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Myosin light chain kinase and Rho-kinase participate in P2Y receptor-mediated acceleration of permeability through the endothelial cell layer

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Abstract

We have shown that P2Y receptor stimulation accelerates macromolecular permeation through the endothelial cell layer. To elucidate the mechanism of this acceleration, we examined the effects of ML-9, a myosin light chain kinase inhibitor, and Y-27632, a Rho-kinase inhibitor, on fluorescein isothiocyanate dextran (FD-4) permeation across the human umbilical vein endothelial cell monolayer. FD-4 permeation was analysed by high-performance liquid chromatography fluorescence detection. A P2Y receptor agonist, 2meS-ATP, enhanced the permeability of FD-4, which was inhibited by pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), a P2Y-receptor antagonist. The 2meS-ATP-induced increase in the permeability of FD-4 was significantly inhibited by ML-9. Also, Y-27632 prevented the 2meS-ATP-induced increase in the permeability of FD-4. Neither ML-9 nor Y-27632 influenced the spontaneous permeation of FD-4. These results suggest that phosphorylation of the myosin light chain may play an important role in the purinergic regulation of macromolecular permeation through the vascular endothelium.

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Introduction

ATP can act as a potent extracellular signalling molecule through interaction with cell membrane receptors. The purinergic receptors (P2X and P2Y) have been identified as receptors that mediate the reaction and many cell types, including the endothelial cells, express them. To date, at least seven ionotropic purine receptors ($P2X_1$–$P2X_7$) and eight 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 (Burnstock 1991; Dalziel & Westfall 1994; Abbracchio & Burnstock 1998; Ralevic & Burnstock 1998). In endothelial cells, it is well known that ATP modulates the generation of nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF) and prostacyclin (PGI_2) (Carter et al 1988; Rongen et al 1994; Patel et al 1996; Qasabian et al 1997; Malmsjo et al 1999; Wihlborg et al 2003). In addition to these, we have found, in rat caudal arterial endothelial cells, that ATP regulates endothelial cell size through intracellular Ca^{2+}, which is derived from Ca^{2+} stores via the P2Y receptor in hypotonic or physiological conditions (Shinozuka et al 2001; Tanaka et al 2003a). Subsequently, we have shown that this purinergic morphological regulation of the endothelial cell contributes to the permeation through the endothelial cell layer (Tanaka et al 2003b, 2004). However, the mechanisms of the P2Y-induced increase in endothelial permeability are not yet understood. As an initial approach to a better understanding of the properties of the purinergic acceleration of endothelial permeation, we investigated whether or not the actin cytoskeletal organization and the integrity of the intercellular junctions contribute to the mechanism.

The tight junction in the intracellular space of endothelial cells is a gate physically separating the lumen and extravascular tissues. The regulation of paracellular permeability seems to be important as a drug delivery system for many medicines that cannot easily permeate the plasma membrane. Recent reports have indicated that the function

of the tight junction is regulated by increasing the phosphorylation of the myosin light chain (MLC), which in non-muscle cells leads to actomyosin contraction (Saito et al 1998; Ma et al 2000; Tinsley et al 2000). An important second messenger that regulates MLC phosphorylation is intracellular Ca^{2+}, which controls the calmodulin-dependent MLC kinase (MLCK) (Wysolmerski & Lagunoff 1990, 1991; Verin et al 1998). Furthermore, Rho, a member of the subfamily of small GTPases, has been demonstrated to be of critical importance in the regulation of tight junction permeability (Moy et al 1996; Dudek & Garcia 2001; Mehta et al 2001; Wojciak-Stothard et al 2001). Increases in MLC phosphorylation also follow the activation of Rho and its target Rho-kinase, which directly phosphorylates MLC (Amano et al 1996; Leung et al 1996; Kureishi et al 1997). In addition, Rho-kinase inhibits both the activity of MLC phosphatase and the increases in MLC phosphorylation (Kimura et al 1996; Kawano et al 1999; Nagumo et al 2000). In this study, we found that MLCK and Rho/Rho-kinase participate in the increase in endothelial permeability using ML-9, an MLCK inhibitor, and Y-27632, a Rho-kinase inhibitor. To the best of our knowledge, this is the first report on the importance of the cytoskeletal networks in the purinergic regulation of the endothelial permeable function.

Materials and Methods

Materials

FD-4 (fluorescein isothiocyanate dextran, MW 4400), 2meS-ATP (2-methylthionadenosine 5′-triphosphate), ML-9 (1-(5-chloronaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine hydrochloride) and Y-27632 ((*R*)-(+)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexane carboxamide dihydrochloride) were obtained from Sigma Chemical Co. (St Louis, MO). Pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) was purchased from Tocris (Bristol, UK). Drug concentrations are expressed as the final molar concentration in PSS (see below) on the Transwell tissue culture inserts.

Cell culture of endothelial cells

HUVECs were purchased from Bio Whittaker (Walkersville, 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 (Bio Whittaker, Walkersville, USA). Cells were plated in 57-cm^2 tissue culture dishes (Cell tight C-1; Sumitomo Bakelite Co. Ltd, Osaka, Japan) 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% ethylenediaminetetraacetic acid (EDTA) solution and seeded onto plates coated with 50 $\mu g\,mL^{-1}$ collagen (type I) (Collagen Research Center, Tokyo, Japan) in 35-mm tissue culture dishes (Cell tight C-1; Sumitomo Bakelite Co. Ltd, Osaka, Japan). All experiments were performed with HUVEC in passages 3–5 and at 4–6 days post-confluence.

Experiment of FD-4 permeation

The transport of FD-4 (MW 4400, 1 $mg\,mL^{-1}$) across endothelial cell monolayers seeded on 6.4 mm Transwell tissue culture inserts (Corning Costar Corporation, NY) was studied. FD-4 was used as a tracer of paracellular transport (Noach et al 1995; Hayashi et al 1999; Sakai et al 1999; Huang & Piantadosi 2002). HUVECs were seeded at a density of 10^5 cells/cm^2 on culture inserts. All experiments were performed in physiological saline solution (PSS) at room temperature (25°C). The composition of PSS was as follows (in mM): NaCl, 140; KCl, 4.0; $CaCl_2$, 2.0; $MgCl_2$, 2.0; HEPES, 10; glucose, 10; pH 7.4 (adjusted with NaOH). 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 2meS-ATP added to the apical side of the inserts (time = 0 min). For the inhibition experiment, endothelial cell monolayers were pre-incubated with 1 μM ML-9 and 10 μM Y-27632 for 15 or 30 min, respectively. Samples (50 μL) 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 analysed by high-performance liquid chromatography (HPLC) with fluorescence detection (excitation and emission filters at 495 and 518 nm). The HPLC system consisted of an analytical Cosmosil 5C18-MS column (Nacalai Tesque Inc., Kyoto, Japan), a delivery pump (LC-10AD, Shimadzu, Kyoto, Japan) equipped with a fluorescence monitor (RF-10A; Shimadzu, Kyoto, Japan), a sample processor (AS-8020; Tosoh, Tokyo, Japan) and a Chromatopac computing integrator (CBM-10A; Shimadzu, Kyoto, Japan). The mobile phase was 5 mM phosphate buffer (pH 7.4)–acetonitrile (88:12), which was maintained at a flow rate of 1 $mL\,min^{-1}$. A standard calibration curve (0.1–5.0 $\mu g\,mL^{-1}$) was constructed by adding a known amount of FD-4 to the PSS. We consistently obtained r^2 values > 0.99.

Statistical analysis

All values are expressed as mean ± s.e.m. Statistical analyses were performed by a two-way analysis of variance, followed by a post-hoc test (Bonferroni's test) using StatView 4.5 (Abacus Concepts, Cupertino, CA). $P < 0.05$ was considered significant.

Results

Effects of 2meS-ATP and PPADS on the permeation of FD-4

The concentration of FD-4 in the basolateral side of the cell culture inserts, which permeated the endothelial cell monolayers, increased over time (Figure 1). In the presence of 10 μM 2meS-ATP, a P2Y receptor agonist, FD-4 permeation was significantly accelerated. Furthermore, the

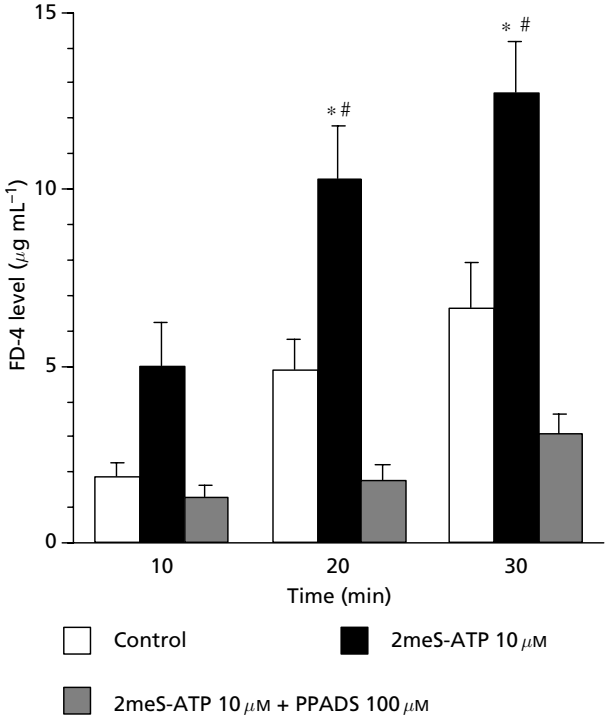

Figure 1 Effects of 10 μM 2-methylthio ATP (2meS-ATP) on the permeation of fluorescein isothiocyanate dextran (FITC-labelled dextran, FD-4; 1 mg mL^{-1}) and the antagonism by 100 μM pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) on the facilitating effect of 2meS-ATP in HUVEC (n = 10–19). Cells were pre-incubated with PPADS for 15 min before stimulation with 2meS-ATP. Data are expressed as mean ± s.e.m. $*P < 0.05$ when compared with the control value; $\#P < 0.05$ when compared with the 2meS-ATP plus PPADS value.

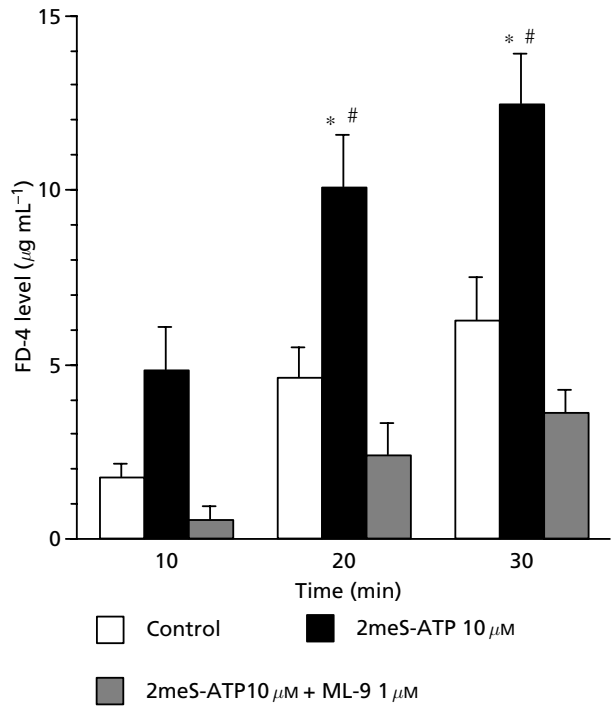

Figure 2 Effects of 1 μM ML-9 on the facilitating effect of 2-methylthio ATP (2meS-ATP; 10 μM) in HUVEC (n = 10–19). Cells were pre-incubated with ML-9 for 15 min before stimulation with 2meS-ATP. Data are expressed as mean ± s.e.m. $*P < 0.05$ when compared with the control value; $\#P < 0.05$ when compared with the 2meS-ATP plus ML-9 value.

2meS-ATP-induced acceleration in the cellular permeation of FD-4 was significantly inhibited at 20 and 30 min by pretreatment with 100 μM PPADS, a P2Y receptor antagonist. PPADS did not influence the permeation of FD-4.

Effect of ML-9 and Y-27632 on the 2meS-ATP-induced increase in the permeation of FD-4

Figures 2 and 3 show the effect of ML-9, an MLCK inhibitor, and Y-27632, a Rho-kinase inhibitor, on the 2meS-ATP-induced increase in the cellular permeation of FD-4. In the presence of 1 μM ML-9, 2meS-ATP-induced acceleration in the cellular permeation of FD-4 was significantly inhibited at 20 and 30 min. Furthermore, in the presence of 10 μM Y-27632, the 2meS-ATP-induced acceleration in the cellular permeation of FD-4 was also significantly inhibited at 10, 20, and 30 min. Neither ML-9 nor Y-27632 influenced the permeation of FD-4 by themselves.

Discussion

P2Y receptors generally act via G-protein coupling to activate phospholipase C (PLC), followed by the formation of inositol 1,4,5-trisphophate (IP_3) and an increase in intracellular Ca^{2+}. We have previously found that U73122, a PLC inhibitor, and thapsigargin, a calcium pump inhibitor, depress both 2meS-ATP-induced increases in the permeation of the endothelial monolayers and increases in intracellular Ca^{2+} (Tanaka et al 2003b). Histamine, serotonin and bradykinin are also G-protein coupled receptors, which activate the PLC–IP_3 pathway in endothelial cells. These substances are known to be able to increase the microvascular permeability (Michel & Curry 1999). Considering our data and these findings, in addition to P2Y receptors, these receptors may regulate permeability by the same mechanisms of PLC- and IP_3-induced Ca^{2+} mobilization. However, the details of the mechanisms are not yet understood. In this study, we showed that the 2meS-ATP-induced increase in the permeability of FD-4 was prevented by both ML-9 and Y-27632. Therefore, MLC and Rho play an important role in the P2Y receptor-mediated regulation of permeability in HUVEC.

Generally, vascular endothelial cells form a selective barrier between the blood and the underlying tissues. Tight junctions are localized at cell–cell contact sites and serve as a paracellular barrier to restrict the movement of ions and proteins across tissue boundaries. At the same time, tight junctions regulate paracellular permeability in endothelial cells (Michel & Curry 1999; DeMaio et al 2001; Wojciak-Stothard et al 2001). In addition, it has

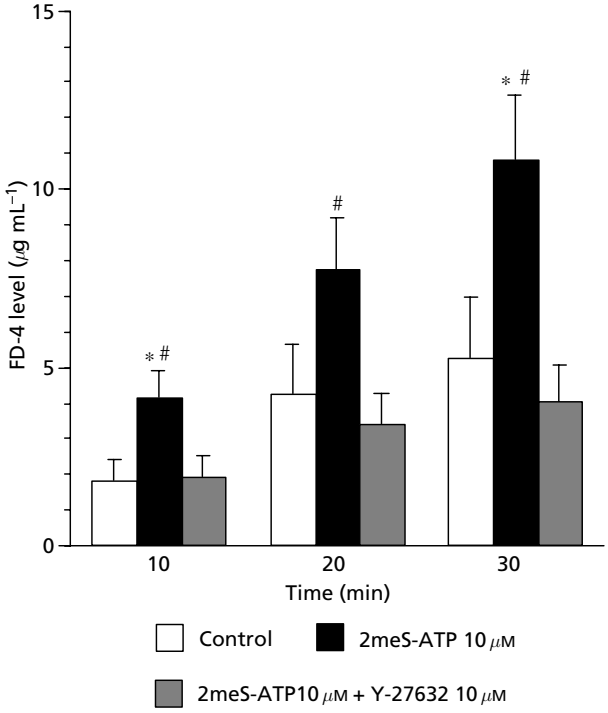

Figure 3 Effects of 10 μM Y-27632 on the facilitating effect of 2-methylthio ATP (2meS-ATP, 10 μM) in HUVEC (n = 10–19). Cells were pre-incubated with Y-27632 for 30 min before stimulation with 2meS-ATP. Data are expressed as mean ± s.e.m. $*P < 0.05$ when compared with the control value; $\#P < 0.05$ when compared with the 2meS-ATP plus Y-27632 value.

been reported that endothelial permeability depends on actomyosin-based cell contractility as well as the integrity of the intercellular junctions (Hordijk et al 1997; Essler et al 1998a, b, 1999). For example, inflammatory mediators, such as thrombin and histamine, accelerate endothelial cell permeation, primarily via actomyosin-derived contraction that is initiated by MLC phosphorylation, and are tightly linked to microfilament reorganization (Wysolmerski & Lagunoff 1991; Moy et al 1993, 1996; Sheldon et al 1993; Garcia et al 1995; Goeckeler & Wysolmerski 1995; Shasby et al 1997; van Nieuw Amerongen et al 1998; Dudek & Garcia 2001; Tiruppathi et al 2002; van Hinsbergh & van Nieuw Amerongen 2002). Recently, there have been reports that thrombin-induced endothelial permeability is regulated by the phosphorylation of MLC, which is mediated by Ca^{2+}/calmodulin-dependent MLCK (Garcia et al 1995; Shasby et al 1997; van Nieuw Amerongen et al 1998; Dudek & Garcia 2001). Furthermore, the small GTPase Rho activation is reported to be important for thrombin- and histamine-induced endothelial cell permeability, and this mechanism has been shown to correlate with the ability of Rho to mediate disassembly of adherence and tight junctions (Dudek & Garcia 2001; Hirase et al 2001; Mehta et al 2001; Wojciak-Stothard et al 2001). Rho-induced phosphorylation of MLC is mediated by Rho-kinases, which leads to phosphorylation of the myosin binding subunit of MLC phosphatase, thereby inhibiting its phosphatase activity and resulting in an increase of MLC phosphorylation (Kimura et al 1996; Amano et al 1997; Essler et al 1998a; Kawano et al 1999; Nagumo et al 2000). The previous studies and our present findings suggest the possibility that the P2Y receptor-regulated endothelial permeation contributes to an increase of actomyosin contractility that leads to the formation of intercellular gaps.

This is the first report of P2Y receptor-mediated endothelial permeability through the Ca^{2+}/calmodulin-dependent activation of MLCK that additionally involves the activation of Rho-kinase. Although further studies that examine the precise mechanism of the P2Y receptor-mediated endothelial permeability are necessary, our data demonstrate a significant role for cytoskeletal dynamics in endothelial permeability function. In the future, if the mechanism for the receptor-regulated vascular permeability can be defined, we may be able to successfully design and apply new drug delivery system technologies.

Conclusion

In conclusion, our results demonstrate that P2Y receptor stimulation accelerates macromolecular permeation through the paracellular pathway of endothelial cells.

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