Comparison of P₂ Receptor Subtypes Producing Dilation in Rat Intracerebral Arterioles

Tetsuyoshi Horiuchi, MD; Hans H. Dietrich, PhD; Kazuhiro Hongo, MD; Ralph G. Dacey, Jr, MD

Background and Purpose—P₂ receptors are important regulators of cerebrovascular tone. However, there is functional heterogeneity of P₂ receptors along the vascular tree, and the functionality of P₂ receptors in small arterioles has not been studied in detail. We investigated the effects of activating P₂₁ and P₂₂ receptors and their underlying dilator mechanisms in rat intracerebral arterioles.

Methods—We used computer-aided videomicroscopy to measure diameter responses from isolated and pressurized rat penetrating arterioles (39.9±1.2 μm) to the natural P₂ receptor agonist ATP in addition to ADP-β-S (P₂₁-selective) and ATP-γ-S (P₂₂-selective) and inhibitors of signaling pathways.

Results—Extraluminal application of ATP-γ-S and ADP-β-S initiated a biphasic response (initial constriction followed by the secondary dilation) similar to ATP-induced responses. Pyridoxal phosphate-6-azophenyl-2',4'-disulphonic acid (0.1 mmol/L; a P₂₁ receptor antagonist) blocked ADP-β-S—but not ATP-γ-S—induced dilation and affected the ATP-mediated dilation at low concentrations. Nω-Monomethyl-L-arginine partially inhibited the dilation of ATP and ADP-β-S but not ATP-γ-S. High K⁺ saline suppressed the dilation of all agonists. Indomethacin had no effect.

Conclusions—Both P₂₁ and P₂₂ receptors are functionally present in cerebral arterioles. ATP stimulates P₂₁ receptors at low concentrations, while high concentrations of ATP activate P₂₂ in addition to P₂₁ receptors. Nitric oxide is involved in P₂₁ but not P₂₂ receptor activation. Potassium channels play an important role in the regulation of P₂ receptor–mediated dilation. (Stroke. 2003;34:1473-1478.)

Key Words: adenosine ■ cerebral circulation ■ nitric oxide ■ potassium channels ■ receptors, purinergic P₂

The importance of purinergic regulation has been recognized in the cerebral circulation. ATP is one of the purines and the natural agonist to control blood flow. Purines released from the parenchyma may be important in regulating microvascular blood flow.¹ Both vasoconstriction and vasodilation can be produced by ATP in the cerebral vasculature.²⁻³ These vasomotor responses depend on distribution of P₂ receptor subtypes. Thus, species, location, and size of vessels greatly influence the vasomotor response to ATP.³

It has been demonstrated that both endothelial P₂₁ and P₂₂ receptors are present and their stimulation dilates rat middle cerebral artery.⁴⁻⁶ In contrast, we² and others³ speculated that P₂₁ receptor may not be present or its function may be silent in the cerebral microcirculation under physiological conditions because 2-methylthio-ATP (2-MeSATP; a P₂₁-receptor agonist) did not dilate the cerebral arteriole effectively. However, P₂₁ and P₂₂ receptors have been detected in primary cultures of rat brain capillary endothelium.¹⁻⁷ In rat middle cerebral artery, P₂₁ releases only nitric oxide (NO), while P₂₂ stimulation liberates both NO and endothelium-derived hyperpolarizing factor (EDHF).⁵ It is of interest that the involvement of NO in response to ATP-induced dilation decreases along the cerebrovascular tree, whereas EDHF seems to be the major contributor to ATP-induced dilation in smaller vessels.⁴

It is not known whether P₂₁ receptors are functionally present in penetrating arterioles, which are important regulators of cerebral microvascular blood flow. Furthermore, the mechanism of P₂₁ receptor–induced dilation has not been studied. Therefore, in rat isolated cerebral arterioles, we evaluated the functional P₂₁ receptor distribution using ADP-β-S (a selective P₂₁ receptor agonist) and ATP-γ-S (a selective P₂₂ receptor agonist) on arteriolar diameter. We compared the results with the natural agonist ATP and also investigated the relaxing factor(s) released by these agonists.

Materials and Methods
The Animal Studies Committee at Washington University approved the experimental protocol for this study. Fifty-six male Sprague-Dawley rats (weight, 350 to 450 g; Harlan, Indianapolis, Ind) were anesthetized with pentobarbital sodium (65 mg/kg IP) and decapitated. The brain was immediately removed and stored in cold physiological salt solution (PSS). The technique for isolation and cannulation of the intracerebral arteriole has been showed in previous studies.⁸⁻¹⁰ In short, the cerebral penetrating arterioles were
Passive Diameter, Tone Diameter, and pH Response in Isolated Rat Intracerebral Arterioles

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>ATP</th>
<th>ATP-γ-S</th>
<th>ADP-β-S</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>19</td>
<td>17</td>
<td>20</td>
</tr>
<tr>
<td>Passive diameter D_{max}, μm</td>
<td>56.4±2.3</td>
<td>60.0±2.0</td>
<td>59.8±2.0</td>
</tr>
<tr>
<td>Tone diameter D_{tone} (%Tone), μm</td>
<td>37.7±2.3 (33.7%)</td>
<td>41.5±1.8 (31%)</td>
<td>40.6±1.8 (32.2%)</td>
</tr>
<tr>
<td>pH 6.8 diameter, μm</td>
<td>46.9±3.0†</td>
<td>53.0±2.5†</td>
<td>52.3±1.9†</td>
</tr>
<tr>
<td>pH 7.65 diameter, μm</td>
<td>28.2±1.7†</td>
<td>32.1±2.0†</td>
<td>29.9±1.4†</td>
</tr>
</tbody>
</table>

The values are mean±SEM, and n is the number of observations. The vessels developed spontaneous tone by constricting more than 30% (%Tone in parentheses) from the maximum passive diameter D_{max} to the tone diameter D_{tone}, * denotes P<0.05 compared with passive diameter) and responded to pH challenge († denotes P<0.05 compared with tone diameter). There were no statistically significant differences in passive, tone or pH diameters between experimental groups.

All vessels (n=56) developed spontaneous tone and responded to pH challenge (Table). There were no significant differences in passive diameter, spontaneous tone, dilation to acidosis, and constriction to alkalinization between vessel groups (ATP, ATP-γ-S, or ADP-β-S) (Table).

Previous studies in our laboratory showed that extraluminal ATP caused a biphasic response consisting of initial constriction followed by secondary dilation.2,10 Extrapluminal ATP-γ-S and ADP-β-S also produced dilation after constriction, as seen with ATP (Figure 1). We previously demonstrated that the initial constriction is caused by smooth muscle cell P2X1 receptors.2 We also showed that this constriction did not affect the subsequent dilation.2 The following results, therefore, focus on the secondary dilation induced by the agonists. Group data for concentration responses of ATP, ATP-γ-S, and ADP-β-S are shown in Figure 1. Dilator responses to ATP-γ-S and ADP-β-S (10 and 100 μmol/L) were significantly more potent than those to ATP, ATP-γ-S, and ADP-β-S at 100 μmol/L produced 60.1±4.3%, 90.2±2.5%, and 90.2±2.5% of maximal dilation, respectively. Furthermore, ATP showed a right-shifted EC_{50} value of 9.69 μmol/L compared with ATP-γ-S (EC_{50}=6.40 μmol/L) and ADP-β-S (EC_{50}=3.72 μmol/L). We compared the control dose-response curves for ATP, ATP-γ-S, and ADP-β-S in Figure 1 with the respective curves in the subsequent protocols and found them to be not statistically different (ANOVA).

Figure 2 illustrates the effects of 0.1 μmol/L PPADS (a P2X1 plus P2Y1 receptor antagonist at this concentration)2 on ATP-, ATP-γ-S-, and ADP-β-S-induced dilation. PPADS had no effect on the baseline vessel diameter. PPADS significantly inhibited dilation to ADP-β-S but not to ATP-γ-S. Interestingly, ATP-mediated dilations at 10 μmol/L to 1 μmol/L were inhibited by PPADS, whereas PPADS did not affect 10 μmol/L ATP- and 0.1 μmol/L ATP- induced
dilation. The initial constrictions of all agonists were abolished by 0.1 mmol/L PPADS (data not shown). 2 In preliminary experiments, 3 μmol/L PPADS inhibited the agonist-induced initial constriction. However, 3 μmol/L PPADS did not affect the secondary dilation.

**Figure 3** shows the effects of 10 μmol/L L-NMMA on ATP-, ATP-γ-S-, and ADP-β-S-induced dilation. The vessels constricted significantly to L-NMMA from 42.2 ± 2.0 to 32.5 ± 1.7 μm. L-NMMA attenuated the dilation of ATP and ADP-β-S but not ATP-γ-S. In preliminary experiments (2 vessels), 100 μmol/L L-NMMA did not produce further inhibition of the dilation.

Figure 4 shows the effects of 10 μmol/L indomethacin on ATP-, ATP-γ-S-, and ADP-β-S-induced dilation. Indomethacin affected neither the control diameter nor dilations to the 3 agonists.

Figure 5 demonstrates the effects of 30 and 80 mmol/L KCl on ATP-, ATP-γ-S-, and ADP-β-S-induced dilation. The high-K⁺ saline (30 and 80 mmol/L) decreased the baseline diameter of the vessels (41.6 ± 2.5 μm [control diameter]: 35.0 ± 2.3 μm [30 mmol/L]: 24.1 ± 2.1 μm [80 mmol/L]) and attenuated the dilation of ATP, ADP-β-S, and ATP-γ-S in a dose-dependent manner. Papaverine (10 μmol/L) fully diluted 3 vessels in the presence of high-K⁺ saline, indicating that high-K⁺ saline did not cause vascular paralysis.

**Discussion**

The purpose of the present study was to clarify the functional contribution of P₂Y₁ receptors in particular to purinergic stimulation with the use of specific agonists/antagonists and to elucidate the relaxing factors released to specific P₂Y receptor stimulation. We found that ADP-β-S and ATP-γ-S potently dilate rat intracerebral arterioles, indicating P₂Y₁ and P₂Y₂ receptor presence, respectively. With the use of the specific P₂Y₁ inhibitor PPADS, low concentrations of ATP stimulated P₂Y₁ receptors, whereas P₂Y₂ receptors were additionally activated by higher concentrations of ATP. NO partially contributed to P₂Y₁ receptor– but not P₂Y₂ receptor–produced dilation. Cyclooxygenase products were not involved. Potassium channels may play a major role in dilation to both P₂Y₁ and P₂Y₂ receptor stimulation.

**Distribution of P₂ Receptors in Cerebral Arterioles**

Extraluminal application of ATP, ADP-β-S, and ATP-γ-S resulted in a biphasic response consisting of the initial constriction followed by the secondary dilation. The initial constriction was inhibited by PPADS (3 μmol/L and 0.1 mmol/L). PPADS is a P₁ receptor antagonist, and its effects depend on concentration, species, and vessel location. In our preparation, 3 μmol/L PPADS inhibits P₂X₁ receptor but not P₂Y receptor. 2 A higher concentration of 0.1 mmol/L PPADS, however, blocks P₂X₁ plus P₂Y₁ receptors but not P₂Y₂ receptors. 2 These results indicate that both ADP-β-S and ATP-γ-S activate P₂X₁ receptors to induce transient arteriolar constriction. We 2 reported that biphasic vasomotor responses
after extraluminal application of agonists not only resulted in the activation of smooth muscle cells (to cause transient constriction) but also activated the endothelium to cause vessel dilation. In cerebral macro- and micro-circulation, P2Y2 receptor subtypes function as an important regulator of cerebral blood flow. However, on the basis of previous studies, P2Y1 receptor subtypes seem to be less important in the regulation of the cerebral microcirculation because 2-MeSATP as a P2Y1 agonist produced no vasodilation in third-order branches of the middle cerebral artery and large penetrating arterioles of the rat. These findings suggested that functional P2Y1 receptors may be absent in the cerebral microcirculation. Recently, our study, which also used 2-MeSATP, confirmed their results. However, we also speculated that 2-MeSATP is not a potent P2Y1 receptor agonist in the cerebral arteriole because 2-MeSATP can also stimulate P2X1 receptors in our preparation. However, we excluded that the P2X1 stimulation has an inhibitory/subtractive effect on ATP-induced secondary dilation. Another explanation for the apparent lack of potency of 2-MeSATP is based on differences in smooth muscle sensitivity to the relaxing factors along the vessel trees, ie, NO.

In the present study ATP-γ-S in addition to ADP-β-S dilated the cerebral arterioles of the rat in a dose-dependent manner. PPADS (0.1 mmol/L) can clearly distinguish P2Y1 from P2Y2 receptor subtypes in our preparation and inhibited ADP-β-S–induced dilation. Thus, P2Y1 receptors are also present in the arteriole and can regulate the vascular tone in the cerebral microcirculation. Furthermore, the water-soluble purinergic agonists we used reached endothelial receptors on the abluminal side to cause endothelium-dependent dilation. Our results are consistent with studies in which P2Y agonists are used intraluminally. However, if endothelial P2Y1 and P2Y2 receptors are heterogeneously distributed on luminal versus abluminal surfaces, it is possible that the effects of the receptor specific agonists we used may differ when applied intraluminally.

ADP-β-S and ATP-γ-S strongly dilated the vessels compared with ATP. It is a possibility that ATP was metabolized by ectonucleotidases to decrease its efficacy. However, we found that ADP as a dephosphorylated ectonucleotidase product dilated penetrating arterioles in a manner similar to that of ATP. Thus, ATP degradation by ectonucleotidases to cause the reduced dilation seems less likely.

Our results show that high concentrations of ATP (10 to 100 μmol/L) activate P2Y2 in addition to P2Y1 receptors, resulting in arteriolar dilation. By contrast, low concentrations of ATP (10 nmol/L to 1 μmol/L) mainly stimulated P2Y1 receptors. These results indicate that ATP acts as both a P2Y1 and P2Y2 receptor agonist, and its function depends on the

**Figure 3.** Effects of 10 μmol/L L-NMMA on vasomotor responses induced by ATP (n=5), ATP-γ-S (n=4), and ADP-β-S (n=6). *P<0.05, significantly different compared with percentage of dilation in presence of L-NMMA.

**Figure 4.** Effects of 10 μmol/L indomethacin on vasomotor responses induced by ATP (n=4), ATP-γ-S (n=4), and ADP-β-S (n=4).
agonist concentration. Sipos et al. discussed the possible relevance of multiple purinergic receptors on the brain endothelium and concluded that in addition to liberating various dilators such as NO and EDHF, multiple receptors could also contribute to “cross-talk” between second messenger systems.

Mechanisms of P2Y1 and P2Y2 Purinoceptor–Induced Vasodilation

It is well known that the activation of endothelial P2Y1 and P2Y2 receptors in the cerebral circulation dilates the vessel via different mechanisms. The stimulation of endothelial P2 receptors dilates vessels through releasing NO, prostanoid, and/or EDHF in the vasculature. However, these relaxing factors may vary from species, size, and location of vessels, even though the P2 receptor subtype is the same. Prostacyclin seems to be a less important factor in response to endothelial P2 receptor stimulation in cerebral circulation.

These findings are consistent with the present study because inhibition of cyclooxygenase in the intracerebral arteriole had no effect in any agonist-induced dilations. Thus, a cyclooxygenase metabolite such as prostacyclin was not involved in P2Y-induced dilation. However, we cannot exclude a possible contribution of cyclooxygenase metabolites to P2Y receptor–induced dilation after NO inhibition because we did not examine the combined effects of L-NMMA and indomethacin.

Our laboratory reported that ATP hyperpolarized the smooth muscle cell, resulting in dilation of the cerebral arteriole, and oxyhemoglobin (a scavenger of NO) partially inhibited ATP-induced dilation. We also reported that an intact endothelium is necessary for the ATP-induced dilation. These findings indicate that endothelial NO production and a hyperpolarization factor caused the dilation of the cerebral arteriole to ATP. In the rat middle cerebral artery, P2Y1 receptor activation produces vasodilation exclusively through NO release, whereas P2Y receptor activation results in vasodilation via NO and EDHF. Interestingly, EDHF rather than NO prominently contributes to dilation of P2Y receptor stimulation in the cerebral microcirculation compared with the macrocirculation.

In the present study NO was partially involved in both ATP- and ADP-β-S–induced (P2Y1 receptor–induced) dilations. This contribution was observed at low but not high concentration of ADP-β-S. By contrast, ATP-γ-S (P2Y2 receptor agonist) dilated the arteriole via a NO-independent pathway. High-K+ saline strongly diminished dilation in response to both P2Y1 and P2Y2 receptor stimulation, suggesting that potassium channels contribute to the vessel dilation. Potassium channels are one of the main contributors to the EDHF-induced dilation. It is possible that NO dilates the vessel via potassium channel stimulation. However, our previous studies demonstrated that NO/potassium channel interactions did not seem to be important in the cerebral arteriole. Thus, NO and potassium channel activation may act as the mediator of P2Y1 receptor independently. On the basis of this finding, the relaxing factors released in response to P2Y receptor stimulation depended on receptor subtypes and agonist concentrations in rat cerebral arterioles.

Intracellular Mechanisms and Mediators Released to Purinergic Stimulation Along the Cerebrovascular Tree

P2Y1 and P2Y2 receptors are G protein coupled. However, the subsequent signal transduction pathway linked to P2Y receptors varies between the cell types even though receptor subtypes on the cells may be identical. Nevertheless, an increase in endothelial intracellular Ca2+ concentration ([Ca2+]i) is seen as an essential intracellular response. In rat middle cerebral artery, Marelli reported that P2Y2 receptor stimulation resulted in a significantly greater increase in [Ca2+]i than P2Y1 receptor stimulation. P2Y1 stimulation produced a purely NO-dependent dilation, while P2Y2 stimulation caused the release of both NO and EDHF. These results suggest that [Ca2+]i thresholds regulate NO- and EDHF-dependent dilation. Our results, however, indicate that in cerebral arterioles P2Y1 stimulation releases both NO and an indomethacin-independent factor, while P2Y2 stimulation was independent of NO. Thus, our results differ from those seen in middle cerebral arteries but agree with studies in pial arterioles, in which P2Y1 stimulation depended partly (approximately one half) on NO. However, if P2Y2 stimulation results in a higher calcium increase than P2Y1, we would also
expect a release of NO, which is calcium dependent. Since we did not find NO involvement after P2Y2 stimulation, a possible explanation is that P2Y2 receptors are directly coupled to phospholipase A2, which could release EDHF.21 Further studies are needed to elucidate the intracellular coupling of P2Y1 and P2Y2 receptors in rat penetrating arterioles.

In summary, the results of the present study provide functional evidence that both P2Y1 and P2Y2 receptors are present and mediate dilation of rat cerebral arterioles via different mechanisms. The P2Y1 receptor dilates via both NO and potassium channels (probably EDHF), whereas the P2Y2 receptor dilates the cerebral arteriole via potassium channel activation but not NO. Cyclooxygenase products are not involved. At low concentrations, the natural agonist ATP stimulates predominantly P2Y1 receptors with subsequent NO release. Higher ATP concentrations stimulate P2Y2 in addition to P2Y1 receptors, releasing a NO- and cyclooxygenase-independent but potassium channel–dependent factor(s). Our results confirm the functional P2Y receptor heterogeneity along the cerebrovascular tree, where stimulation of similar P2Y receptors results in the release of different dilators.

Acknowledgments
This study was supported by National Institutes of Health grants HL57540 and NS30555.

References
Comparison of P2 Receptor Subtypes Producing Dilation in Rat Intracerebral Arterioles
Tetsuyoshi Horiuchi, Hans H. Dietrich, Kazuhiro Hongo and Ralph G. Dacey, Jr

*Stroke.* 2003;34:1473-1478; originally published online May 1, 2003;
doi: 10.1161/01.STR.0000071527.10129.65
*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://stroke.ahajournals.org/content/34/6/1473

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published
in *Stroke* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office.
Once the online version of the published article for which permission is being requested is located, click
Request Permissions in the middle column of the Web page under Services. Further information about this
process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Stroke* is online at:
http://stroke.ahajournals.org//subscriptions/