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P_1-/P_2-Purinergic Receptors on Cultured Rabbit Retinal Müller Cells

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Abstract: Adenosine 5′-triphosphate (ATP) and its metabolic products function as neurotransmitters or neuromodulators under the control of P_1/P_2-purinergic receptors. To determine the presence of these receptors on retinal Müller cells, spectrofluorometry was carried out on intracellular calcium mobilization, using Fura-2 images. Müller cells were cultured from adult rabbit retinas. Cytosolic calcium ($[Ca^{2+}]_i$) increased dose dependently with the application of ATP. This response was not blocked when a calcium channel blocker, nifedipine, was present, but this response was blocked, for the most part, when a P_2 receptor antagonist, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) was present. Increase in $[Ca^{2+}]_i$ was noted by the A_1 or A_2 agonist, which was blocked completely by each antagonist. Response to the A_1 agonist was apparent only at high concentrations. Increase in $[Ca^{2+}]_i$ was seen in some cells following administration of the P_{2x} agonist, methylene ATP, only at a high concentration (100 μM) but not in the presence of PPADS (50 nM). The greatest increase in $[Ca^{2+}]_i$ was induced by a P_{2y} agonist, methyl thio ATP at 1 to 10 μM, which was completely blocked by PPADS. Cultured Müller cells are thus shown quite likely to possess the P_1-/P_2-purinergic receptors including A_2 and P_{2y}. **Jpn J Ophthalmol 1998;42:33–40** © 1998 Japanese Ophthalmological Society

Key Words: Adenosine, ATP, calcium transients, Müller cells, P_1-/P_2-purinergic receptor, retinal glia.

Introduction

Retinal Müller cells are glial cells that are unique for their many physiologic and pathophysiologic functions essential for retinal neurons.[1–4] As with astrocytes in the brain,[5–9] retinal Müller cells may be involved in glioneuronal or neuroglial interactions mediated by neurotransmitter receptors. Neurotransmitter receptors and receptor-linked events in Müller cells have been studied only sparingly.[10–14]

Adenosine 5′-triphosphate (ATP) and its metabolic products in the central nervous system act as neurotransmitters or neuromodulators mediated by adenosine (P_1) and/or purinergic (P_2) receptors.[15,16] Two subclasses of extracellular P_1 receptors, A_1 and A_2, have been shown to be present in the vertebrate retina.[17–21] However, the presence of P_1-/P_2-purinergic receptors on Müller cells has yet to be confirmed. The ATP causes the formation of cytosolic calcium transients in astrocytes.[22] Thus, to confirm whether these receptors are present in cultured Müller cells, spectrofluorometric study was carried out on intracellular calcium mobilization using images obtained by Fura-2.

Materials and Methods

Cell Culture

Müller cells were cultured as reported previously.[23] In brief, in Hank's medium solution (containing 140 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM KH_2PO_4, and 22 mM glucose), New Zealand white rabbit eye specimens were cut 2 mm away from the limbus so as to remove the anterior part and vitreous. The retina was carefully detached, and the vascular and medullated portions were removed to avoid any contamination of astrocytes and oligodendrocytes. The residual retina was cut into 0.25 × 0.25-mm pieces under a biomicroscope and then cen-

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trifuged at 500 rpm for 5 minutes and suspended on culture plates containing Dulbecco minimum essential medium (Gibco, Tokyo, Japan) supplemented with 10% fetal bovine serum (Gibco). After a 7-day incubation in 5% CO_2 in air at 36.5°C, the suspended tissue was collected, centrifuged, pelleted, and resuspended in fresh medium and then subcultured onto glass coverslides. Most explants adhered to the surface of the coverslides within 5 days. By previous immunocytochemical and electron microscopic study, the high purity of Müller cell cultures had been confirmed.[23,24] The 10- to 30-day-old semiconfluent culture cells were used.

Cytosolic Calcium Concentration ($[Ca^{2+}]_i$)

The $[Ca^{2+}]_i$ was measured using the Argus-100/CA system (Hamamatsu Photonics, Hamamatsu, Japan) as described previously.[11,13,14] Cells on the coverslides were washed with KRH solution containing 120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $KHPO_4$, 0.5 mM $CaCl_2$, 6 mM glucose, and 25 mM N-2-hydroxyethylpiperazine-N′-ethanesulfonic acid, followed by an incubation with an indicator of free calcium ions, 5 μM Fura-2-acetoxymethylester (Fura-2AM, Dojin Chemical Co., Kumamoto, Japan), at room temperature for 30 minutes. Each coverslide was washed with KRH for the removal of excessive Fura-2AM. The cells on each coverslide were then transferred to a culture dish with a silicone wall (Flexiperm, Heraus, Germany), placed in 25 μL of KRH solution, and examined under an inverted microscope (Olympus IMT-2, Tokyo, Japan). Dye excitation was conducted at 340 nm and 380 nm with epifluorescence using a filter exchanger (Olympus OSP-exch, Tokyo, Japan). Fluorescence signals were stored and analyzed in a fluorescence microscope/video-camera system equipped with an intensified charged-coupled camera (Argus 100/CA). The ratio of fluorescence intensity during excitation at 340 nm to that during excitation at 380 nm (F340/F380) was expressed in a pseudocolor map. The $[Ca^{2+}]_i$ calibration was conducted as previously indicated.[14] An F340/F380 increase of approximately 15% from the baseline was considered a positive response in each cell. Measurement was made every 3 seconds. To confirm the presence of the P_1/P_2-purinergic receptor subclass on cultured rabbit retinal Müller cells, increase in $[Ca^{2+}]_i$ following the administration of each agonist with or without the antagonist (Table 1) was measured over a period of 30 seconds. The agonists and antagonists, except for N6-cyclopentyladenosine (CPA) were purchased from Research Biochemicals International (Natick, MA, USA); CPA was purchased from Sigma (St. Louis, MO, USA). Each agonist was prepared in twofold concentration to be examined and applied by puffing 25 μL of bathing solution onto each coverslide. For the examination of antagonist effects, agonist puffing was carried out in KRH solution containing the antagonist. Calcium-free ethylene glycol bis (β-aminoethylether)-N,N,N′,N′-tetraacetic acid (5 mM, EGTA) containing KRH solution was prepared to examine the increase in $[Ca^{2+}]_i$ not due to calcium influx. In some experiments, cells preincubated by a calcium channel blocker, nifedipine (Sigma), at 20 μM were used to exclude possible effects of voltage-dependent calcium channels. At least five coverslides were used for each agonist concentration; using five coverslides, 56 to 188 cells were examined in each set of experiments.

Table 1. Agonists and Antagonists of the P_1-/P_2-Purinergic Receptor Used in the Present Study

	Agonist (Concentration Examined)	Antagonist (Concentration Used)
P_1		
A_1	CPA (0.1, 1, 5 μM)	DPCPX (10 nM)
A_2	CPCA (1,10,100 μM)	DMPX (50 nM)
P_2		
P_{2x}	α,β-methylene ATP (1,10,100 μM)	PPADS (50 nM)
P_{2y}	2-methyl thio ATP (0.1, 1, 10 μM)	PPADS (50 nM)

CPA = N6-cyclopentyladenosine; DPCPX = 8-cyclopentyl-1,3-dipropylxanthine; CPCA = 5′(N-cyclopropyl) carboxamidoadenosine; DMPX = 3,7-dimethyl-1-propargylxanthine; PPADS = pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid.

Results

Resting $[Ca^{2+}]_i$ was 101 ± 53 nM for 40 cells selected randomly. The $[Ca^{2+}]_i$ transients were produced in cultured Müller cells following ATP administration at 1 μM, generally with 3 seconds (Figure 1). Increase in $[Ca^{2+}]_i$ with a substantial latent period of 3–6 seconds was observed in some cells. Response intensity was usually in proportion to agonist concentration but was slightly different for each cell. Following the increase, $[Ca^{2+}]_i$ remained constant or slowly resumed its initial value. In this study, we examined the percentage of responding cells. As shown in Figure 2, response was dose dependent from 0.001 to 1 μM. The ATP (0.1 μM)-induced increase in $[Ca^{2+}]_i$ was inhibited in most cells by a 10-minute prior incubation with pyridoxalphosphate-6-

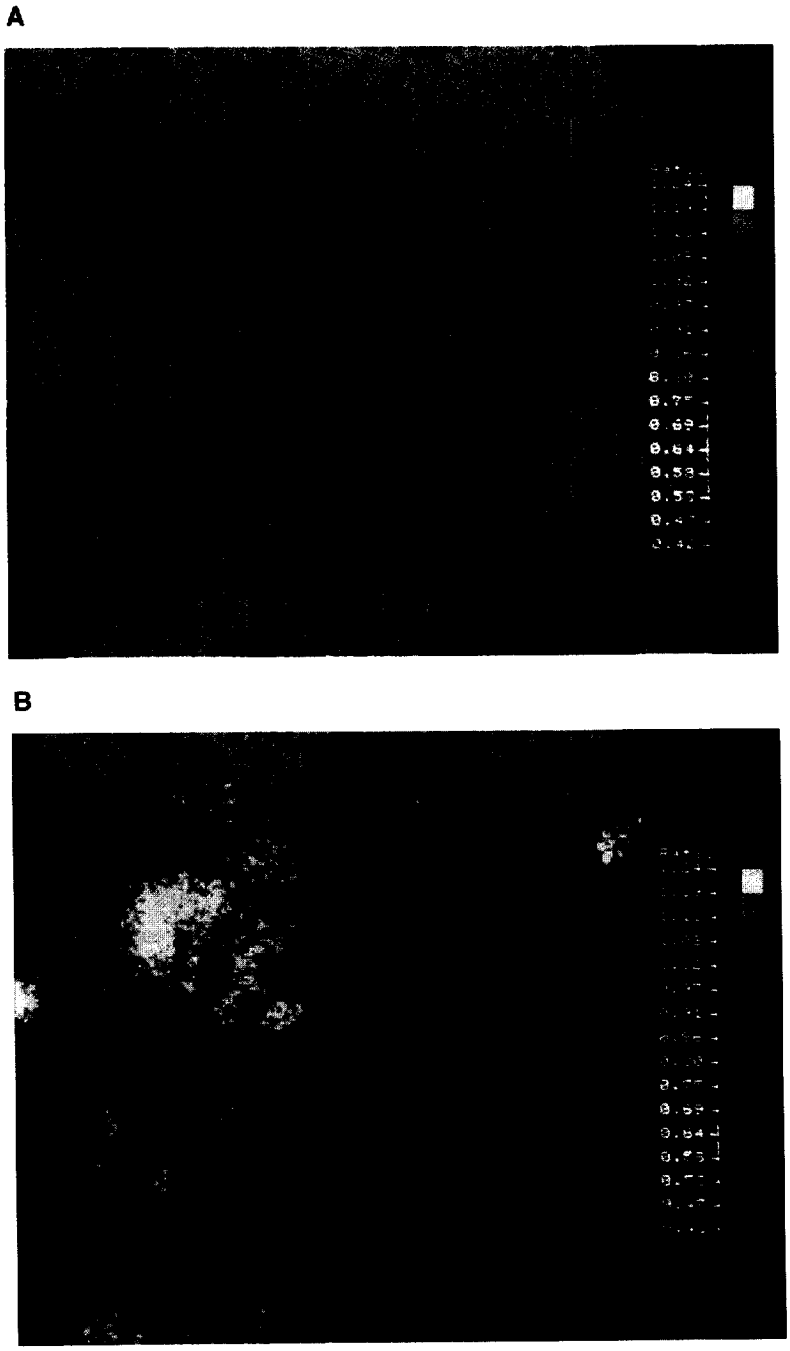

Figure 1. Fluorescence ratio images (340/380 nm) of cultured Müller cells before (**A**) and 3 seconds after (**B**) adding 1 μM ATP depicted in a pseudocolor map. The ratio of $[Ca^{2-}]_i$ is illustrated along with a color bar scale (×300).

azophenyl-2′,4′-disulfonic acid (PPADS) at 50 nM, a strong P_2 antagonist, but it was not completely blocked in some cells (Figure 3). No positive response to ATP at 0.01 or 0.1 μM could be clearly detected in most cells in calcium-free–EGTA-containing solution. However, at least in half cells, a slight but definite increase in $[Ca^{2+}]_i$ was induced following ATP administration at 1 μM in the above condition. The ATP administration at 0.01 to 1 μM induced transients even following the 10-minute prior incubation with nifedipine (100 μM). One hundred twenty-one to 126 cells were examined in each ATP concentration, and the percentage of responding cells was essentially the same as shown in Figure 2.

Jpn J Ophthalmol
Vol 42: 33–40, 1998

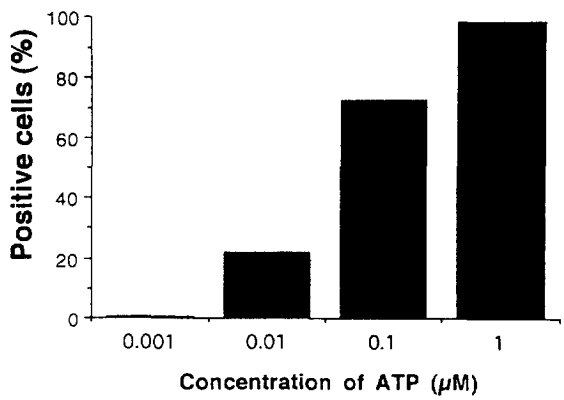

Figure 2. Rate of cells with calcium transients following administration of ATP at each concentration in Müller cells. The sample size in each concentration (from left to right) is 98, 165, 56, and 174 cells, respectively.

Positive cells were 20% of the total number of cells in ATP administration at 0.01 μM, 74% at 0.1 μM, and 100% at 1 μM.

The ATP-induced response appeared to persist to some extent in the presence of P_2 antagonist, and thus the response to P_1 agonists was examined. Positive response to CPA, an A_1 agonist, could be detected in only a few cells at high concentration (1 and 5 μM [Figure 4]), which was completely blocked by 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) at 10 nM. Positive response to 5′-(N-cyclopropyl)-carboxamidoadenosine (CPCA) at 10 μM, an A_2 agonist, was observed in half the cells (Figure 4). One example of this experiment is shown in Figure 5. An increase in $[Ca^{2+}]_i$ with time latency was also evident. The CPCA-induced response was completely blocked by 3,7-dimethyl-1-propargylxanthine (DMPX) at 50 nM. The DPCPX partly blocked the $[Ca^{2+}]_i$ increase induced by ATP (>0.1 μM), but it completely prevented a cytosolic calcium transient increase induced by CPA (>0.1 μM). For the A_2 receptor agonist and antagonist, the results were virtually the same. The DMPX at 50 nM only partially blocked an increase in $[Ca^{2+}]_i$ caused by ATP (>0.1 μM), and it prevented transient formation induced by CPCA (>10 μM).

Methylene ATP at 1.0 μM, a P_{2x} agonist, failed to induce a positive response, but at 10 μM a positive response was apparent in some cells. Methylene ATP at high concentration (100 μM) caused a $[Ca^{2+}]_i$ increase in nearly 60% of the cells (Figure 4). No methylene ATP-induced response was observed in the presence of PPADS at 50 nM. Response was greatest at 1 and 10 μM methyl thio ATP, a P_{2y} agonist. Figure 6 shows an example of such an experiment. This proves that a response occurred immediately after administration and that the time course of the increase was essentially the same in most cells. Methyl thio ATP-induced response was blocked completely by PPADS. Positive response to P_1-/P_2-purinergic receptor agonists is summarized in Figure 4. No antagonist used alone caused an increase in $[Ca^{2+}]_i$.

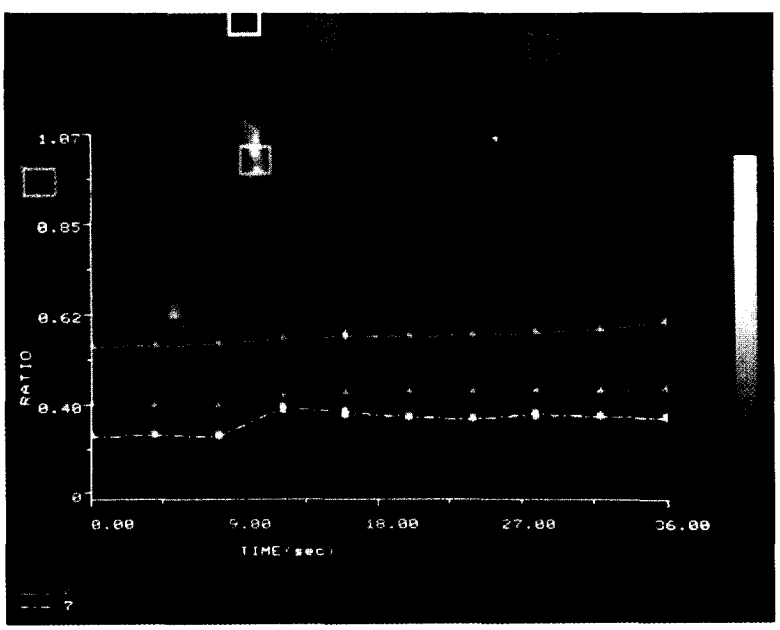

Figure 3. Diagram showing temporal change in each cell following ATP administration at 1 μM in the presence of 50-nM PPADS. The diagram is superimposed on the cell image. The temporal change in each cell indicated by a colored square is illustrated by the same color in the diagram. The abscissa axis indicates time in seconds, and the ordinate axis indicates the ratio. The arrow indicates the time at which ATP was added. Most cells showed no response (×150).

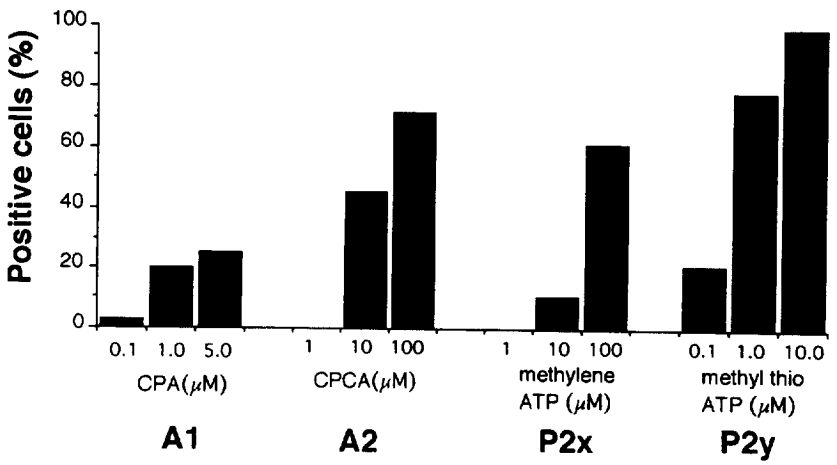

Figure 4. Rate of positive cells in response to each agonist. The sample size in each concentration (from left to right) is 121, 156, and 144 in CPA; 80, 188, and 181 in CPCA; 120, 122, and 168 in methylene ATP; and 160, 142, and 78 cells in methyl thio ATP, respectively.

Discussion

The Ca^{2+}-dependent neurogenic release of ATP into the central peripheral nervous systems has been well documented. The extracellular hydrolysis of ATP leads to adenosine 5′-diphosphate (ADP), adenosine monophosphate (AMP), and adenosine formation. The ATP and its hydrolysis product activate P_1-/P_2-purinergic receptors and function as neurotransmitters or neuromodulators.[15,16] Immunocytochemical studies indicate adenosine to be abundantly present in retinal neurons,[17] and biochemical and pharmacological data confirm two major subclasses of P_1 adenosine receptors, A_1 and A_2, to be present in the retina.[17–21] Endogenous adenosine, preferentially linked to the P_1 receptor, modulates the light-induced release of acetylcholine.[25] Neal and Cunningham[25] noted ATP to decrease and the novel selective P_2 antagonist, PPADS[27] to increase this release of acetylcholine from the retina, suggesting its action quite likely to be mediated by the P_2 receptor. These findings conclusively demonstrate the presence of the P_1 and P_2 receptors in retinal neurons.

Receptor-linked events are no longer considered specific for neurons,[4,13,28] and neuroglial, glioneuronal, or glioglial interactions can be made to occur through calcium signaling using glial receptors.[7,9]

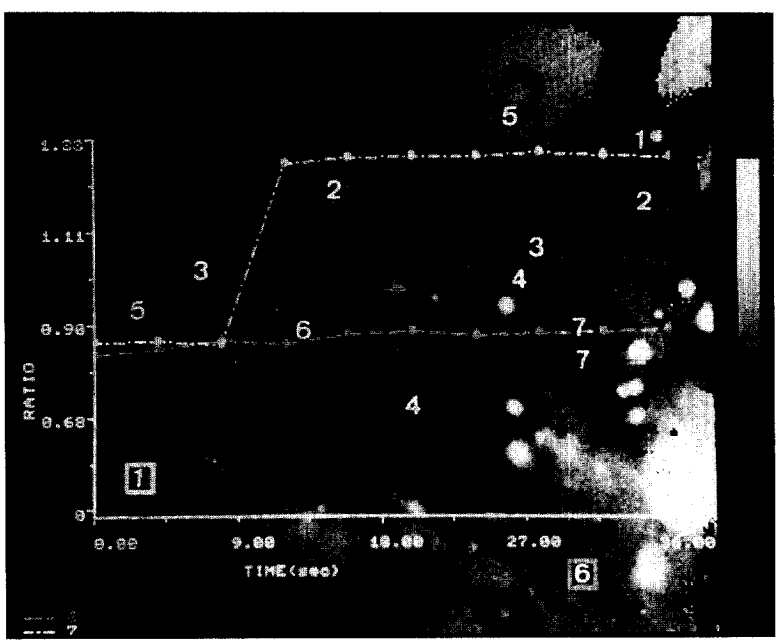

Figure 5. Temporal change in each cell following CPCA administration at 10 μM. The diagram showing temporal change in the ratio is superimposed on the cell image (see Figure 3). Temporal change for cells indicated by squares numbered 1–7 is shown by lines with the same numbers on the diagram. Four (Nos. 1–4) of six cells were positive (×150).

Jpn J Ophthalmol
Vol 42: 33–40, 1998

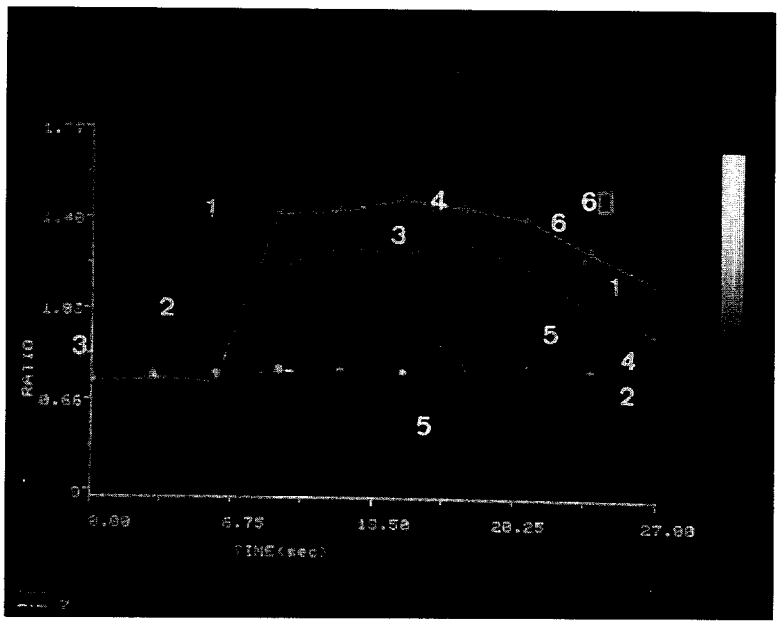

Figure 6. Temporal change in each cell following methyl thio ATP administration at 1 μM. The diagram showing temporal change in the ratio is superimposed on the cell image (see Figure 3). Temporal change for cells indicated by squares numbered 1–7 is shown by lines with the same numbers on the diagram. A rapid increase in $[Ca^{2+}]_i$ was evident in three (Nos. 1, 3, and 6) of six cells (×150).

The A_2 receptor subtype has been shown apparently to predominate over the A_1 receptor in primary astrocyte cultures,[5] and the P_2 receptor may be essential for the action of calcium channels in astrocytes.[29]

In cultured Müller cells, ATP was shown in this study to increase $[Ca^{2+}]_i$—this being mediated mainly by calcium influx as indicated by the absence of a 0.01–0.1-μM ATP-induced increase in $[Ca^{2+}]_i$ in calcium-free EGTA-containing solution. In approximately half the cells, however, an increase in $[Ca^{2+}]_i$ occurred following 1-μM ATP administration in calcium-free–EGTA-containing solution, and in some cells an increase in $[Ca^{2+}]_i$ induced by 0.01–1-μM ATP administration occurred with time latency. Release of calcium ions from an intracellular store would probably be an explanation for such an event as suggested by Keirstead and Miller.[30] A calcium-permeable channel on Müller cells has been identified.[31] The voltage-dependent L-type channel previously detected on Müller cells was nifedipine sensitive.[13] To exclude the possibility of direct stimulation of the calcium channel, the voltage-dependent channel blocker, nifedipine, was used, but it made no apparent contribution to calcium-transient formation induced by ATP in Müller cells in the present study.

Some cultured Müller cells appeared, in this study, to respond more quickly than others. This may occur due to slow diffusion; however, delayed response was not observed on every occasion, even in the same experimental condition. As another explanation, this is possibly owing to the particular site of a receptor on a Müller cell and/or differences in affinity for receptors. Some Müller cells may possibly possess no P_1-/P_2-purinergic receptor at all and receive current signals from nearby responding cells. This would be mediated by cell-to-cell communication through gap junctions[32] or other cell junctions.[24]

Adenosine exerts strong action on the P_1 receptor, while ATP does so on the P_2 receptor.[33] Three P_1 receptors, A_1, A_2, and A_3, have been identified.[15] Only a few cell types possess the A_3 subtype. Both the A_1 and A_2 receptors were shown to be present here in cultured Müller cells. Blazynski[18] noted A_1 receptor sites to be localized mainly in the inner retina and A_2 receptor sites in the outer retina, suggesting different functions.

The present study also shows the P_2 receptor, especially P_{2y}, quite likely to be present on Müller cells. Although the P_2 receptors have yet to be found in the retina, Neal and Cunningham[26] suggest that P_2 receptors are present on glycinergic amacrine cells. As also shown here, Kastritsis, Salm, and McCarthy[34] noted that stimulation of the P_{2y} receptor leads to calcium mobilization on type 1 astrocytes.

The functions of P_1-/P_2-purinergic receptors on Müller cells remain unknown. Various putative functions related to mitotic processes, intercellular communication, cell morphology, cell proliferation, energic metabolism, and secretion of trophic/active factors[5,9] have been proposed for neurotransmitter receptors on astrocytes. Kawasaki and Wakakura[14]

reported non-N-methyl-D-aspartate receptors to be expressed to a great extent on Müller cells under pathological rather than normal physiological conditions. The ATP at 1 μM induces cell death of cultured retinal neurons[35] and thus possibly may function as a neurotoxicant as does glutamate.[16] The P_1/P_2-purinergic receptors may accordingly express action not only under certain physiological but also pathological conditions. Stimulation of vascular A_2 receptors induces retinal vasodilation with consequent increase in blood flow.[36–38] It follows then that the same receptors on Müller cells may serve to preserve cellular function by warding off insult such as that of anoxia, ischemia, and hypoglycemia. The A_1 receptor agonist inhibits adenyl cyclase, resulting in lower concentrations of intracellular cyclic AMP, while the A_2 receptor agonist stimulates adenyl cyclase, resulting in an increase.[39] The P_{2y} receptors are metabotropic and coupled to phospholipase A_2.[40] These differences may possibly be an indication of functional differences.

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Jpn J Ophthalmol
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