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ATP participates in the regulation of microvessel permeability

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Abstract

We demonstrated previously that stimulation of the P2Y receptor enhanced the macromolecular permeability of cultured endothelial cell monolayers via the paracellular pathway. To determine whether the P2Y receptor participates in the regulation of permeability in intact microvessels, we have examined the effects of exogenous and endogenous ATP on the permeation of the surface tissue of perfused rat tail caudal artery using a fluorescein isothiocyanate-dextran (FD-4; MW 4400; 1.0 mg mL^{-1}). The permeation of FD-4 was assessed by a confocal fluorescence imaging system. We found that 2-methylthioadenosine 5′-triphosphate, a P2Y receptor agonist, enhanced the fluorescence intensity of FD-4 in the surface of the rat caudal artery tissue and that it was inhibited by pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid, a P2 receptor antagonist. Also, noradrenaline, a sympathetic neurotransmitter, and bradykinin, an inflammatory autacoid, enhanced the fluorescence intensity of FD-4 in the surface tissue of the rat caudal artery. The enhancement by noradrenaline was significantly inhibited by the P2 receptor antagonist. In addition, noradrenaline and bradykinin caused the release of ATP, ADP, AMP and adenosine from the endothelium of the rat caudal artery. These results indicated that the exogenous and endogenous ATP increased the macromolecular permeability of blood capillaries via the P2Y receptor. Such purinergic regulation of endothelial permeability may function in physiological and pathophysiological conditions.

Introduction

Microvessels are formed by a monolayer of endothelial cells that restricts the movement of small polar molecules and macromolecules between the blood and the underlying tissue. Microvessels have many functions, one of which is modulation of microvascular permeability. Many studies have shown that the endothelial barrier function and endothelial permeability are modulated by various intracellular signalling pathways. For example, in endothelial monolayers of coronary origin (Watanabe et al 1992; Hempel et al 1996) as well as in microvessels from the coronary system (Huxley et al 1997), hamster cheek pouch (Gawlowski & Duran 1986), and adipose tissue (Sollvei & Fredholm 1981), stimulation of the cAMP/protein kinase A pathway causes an increase in endothelial permeability. In addition, we found that in rat caudal arterial cultured endothelial cells ATP increased intracellular Ca^{2+} and caused a change in the cell shape via P2Y receptors (Tanaka et al 2003a). We reported that this contributed to the permeability of the endothelial cell layers (Tanaka et al 2003b; 2004). Also, ATP has been reported to increase the hydraulic conductivity in frog mesenteric microvessels, a property that was dependent on the release of Ca^{2+} from intracellular stores (Glass & Bates 2004).

Alteration of the actin–myosin based contractile machinery, which is triggered by the phosphorylation of myosin light chains (MLC), is an important and well-known mechanism for controlling endothelial barrier function (Verin et al 2001; Wang et al 2001). The phosphorylation of MLC is regulated by a Ca^{2+}-dependent MLC kinase (MLCK) and a MLC phosphatase. Indeed, we observed that ML-9, a MLCK

inhibitor, increased P2Y receptor-mediated stimulation of endothelial permeability in cultured monolayers of rat caudal artery endothelial cells, suggesting that the Ca^{2+}/MLCK pathway plays an important role in purinergic regulation of macromolecular permeability (Tanaka et al 2005). Based on these results, it appears that Ca^{2+} is a key downstream mediator through which the P2Y receptor regulates endothelial permeability.

It is generally accepted that several receptor agonists cause the release of ATP from various tissues (Yang et al 1994; Katsuragi et al 1996a, b, 2002; Tamesue et al 1998). In addition, in the vascular endothelium, ATP is released in response to mechanical and chemical stimuli. For example, point mechanical stimuli in corneal endothelial cells (Gomes et al 2005), hypotonic stress in human umbilical vein endothelial cells (Hirakawa et al 2004) and rat caudal arterial endothelial cells (Shinozuka et al 2001), shear stress in human pulmonary arterial endothelial cells (Yamamoto et al 2003), hyperoxia-induced oxidative stress in human lung microvascular endothelial cells (Ahmad et al 2004), β-blockers in glomerular endothelial cells (Kalinowski et al 2003), and α_1-agonists in rat caudal arterial endothelial cells (Shinozuka et al 1994) cause the release of ATP. Despite this evidence, whether P2Y receptor-mediated enhancement of permeability occurs in intact microvessels has not been determined.

We have carried out this study to confirm that ATP enhanced the permeability of intact rat tail microvessels. We investigated whether the endogenous release of ATP in the tail contributed to hyperpermeability. To help clarify the purinergic enhancement of permeability, we investigated the effects of ATP on the permeability of surface tissue from perfused rat caudal artery to fluorescein isothiocyanate dextran (FD-4). To the best of our knowledge, this is the first report on the modulation of intact microvessel permeability by endogenous ATP.

Materials and Methods

Materials

FD-4 (fluorescein isothiocyanate dextran, MW 4400), 2-methylthioadenosine 5′-triphosphate (2meS-ATP), and (−)-noradrenaline bitartrate salt were purchased from Sigma Chemical Co. (St Louis, MO). Pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) was from Tocris (Bristol, UK) and bradykinin was from Peptide Institute, Inc. (Osaka, Japan). The drug concentrations were expressed as the final molar concentration in the perfusate or Krebs solution in the organ chambers.

Animals

Male Wistar rats (SLC, Hamamatsu, Japan) aged 9–12 weeks were used in accordance with the Guidelines for Animal Experimentation of Mukogawa Women's University, which are based on the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science. The caudal artery was removed from rats that had been anaesthetized with pentobarbital sodium (40 mg kg^{-1}, i.p.) and exsanguinated. Arteries were immediately placed into continuously oxygenated Krebs–Henseleit solution (composition (mM): 118.4 NaCl, 4.7 KCl, 2.2 $CaCl_2$, 1.2 KH_2PO_4, 1.2 Mg SO_4, 25.0 $NaHCO_3$, 5.6 glucose, pH 7.4) and cleaned of adhering connective tissue.

Analysis of FD-4 permeation of the surface tissue of rat caudal artery

The transport of FD-4 (1.0 mg mL^{-1}) in the presence and absence of 2meS-ATP (30 μM), noradrenaline or bradykinin into the surface tissue of rat caudal artery was studied as follows. Caudae were perfused with physiological saline solution (PSS (mM); 10 HEPES, pH 7.4, 140 NaCl, 4 KCl, 2 $CaCl_2$ and 10 glucose) at 25°C. Caudae were cut and cannulated into the caudal arteries with polyethylene tubing. Caudal preparations were perfused in chambers with PSS at a constant rate of 4.0 mL min^{-1} using a peristaltic pump (SJ-1220, Atto, Tokyo, Japan). After an equilibration period of 60 min, caudae were perfused with PSS containing FD-4 with or without 2meS-ATP, noradrenaline and bradykinin. Caudal arteries were then perfused for 15 min with PSS with no addition or supplemented with 100 μM PPADS at a constant rate of 4.0 mL min^{-1}, followed by 30 min with PSS containing FD-4 and either 2meS-ATP, noradrenaline or bradykinin. Chambers containing the caudae were placed on the stage of an inverted microscope coupled to a Nipkow disk confocal scanner (CSU10; Yokogawa Electric Corp., Tokyo, Japan) and the ARGUS-50 imaging system (Hamamatsu Photonics, Hamamatsu, Japan). An argon–krypton laser (Omnichrome, Chino, CA) was used to emit an excitation wavelength of 488 nm. The emitted light was collected with a 510-nm long-pass dichroic reflector and a 515-nm long-pass emission filter through a planfluor objective (× 4). Fluorescence images were obtained using an ICCD camera (C2400–87; Hamamatsu Photonics, Hamamatsu, Japan). To quantify permeation of the caudal artery, images were taken at a resolution of 195 × 130 pixels every 5 s. The fluorescence intensity was calculated by dividing the peak fluorescence intensity (F) during the experiment by the average fluorescence intensity at the beginning of each experiment (F_o).

Measurement of the release of adenyl purines from rat caudal artery

The caudal artery was suspended in water-jacketed organ chambers containing 2.0 mL Krebs solution at 37°C and allowed to equilibrate for 1 h. In the release experiments, the caudal arteries (approximately 11 cm in length and 10 mg wet weight) were cut open lengthwise (open preparation). The medium was replaced every 5 min during the second half of the equilibration period. After a 60-min equilibration period, the bathing solution was rapidly collected every 5 min by draining the organ chamber. After the first samples were taken (basal overflow), the

tissues were stimulated with noradrenaline (1.0 μM) or bradykinin (1.0 μM) for 5 min, and the bathing solutions (noradrenaline- or bradykinin-induced overflow) were collected. The collected sample solutions were immediately acidified to pH 4 with 0.36 mL citrate-phosphate buffer solution and placed on ice. Chloroacetaldehyde and internal standard (α,β-methylene adenosine 5′-diphosphate) were added to each acidified sample, and the mixture was then incubated for 40 min in a dry bath at 80°C. The reaction was terminated by placing the samples on ice. The resulting ethenonucleotides and ethenoadenosine were analysed by high-performance liquid chromatography (HPLC) with fluorescence detection as described by Kawamoto et al (1998).

Statistical analysis

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

Results

Effects of 2meS-ATP and PPADS on the permeation of rat caudal artery by FD-4

The amount of FD-4 permeating the surface tissue of rat caudal artery did not change in the absence of 2me-SATP (Figure 1, 0 min). In the presence of 30 μM 2meS-ATP, a P2Y receptor agonist, there was a significant increase in the amount of FD-4 permeating the surface of the rat caudal artery at 15 min. This was also observed at 30 min (control experiment, Figure 1 left panel). This 2meS-ATP-enhanced permeation of FD-4 at 30 min was significantly inhibited by pretreatment with 100 μM PPADS, a P2 receptor antagonist (PPADS experiment, Figure 1 right panel). Alone, PPADS did not affect the permeation of FD-4.

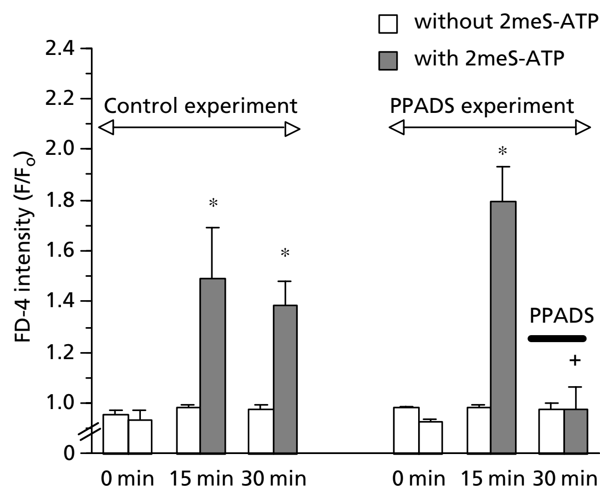

Figure 1 Effects of 30 μM 2meS-ATP and 100 μM PPADS on the permeability of the surface tissue of rat caudal artery to FD-4 (1.0 mg mL^{-1}). Rat caudal artery was pre-incubated with or without PPADS for 15 min before stimulation with 2meS-ATP. Data are expressed as means ± s.e.m. (n = 5–6). *$P < 0.05$ compared with no 2meS-ATP. $^{+}P < 0.05$ compared with the value with 2meS-ATP in the PPADS experiment (PPADS experiment).

Effects of noradrenaline and bradykinin on the overflow of purines from the caudal artery and its permeation by FD-4

In the rat caudal artery, 1.0 μM noradrenaline significantly increased the overflow of ATP, ADP, AMP and adenosine from caudal artery segments that had been opened lengthwise (Figure 2A). Bradykinin (1.0 μM) also increased the overflow of adenine nucleotides and adenosine (Figure 2A). Furthermore, addition of noradrenaline and bradykinin significantly enhanced the permeation of rat caudal artery by FD-4 (Figure 2B).

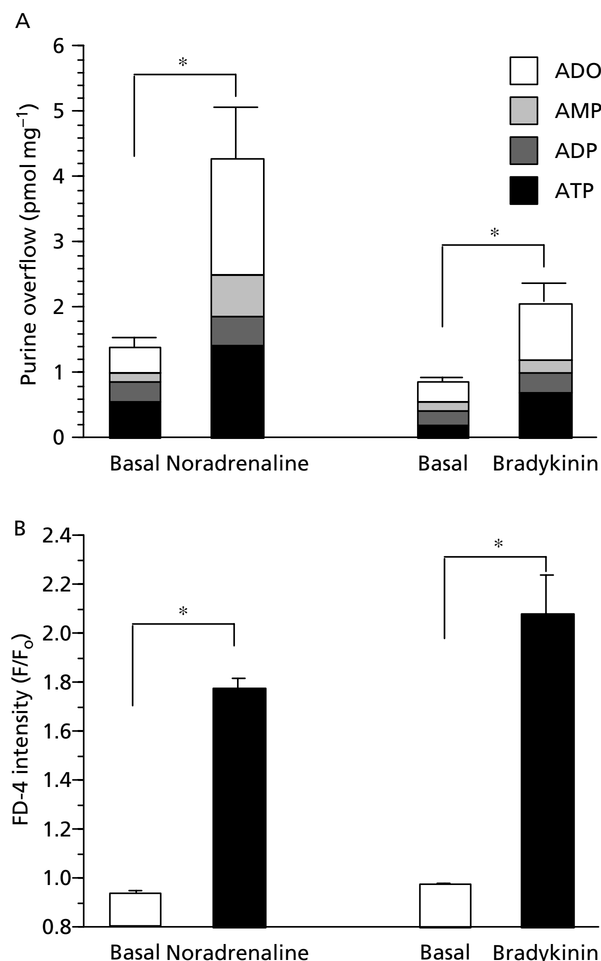

Figure 2 Comparison of the effects of 1.0 μM noradrenaline or 1.0 μM bradykinin on purine overflow (A) and the permeability to FD-4 (B) of the surface tissue from rat caudal artery. Data are expressed as means ± s.e.m. (n = 5–6). *$P < 0.05$ compared with the basal value.

Effects of PPADS on the permeation of rat caudal artery by noradrenaline and bradykinin

Figure 3 shows the effects of endogenous adenyl purines on the permeation of rat caudal artery by FD-4. The amount of FD-4 that permeated the surface tissue of rat caudal artery did not change in the absence of noradrenaline (Figure 3A, 0 min). Addition of 1.0 μM noradrenaline significantly enhanced the permeation of rat caudal artery by FD-4 at 15 min. This enhancement of permeation by FD-4 was also obtained at 30 min (Figure 3A, control experiment). Furthermore, the noradrenaline-enhanced permeation of rat caudal artery by FD-4 was significantly inhibited by pretreatment with 100 μM PPADS, a P2 receptor antagonist (Figure 3A, PPADS experiment). On the other hand, the amount of FD-4 that permeated the surface tissue of rat caudal artery did not change in the absence of bradykinin (Figure 3B, 0 min). Addition of 1.0 μM bradykinin significantly enhanced the permeation of rat caudal artery by FD-4 at 15 min. This enhancement of permeation by FD-4 was also obtained at 30 min (Figure 3B, control experiment). However, there were no significant differences between the permeation in the absence and presence of PPADS, a P2 receptor antagonist (Figure 3B, PPADS experiment).

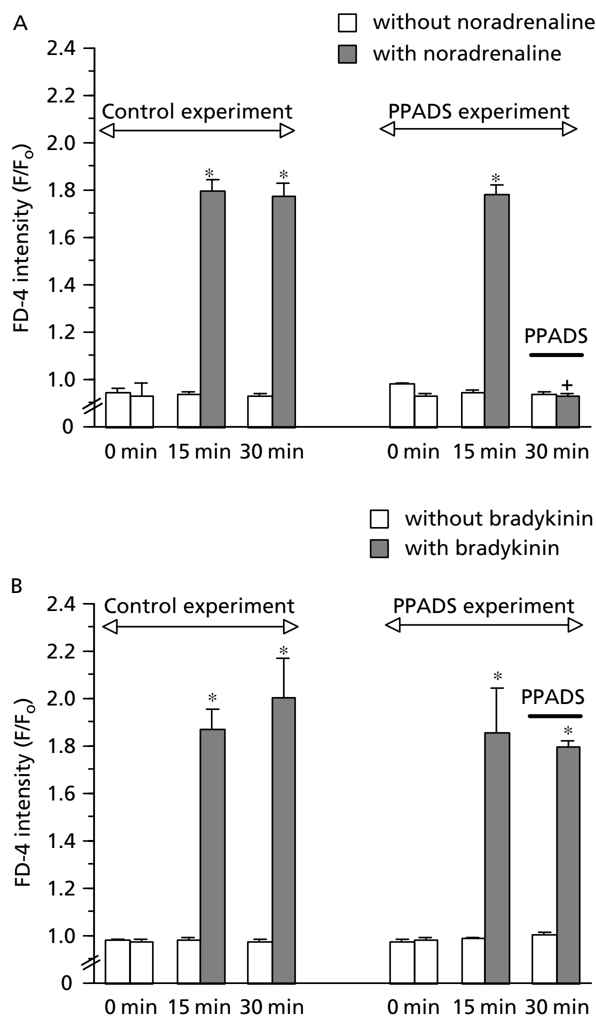

Figure 3 Effects of 1.0 μM noradrenaline (A) or 1.0 μM bradykinin (B) on the permeability of the surface tissue of rat caudal artery to FD-4 (1.0 mg mL^{-1}) to the surface tissue from rat caudal artery and the inhibition by 100 μM PPADS. Rat caudal artery was pre-incubated with PPADS for 15 min before stimulation with noradrenaline or bradykinin. Data are expressed as means $\pm$ s.e.m. (n = 5–6). $^{*}P < 0.05$ compared with the value in the absence of noradrenaline or bradykinin value. $^{+}P < 0.05$ compared with the value for noradrenaline or bradykinin alone in the PPADS experiment.

Discussion

2meS-ATP, a P2Y receptor agonist, enhanced the FD-4 fluorescence in the surface of the caudal artery from rat tail. This enhancement was prevented by PPADS, a P2 receptor antagonist. FD-4 and 2meS-ATP were continuously perfused at constant rate, and so there are two possible explanations for this finding. Firstly, permeability to FD-4 in the capillary was enhanced so that the amount of FD-4 diffusing across the tissue surface increased. Secondly, there was an increase in the microcirculation as a result of vasodilation. It is well known that stimulation of vascular endothelial P2Y receptors ($P2Y_1$, $P2Y_2$ and probably $P2Y_4$) results in the release of endothelium-derived relaxing factor and causes potent vasodilation (Dalziel & Westfall 1994; Abbracchio & Burnstock 1998). Vasodilation by 2meS-ATP usually decreases the perfusion pressure and produces an increase in perfusion flow in the microcirculation. However, in this study, 2meS-ATP did not cause a reduction but rather an increase in the perfusion pressure, a change that was abolished by PPADS (data not shown). It is also well known that stimulation of P2X receptors on vascular smooth muscle results in potent vasoconstriction. Indeed, Saiag et al (1987) reported that ATP induced contraction of rat caudal artery. We found that 2meS-ATP caused similar effects (data not shown), which was not surprising because 2meS-ATP can stimulate not only P2Y but also P2X receptors (Dalziel & Westfall 1994; Ralevic & Burnstock 1998). In addition, PPADS can act as an antagonist of both P2X and P2Y receptors (Ralevic & Burnstock 1998). This evidence suggested that an increase in the microcirculation could not account for the enhancement of surface permeation of FD-4. Rather, the enhancement by 2meS-ATP would weaken by its induction of vasoconstriction.

FD-4 is used as a tracer for paracellular transport (Noach et al 1995; Hayashi et al 1999; Sakai et al 1999; Huang & Piantadosi 2002), and so the results indicated that the P2Y receptor participated in the enhancement of macromolecular permeability in intact microvessels in the rat tail. This was consistent with our previous results that P2Y receptor agonists increased the permeability of confluent monolayers of cultured caudal arterial endothelial cells to FD-4 (Tanaka et al 2003b, 2004, 2005). Ligand binding by the P2Y receptor on the endothelial membrane resulted in activation of phospholipase C, leading to

production of inositol 1,4,5-trisphosphate, which caused the release of intracellular Ca^{2+}. This, in turn, caused changes in the shape of the cells via Ca^{2+}/calmodulin-dependent activation of MLCK (Tanaka et al 2005). Activation of the contractile apparatus caused changes in cell shape, which may have opened intercellular gaps, changing the barrier function and increasing the paracellular permeability. In this study, we demonstrated that activation of the P2Y receptor played a key role in increased permeability in not only cultured endothelial monolayers but also intact vessels, such as caudal microvessels.

Several other receptors are also reported to regulate vascular permeability, including the adenosine receptor, leukotriene 2 receptor, atrial natriuretic peptide-C receptor, thrombin receptor, bradykinin and histamine receptors (Minnear et al 1993; Ehringer et al 1996; Hempel et al 1996; Nguyen et al 1997; Hui et al 2004). Bindewald et al (2004) reported that β-adrenoceptor stimulation increased the permeability of endothelial cells from coronary artery, although the opposite effect was observed in aortic artery endothelial cells. They suggested that in macrovascular aortic endothelial cells, cAMP mediates stabilization of the barrier function, whereas in microvascular coronary endothelial cells, cAMP induces disintegration of cell adhesion structures, which overrides its stabilizing effect. Investigation of the different roles of each receptor in the barrier properties of macrovascular and microvascular endothelial cells should provide valuable insight into the regulation of endothelial cell permeability in the circulation of various organs. In particular, understanding of the local distribution, properties and interactions of receptors that regulate endothelial permeability may allow the manipulation of the absorption and distribution of drugs by pharmacologic agents i.e. pharmacological drug delivery system. It is generally accepted that ATP is released from endothelial cells in response to various stimuli and that it exists in the extracellular space. Shinozuka et al (1994, 1995) demonstrated that there was an α_1-adrenoceptor-mediated mechanism for the release of ATP from intact vascular endothelium and cultured caudal artery endothelial cells from Wistar rats, Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR). In those studies, they found that 1.0 μM noradrenaline induced the release of approximately 20 μM ATP (based on tissue volume). That led us to suspect that noradrenaline, a sympathetic neurotransmitter, enhanced endothelial permeability in the rat tail by causing the extracellular release of endogenous ATP. Indeed, 1.0 μM noradrenaline increased the fluorescence intensity of FD-4 in the surface of rat tail tissue, and the increase was prevented by PPADS. We also confirmed that 1.0 μM noradrenaline elicited the release of ATP, ADP, AMP and adenosine from the isolated rat caudal artery. Noradrenaline also markedly increased the perfusion pressure in these arteries (data not shown). Therefore, the noradrenaline-induced increase in FD-4 intensity did not appear to be due to increased blood flow due to vasodilation. Borges et al (1994) also reported that α-adrenoceptor stimulation increased the permeability of monolayers of bovine brain microvessel endothelial cells. Therefore, noradrenaline from sympathetic nerves may play a role in the regulation of capillary permeability by causing the release of endogenous ATP, although these physiological significances are not clear.

In response to inflammatory stimuli, the endothelial barrier becomes less restrictive, resulting in increased permeability to water and protein. Bradykinin is a well-known extracellular inflammatory mediator and increases transendothelial permeability to macromolecules such as albumin (Carl et al 1996; Aschner et al 1997; Mackic et al 1999). Indeed, in this study, we found that bradykinin enhanced the fluorescence intensity of FD-4 in the surface of the rat caudal artery. We also found that bradykinin induced the release of ATP, ADP, AMP and adenosine from the caudal artery, although the stimulation of ATP release by bradykinin has been shown by Yang et al (1994) in cultured cardiac endothelial cells. On the other hand, bradykinin is well-known to cause vasodilatation by releasing endothelium-derived relaxing factor from endothelial cells (Pelc et al 1991). This information led us to the hypothesis that vasodilation, increased endothelial permeability, and the release of ATP contributed to the enhancement of the FD-4 fluorescence intensity by bradykinin. This accounted for our finding that, although less adenyl purines were released by bradykinin than noradrenaline, bradykinin caused a larger enhancement of the intensity of FD-4. In other words, bradykinin may have caused a greater increase in the permeability due to activation of P2Y receptor by released ATP combined with a direct increase in the permeability. Green et al (1993) showed that ATP greatly enhanced bradykinin-induced plasma extravasation in the knee joint of the rat. Abbracchio & Burnstock (1998) also suggested that ATP was an inflammatory component in a variety of neurological disorders, including trauma, ischaemia, sclerosis and various encephalopathies. However, in this study, there were no significant differences between the permeability of FD-4 in the absence and presence of PPADS, although PPADS slightly inhibited the enhancement of permeability by bradykinin. The reason why PPADS did not antagonize the bradykinin-induced acceleration of FD-4 permeability in the microcirculation of rat caudal tissues was not clear. As a possibility, in microvascular endothelial cells, there may not be cross talk between intracellular signalling pathways via ATP and bradykinin receptors like in rat knee joint. Furthermore, bradykinin has been reported to play a part in the formation of PAF or PAF-like lipid and superoxide by cultured endothelial cells, which play a role in its pro-inflammatory effects in microvascular endothelium (Holland et al 1990; Suzuki et al 1991; Shimizu et al 1994; Shigematsu et al 1999). Accordingly, other factors besides endogenous ATP may be related to the enhancement of bradykinin-induced permeability. The participation of these receptors in microvascular permeation requires further study.

The results indicated a possibility that ATP may play a role as an endogenous pro-inflammatory mediator. Abbracchio & Burnstock (1998) suggested that ATP was an inflammatory component in a variety of neurological disorders, including trauma, ischaemia, sclerosis and

various encephalopathies. Sufficient characterization of the specific receptor subtypes of ATP that regulate capillary permeability may allow the development of novel anti-inflammatory drugs.

Conclusion

2meS-ATP increased the permeability of intact rat tail microvessels to FD-4. The increased permeability appeared to be mediated by the P2Y receptor since it was blocked by PPADS. Noradrenaline, a sympathetic neurotransmitter, and bradykinin, an inflammatory mediator, increased the permeability of intact rat tail microvessels to FD-4. Both of these markedly increased the release of ATP and its metabolites from rat caudal artery, and so the increase in microvessel permeation appeared to be mediated by endogenous ATP. The results led us to speculate that the P2Y receptor-mediated enhancement of microvascular permeability not only regulated the physiological movement of small polar molecules and macromolecules but also participated in the inflammatory process.

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