P2Y Receptor-Mediated Ca\(^{2+}\) Signaling Increases Human Vascular Endothelial Cell Permeability

Naoko Tanaka\(^1\), Kumiko Kawasaki\(^1\), Namie Nejime\(^1\), Yoko Kubota\(^1\), Kazuki Nakamura\(^1\), Masaru Kunitomo\(^1\), Koichi Takahashi\(^2\), Michio Hashimoto\(^3\), and Kazumasa Shinozuka\(^1,\*\)

Departments of \(^1\)Pharmacology and \(^2\)Pharmaceutics, School of Pharmaceutical Sciences, Mukogawa Women’s University, Nishinomiya 663-8179, Japan

\(^3\)Department of Physiology, Shimane Medical University, Izumo, Shimane 693-8501, Japan

Received April 11, 2003; Accepted March 31, 2004

Abstract. We investigated the effects of P2-receptor agonists on cell size, intracellular calcium levels ([Ca\(^{2+}\)])\(_i\), and permeation of FITC-labeled dextran (FD-4) as well as the relationship between these effects in human umbilical vein endothelial cells (HUVEC). FD-4 concentration, cell size, and [Ca\(^{2+}\)])\(_i\) were analyzed by HPLC with fluorescence, phase contrast microscopic imaging, and fluorescent confocal microscopic imaging, respectively. The P2Y\(_1\)-receptor agonists 2-methylthio ATP (2meS-ATP) and ADP decreased cell size and increased [Ca\(^{2+}\)])\(_i\) in HUVEC. The P2Y\(_2\)-receptor agonist UTP increased [Ca\(^{2+}\)])\(_i\), but did not influence cell size. The P2X-receptor agonist \(\alpha,\beta\)-methylene ATP did not induce either response. The decrease in size and increase in [Ca\(^{2+}\)])\(_i\) by 2meS-ATP were blocked by pyridoxal phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS, P2Y\(_1\)-antagonist), thapsigargin (Ca\(^{2+}\)-pump inhibitor), and U73122 (phospholipase C inhibitor). Furthermore, 2meS-ATP (P2Y\(_1\)-receptor agonist) enhanced permeation of FD-4 through the endothelial cell monolayer. The 2meS-ATP-induced enhancement of the permeation was also prevented by PPADS, thapsigargin, and U73122. These results indicate that activation of P2Y receptors induces a decrease in cell size, an increase in [Ca\(^{2+}\)])\(_i\), and may participate in facilitating macromolecular permeability in HUVEC.

Keywords: cell size, intracellular Ca\(^{2+}\), permeability, P2Y receptor, human umbilical vein endothelial cell

Introduction

ATP has been increasingly recognized as an important extracellular signaling molecule that exerts a range of effects in various tissues, including blood vessels. ATP regulates a wide range of functions in nervous, smooth muscle, and endothelial cells via direct stimulation of membrane P2X and P2Y receptors. To date, at least 7 ionotropic purine receptors (P2X\(_1\)–P2X\(_7\)) and 8 G protein-coupled purine receptors (P2Y\(_1\), P2Y\(_2\), P2Y\(_3\), P2Y\(_4\), P2Y\(_6\), P2Y\(_8\), P2Y\(_11\), and P2Y\(_14\)) have been identified in various animal species (1–4). In endothelial cells, ATP generally causes an increase in intracellular calcium levels ([Ca\(^{2+}\)])\(_i\)) via activation of P2Y receptors, which are coupled to phospholipase C and inositol 1,4,5-trisphosphate (IP\(_3\))-induced Ca\(^{2+}\) mobilization (4). We recently found that stimulation of P2Y receptors facilitates regulatory volume decrease (RVD) under hypotonic conditions in an intracellular Ca\(^{2+}\)-dependent manner (5). Furthermore, we also found that ATP decreased the size of endothelial cells under conditions of physiological iso-osmotic pressure via a P2Y receptor coupled to phospholipase C and IP\(_3\)-induced Ca\(^{2+}\) mobilization (6). However, the physiological role of cell size regulation via the P2Y receptor under physiological conditions remains unknown. Hayashi et al. found that opening of the tight junctions is dependent on increases in [Ca\(^{2+}\)])\(_i\), through interaction with phospholipase C in the membrane and that this process enhances permeability in rodent colonic epithelial cells (7). In bovine pulmonary artery endothelial cells, activation of store-operated Ca\(^{2+}\) entry is...
associated with intercellular gap formation and increased barrier transport of macromolecules (8). These findings led us to hypothesize that opening of tight junctions in endothelial cells may occur as a result of P2Y-receptor stimulation and that endothelial cell shrinkage may participate in the mechanism for increasing endothelial macromolecule permeability.

In the present study, we investigated the effects of P2Y-receptor agonists on cell size, \([Ca^{2+}]_i\), and permeation of FITC (fluorescein isothiocyanate)-labeled dextran (FD-4) under normal isotonic conditions in cultured human vascular endothelial cells. Particular attention was focused on the relationship between these three effects in order to elucidate the possible physiological roles of purinergic signaling. Preliminary accounts of this work have been presented (9).

Materials and Methods

Cell culture of endothelial cells

Human umbilical vein endothelial cells (HUVEC) were purchased from Bio Whittaker (Walkersville, MD, USA) and were cultured in EBM-2 medium supplemented with 5% fetal bovine serum, hydrocortisone, endothelial growth factor, epidermal growth factor, human fibroblast growth factor, insulin-like growth factor, and ascorbic acid. Cells were plated out in 57-cm\(^2\) tissue culture dishes (Cell tight C-1; Sumitomo Bakelite Co., Ltd., Osaka) and incubated at 37°C in a 95% air/5% CO\(_2\) atmosphere. Cells were removed from the flasks with 0.025% trypsin/0.01% EDTA solution and seeded onto plates coated with collagen in 35-mm tissue culture dishes (Cell tight C-1). All experiments were performed with HUVEC in passages 3 – 5 and at 4 – 6 days post-confluence.

Measurement of \([Ca^{2+}]_i\), and cell surface area

The standard extracellular solutions for the measurements of \([Ca^{2+}]_i\), and cell surface area were physiological saline solution (PSS) of the following composition: 140 mM NaCl, 4.0 mM KCl, 2.0 mM CaCl\(_2\), 2.0 mM MgCl\(_2\), 10 mM HEPES, 10 mM glucose, pH 7.4 (adjusted with NaOH). Ca\(^{2+}\)-free PSS was made by substituting the CaCl\(_2\) of the PSS with 1 mM EGTA. Primary cultured endothelial cells on type I collagen-coated glass coverslips were incubated with 5 \(\mu\)M Calcium Green 1/AM for 30 min at 37°C. Cells were rinsed three times with PSS and then incubated in PSS for an additional 15 min at 37°C in a 95% air/5% CO\(_2\) atmosphere in order to complete hydrolysis of any intact ester linkages in the intracellular Calcium Green 1/AM. Loaded coverslips were placed onto the stage of an inverted microscope coupled to a Nipkow disk confocal scanner (CSU10; Yokogawa Electric Corp., Tokyo) and the ARGUS-50 imaging system (Hamamatsu Photonics, Hamamatsu). An argon-krypton laser (Omnichrome, Chino, CA, USA) emitted an excitation wavelength of 488 nm. Emitted light was collected with a 510-nm long-pass dichroic reflector and a 515-nm long-pass emission filter through a planfluor objective (×100 or ×20; NA = 1.4, 0.5, respectively). Fluorescence images were obtained using an ICCD camera (C2400-87, Hamamatsu Photonics). To quantify \([Ca^{2+}]_i\), in the endothelial cells, images were taken at a resolution of 195 x 130 pixels every 5 s. Fluorescence intensity was calculated by dividing peak fluorescence intensity (F) during the experiment by the fluorescence intensity at the beginning of each experiment (F\(_0\)).

For measurement of cell size, HUVEC were seeded onto 35-mm culture dishes and grown to confluence. Cells were rinsed three times with PSS and then incubated in PSS for 45 min at 37°C in a 95% air/5% CO\(_2\) atmosphere, after which PSS containing P2 agonists was added to the culture dishes for 3 min. To quantify cell size, images with phase-contrast microscope were taken before and after addition of each P2 agonist. Endothelial cell size was measured using National Institutes of Health imaging software (NIH Image 1.61; Yodosha, Tokyo).

Transport of FD-4 experiment

The transport of FD-4 (MW 4400, 1 mg/mL) across endothelial cell monolayers seeded on 6.4-mm Transwell tissue culture inserts (Corning Costar Corporation, Corning, NY, USA) was studied. Endothelial cell monolayers were equilibrated in warm PSS for 30 min before the transport experiments. The inserts were placed in wells containing PSS and then in PSS containing FD-4 with or without 2-methylthio ATP (2meS-ATP) added to the apical side of the inserts (time = 0 min). For the inhibition experiment, endothelial cell monolayers were preincubated with 100 \(\mu\)M PPADS, 1 \(\mu\)M U73122, and 1 \(\mu\)M thapsigargin for 15 min. Samples (50 \(\mu\)L) were collected at 10-, 20-, or 30-min from the basolateral side, and then fresh PSS was added. The amount of FD-4 in the samples was analyzed by HPLC with fluorescence detection (excitation and emission filters at 495 and 518 nm). The HPLC system consisted of an analytical Cosmosil SC18-MS column (Nacalai Tesque, Inc., Kyoto), a delivery pump (LC-10AD; Shimadzu, Kyoto) equipped with a fluorescence monitor (RF-10A, Shimadzu) a sample processor (AS-8020; Tosoh, Tokyo), and a chromatopac computing integrator (CBM-10A, Shimadzu). The mobile phase was 5 mM phosphate buffer (pH 7.4)-acetonitrile (88:12) at a flow rate of 1 mL/min. A standard calibra-
tion curve (0.1 – 5.0 μg/mL) was constructed by adding a known amount of FD-4 to PSS. We consistently obtained r² values greater than 0.99.

**Materials**

FD-4, α,β-methylene ATP, ADP, ATP, 2meS-ATP, UTP, thapsigargin (TSG), U73122, and bis-(O-aminophenoxy)ethane-N,N',N″-tetraacetic acid acetoxymethyl ester (BAPTA-AM) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) was purchased from Tocris (Bristol, UK). Calcium Green 1/AM (Molecular Probes, Eugene, OR, USA) was used as an intracellular Ca²⁺ probe. Drug concentrations are expressed as the final molar concentration in PSS on the plates.

**Statistics**

All values are expressed as the mean ± S.E.M. Student’s paired t-test and ANOVA were followed by Bonferroni modification of the t-test. An unpaired Student’s t-test evaluated the other data for statistical significance. P < 0.05 was considered significant. Statistical analyses were performed using StatView 4.5 (Abacus Concepts, Cupertino, CA, USA).

**Results**

*Effects of P2 agonists on cell size and [Ca²⁺], in endothelial cells*

As shown in Fig. 1, treatment with 10 μM 2meS-ATP, a P2Y₁-receptor agonist, significantly decreased cell size (n = 15) and increased [Ca²⁺], (n = 7) to 86 ± 2% and 214 ± 6% of the values before 2meS-ATP addition, respectively. Similarly, ADP, another P2Y₁-receptor agonist, at a concentration of 10 μM also significantly decreased cell size (n = 15) to 93 ± 2%, and increased [Ca²⁺], (n = 7) to 192 ± 4% (Fig. 1: a and b). However, a P2Y₂-receptor agonist, UTP, at a concentration of 10 μM increased [Ca²⁺], but had no effect on cell size. Furthermore, the P2X-receptor agonist α,β-mATP (100 μM) had no effect on either cell size or [Ca²⁺], in endothelial cells (Fig. 1: a and b). Figure 2 shows the concentration-dependent effects of 2meS-ATP on cell size and [Ca²⁺], in HUVEC (n = 14); treatment with 1, 10, and 100 μM 2meS-ATP significantly decreased cell size and increased [Ca²⁺], in a dose-dependent manner (Fig. 2: a and b). UTP at 100 μM did not produce more response than that at 10 μM (data not shown).

*Effects of PPADS, U73122, and TSG on 2meS-ATP-induced decrease in cell size and increase in [Ca²⁺]*

The 2meS-ATP-induced decrease in cell size was significantly inhibited by pretreatment with 100 μM PPADS, which is a P2Y₁-receptor antagonist, for 15 min (Fig. 3a). Furthermore, 10 μM U73122, a phospholipase C inhibitor, and 1 μM TSG, a calcium pump inhibitor, also inhibited the decrease in cell size (Fig. 3a). The increase in [Ca²⁺], induced by 2meS-ATP was also significantly inhibited in the presence of 100 μM PPADS (Fig. 3b). U73122 (10 μM) and TSG (1 μM) also inhibited the 2meS-ATP-induced increase

![Fig. 1. Effects of 10 μM 2meS-ATP, ADP, and UTP and 100 μM α,β-mATP on cell size (a) and [Ca²⁺], (b) in HUVEC (n = 15). Data are expressed as the mean ± S.E.M. *P<0.05, compared with values before adding P2 agonists (Student’s paired t-test).](image-url)
in [Ca^{2+}] (Fig. 3b).

**Effects of removal of external Ca^{2+} and BAPTA-AM on 2meS-ATP-induced decrease in cell size and increase in [Ca^{2+}].**

Pretreatment with Ca^{2+}-free medium for 15 min, the 2meS-ATP-induced decrease in cell size was not affected (Fig. 4a). On the other hand, pretreatment with BAPTA-AM (10 μM), an intracellular Ca^{2+} chelator, abolished the 2meS-ATP-induced decrease in cell size. Furthermore, the increase in [Ca^{2+}], induced by 2meS-ATP was not affected by pretreatment with Ca^{2+}-free medium (Fig. 4b). BAPTA-AM (10 μM) also inhibited the 2meS-ATP-induced increase in [Ca^{2+}] (Fig. 4b).

---

**Fig. 2.** Concentration-dependent effects of 2meS-ATP on cell size (a) and [Ca^{2+}] (b) in HUVEC (n = 14). Data are expressed as the mean ± S.E.M. *P<0.05, compared with values before adding 2meS-ATP (Student’s paired t-test).

**Fig. 3.** Effects of 100 μM pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), 10 μM U73122, and 1 μM thapsigargin (TSG) on 2meS-ATP-induced decrease in cell size (a) (n = 10) and 2meS-ATP-induced increase in [Ca^{2+}] (b) (n = 5) in HUVEC. Cells were preincubated with PPADS, U73122, and TSG for 15 min before stimulation with 2meS-ATP. Data are expressed as the mean ± S.E.M. *P<0.05, compared with values before adding 2meS-ATP (Student’s paired t-test). P<0.05, compared with the control value after adding 2meS-ATP (Bonferroni modification of the t-test).
Effects of 2meS-ATP, PPADS, U73122, and TSG on the permeation of FD-4

The concentration of FD-4 in the basolateral side of the cell culture inserts, which is a parameter of permeation through the endothelial cell monolayers, increased markedly in the presence of 10 μM 2meS-ATP, as shown in Fig. 5. Furthermore, the 2meS-ATP-induced increase in cellular permeation of FD-4 was inhibited by pretreatment with 100 μM PPADS, 1 μM U73122, and 1 μM thapsigargin (TSG) (20 min, 30 min).

Discussion

All cells have a mechanism by which cell volume is adjusted depending on changes in external conditions. For example, under hypotonic conditions imposed by decreased extracellular osmolarity, cells can adjust their volume after transient osmotic swelling by a well-known mechanism called regulatory volume decrease (RVD). Recent evidence indicates that stimulation of P2 receptors facilitates RVD in human salivary gland duct cells (10), human hepatocytes (11), necturus erythrocytes (12), rat hepatoma cells (13), and a human epithelial cell line (14). We also found that ATP facilitates RVD via P2Y receptors in endothelial cells of the rat caudal artery (5). Interestingly, stimulation of P2 receptors decreases the size of endothelial cells of
the rat caudal artery not only under hypotonic conditions but also under the isotonic physiological conditions (6). Therefore, P2-receptor agonists such as ATP appear to be a widespread patho-physiological or physiological modulator of cell size regulation in endothelial cells.

In the present study, it was shown that the cell size of HUVEC was decreased by the presence of 2meS-ATP and ADP under isotonic conditions. Furthermore, the decrease in cell size induced by 2meS-ATP was blocked by PPADS, which is a P2Y1-receptor antagonist. However, α,β-mATP had no effect at all on cell size. 2meS-ATP, ATP, and ADP are all known P2Y1-receptor agonists. UTP is known to stimulate P2Y2 and P2Y4, not P2Y1; and α,β-mATP is a P2X-receptor agonist (4). Northern blot analysis recently demonstrated the distinct expression of P2Y1, P2Y2, P2Y11, and P2X4 mRNA in HUVEC (15, 16). P2Y1 and P2Y2 are coupled to stimulation of phosphoinositide pathways, and P2Y11 is coupled to stimulation of both phosphoinositide and adenyllyclase pathways. P2Y11 agonists, in order of potency, are ATP > 2meS-ATP > ADP (17). White et al. also reported that ATP and UTP are Ca2+-mobilizing agonists of P2Y11 receptors (18). In addition, P2Xs, a subtype of ATP-operated cation channels, is insensitive to α,β-mATP. These findings, together with our observations, suggest that the P2 receptor associated with the decrease in cell size may be P2Y1, P2Y2, P2Y11, or P2X4. However, UTP (1 and 10 μM) had no effect on cell size, and thus the role of P2Y2 in the decrease in cell size appears to be extremely small.

On the other hand, stimulation of endothelial cells with 2meS-ATP, ADP, and UTP increased [Ca2+]i in HUVEC, while α,β-mATP had no effect. Furthermore, the increase in [Ca2+]i induced by 2meS-ATP was blocked by PPADS. Therefore, 2meS-ATP exhibits similar pharmacological effects on cell size and [Ca2+]i. P2Y receptors generally act via G-protein coupling in order to activate phospholipase C, followed by the formation of IP3 and an increase in intracellular Ca2+ (3, 4). Our results demonstrated that U73122, a phospholipase C inhibitor, and TSG, a calcium pump inhibitor, depress both the 2meS-ATP-induced increase in [Ca2+]i and the decrease in endothelial cell size. This indicates that the 2meS-ATP-induced decrease in endothelial cell size is mediated by activation of P2Y receptors, but probably not P2X receptors, and induces both a decrease in cell size and an increase in [Ca2+]i, which is derived from Ca2+ stores. Although TSG by itself slightly but significantly increased [Ca2+]i, it did not change the cell size (data not shown). Since TSG-induced increase in [Ca2+]i is clearly lower than that induced by P2Y agonist, the Ca2+ concentration may not be enough to change cell size. On the other hand, intracellular Ca2+ localization coupled with cell size change may be a specific compartment for the P2Y-receptor mechanism. Therefore, Ca2+ released by UTP also may not link to the mechanisms for decreasing the cell size. Otherwise, the amount of increase of Ca2+ induced by UTP may be too low to decrease the cell size. These are speculations and must be elucidated by further study.

After pretreatment with Ca2+-free medium, 2meS-ATP-induced decrease in cell size and increase in [Ca2+]i were not affected. However, treatment with BAPTA-AM, an intracellular Ca2+ chelator, abolished the 2meS-ATP-induced decrease in cell size. The 2meS-ATP-induced increase in [Ca2+]i was also significantly inhibited. This strongly suggests that the 2meS-ATP-induced decrease in endothelial cell size is mediated by [Ca2+]i derived from Ca2+ stores.

We also observed an increase in permeation of FD-4 across the endothelial monolayer when cells were exposed to 2meS-ATP, which indicates a correlation between changes in cell size and transendothelial FD-4 flux. Shepard et al. reported that endothelial cell shrinkage might be an important mechanism that is related to endothelial macromolecule permeability (19). It was also found that opening of the tight junctions is dependent on increased [Ca2+]i, through interaction with phospholipase C in the membrane and that this process enhances permeability in rodent colonic epithelial cells (7). It is possible that decrease in cell size might be related to increases in the intercellular space. Therefore, regulating the size of endothelial cells by extracellular ATP may be an important mechanism for increasing endothelial macromolecule permeability.

Permeation of FD-4 across endothelial cell monolayers was obviously increased by 2meS-ATP, and the 2meS-ATP-induced increase in the permeation of FD-4 was inhibited by pretreatment with PPADS, U73122, and TSG. These findings indicate that 2meS-ATP increases the permeability of endothelial monolayers through Ca2+-dependent mechanisms via activation of P2Y receptors.

Several studies have also indicated that calcium-dependent mechanisms in endothelial cells modulate acute changes in the permeability of venular microvessels to water and macromolecules (20–27). He et al. (28) suggested that Ca2+ entry through conductive pathways modulate receptor-mediated increases in cytoplasmic Ca2+ concentration and increased microvessel permeability. Taken together, these findings indicate that extracellular ATP may increase microvessel permeability through activation of P2Y1 receptors in HUVEC. Increased microvessel permeability is believed to elevate absorption and/or distribution of highly polar.
drugs and macromolecules. The findings of the present study indicate that purinergic receptors may participate in the absorption/distribution process of drugs by regulating endothelial cell size.

Acknowledgments

The authors would like to thank Ms. Takako Masumoto and Ms. Asami Takei for their invaluable technical assistance. This research was supported in part by a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Science, Sports, and Culture of Japan.

References