^{2+}; (3) use non-hydrolyzable nucleotide analogues, such as ATPγS; and (4) use inhibitors such as suramin or Evans blue (Bültmann et al., 1995, Chen and Lin, 1997). In the present study, most of the available methods were used to inhibit ectonucleotidase activity. Depletion of extracellular Ca^{2+} (Fig. 5A), the pretreatment with Ni^{2+} (Fig. 3B) and Evans blue (Fig. 3) were not able to end the augmenting effect of α,β-MeATP on the UTP contraction. In addition, the UTP contraction was not affected by gently mixing the UTP-containing solution in an organ bath in order to rinse out the degraded molecules (UDP or UMP) and to provide fresh UTP around the tissue (data not shown). In addition, ATPγS, one of the non-hydrolyzable nucleotides and also a potent agonist for $P2Y_4$ receptors, induced a tonic contraction in rabbit basilar artery, and α,β-MeATP augmented the contractile response to ATPγS (Fig. 4A, B). These results indicate that the augmenting effect of α,β-MeATP on the UTP contraction cannot be explained solely by ectonucleotidase inhibition. In addition, there was no additivity between UTP and ATPγS contractions, suggesting that UTP contraction is mediated by the $P2Y_4$ receptor that is shared with ATPγS.

4.3. The site of α,β-methylene-ATP action

P2Y families are defined by the seven-transmembrane structure, activation of which is known to induce increases in $[Ca^{2+}]_i$ and force of smooth muscle through receptor/G protein-coupled mechanisms, including phospholipase C activation and phosphoinositides breakdown. The increase in $[Ca^{2+}]_i$, which is generally driven by two sources, namely the IP_3-induced release of cytosolic Ca^{2+} (from an intracellular storage site), and the influx of extracellular Ca^{2+} (capacitative Ca^{2+} entry), initiates the force development of smooth muscle. In our study, UTP also induced increases in $[Ca^{2+}]_i$ from two sources (Ca^{2+} influx and Ca^{2+} release) in the same manner as the other G protein-regulated vasoconstrictor agonists. The fact that both Ca^{2+}-increasing pathways are augmented by pretreatment with α,β-MeATP strongly indicates that the site of action of α,β-MeATP is at the receptor level, but it is not the intracellular mechanism.

However, in our study, the excess concentration of UTP (over 1 mM) induced a decrease in $[Ca^{2+}]_i$ after the first transient peak of $[Ca^{2+}]_i$. In HL-60 cells, the P2U receptor activation by the overdose of UTP inhibited capacitative Ca^{2+} entry by a protein kinase C (PKC)-mediated mechanism (Lee et al., 1997). Also, in vascular smooth muscle cells, PKC activates the plasma membrane Ca^{2+} pump to decrease the $[Ca^{2+}]_i$ (Broad et al., 1999). Therefore, a decrease in $[Ca^{2+}]_i$ at a high concentration of UTP (Fig. 6C) may be explained by the activation of the PKC pathway, which occurs downstream of the P2Y receptor/G protein-coupled phosphatidyl inositol cascade.

In addition to an increase in $[Ca^{2+}]_i$, it has been well established that the force of smooth muscle is also regulated by Ca^{2+}-sensitivity of the contractile apparatus, a mechanism which involves some regulators, such as the activation of PKC, the inhibition of myosin light-chain phosphatase and Rho-kinase (Somlyo and Somlyo, 2000). Our finding that UTP induced tonic contraction without a significant increase in $[Ca^{2+}]_i$ at 100 μM (Fig. 6) can be explained by the Ca^{2+}-sensitization. Even in the absence of extracellular Ca^{2+}, full activation of the P2Y receptor facilitated by α,β-MeATP induced a potent tonic UTP contraction. The disproportionately large additive contraction in association with such a small $[Ca^{2+}]_i$ increase suggests a potent Ca^{2+}-sensitization by the UTP/P2Y-receptor coupling in the rabbit basilar artery.

Multiple signal transduction pathways and second messengers are considered to be involved in Ca^{2+}-independent contraction in smooth muscle. An experiment with the skinned preparation in which the $[Ca^{2+}]_i$ is kept constant (Kureishi et al., 1997) is required to elucidate the signal transduction involved in a Ca^{2+}-independent pathway in UTP-contraction; however, a skinned preparation is not available for this study because the intra- and extracellular solution contains a considerable amount of nucleotides (ATP and GTP) (Chen et al., 1997), which stimulate and/or desensitize the P2Y-receptor coupling.

4.4. Physiological relevance of UTP contraction

Some inhibitory regulatory functions indicated for the P2Y receptor may be involved, such as: (1) rapid degradation of nucleotides by extracellular ectonucleotidase activity (Malmsjö et al., 2000); (2) desensitization by PKC-mediated pathway downstream below receptor/G protein activation (Wilkinson et al., 1994); and (3) agonist-dependent sequestration of the $P2Y_2$ receptor (Garrad et al., 1998; Stromek and Harden, 1998). α,β-MeATP revealed one of the inhibitory mechanisms for UTP contraction in this study. UTP can also be released from injured cells, resulting from a variety of pathological conditions, such as trauma, hypoxia and inflammation. Under such pathological conditions, P2Y receptors may be protected from excessive UTP exposure by some inhibitory regulation systems, which depend on ectonucleotidase or α,β-MeATP-sensitive mechanisms.

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