Astrocytes, but Not Neurons, Exhibit Constitutive Activation of P2X7 Receptors in Mouse Acute Cortical Slices under Non-stimulated Resting Conditions

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Received July 30, 2014; accepted September 10, 2014

We previously demonstrated that the P2X7 receptor (P2X7R), a purinergic receptor, expressed by mouse cultured cortical astrocytes is constitutively activated without any exogenous stimulus, differing from the case of neurons. It is well known that astrocytic morphology differs between in vitro and in vivo situations, implying different functionalities. Brain acute slices are widely accepted as an in vitro experimental system that reflects in vivo cell conditions better than in vitro cell culture ones. We examined whether astrocytic P2X7Rs exhibited constitutive activation in mouse cortical slices. In acute cortical slices, P2X7R-immunoreactivity was detected in both glial fibrillary acidic protein-immunopositive astrocytes and microtubule-associated protein 2-immunopositive neurons. Astrocytic, but not neuronal, spontaneous uptake of propidium iodide, an indicator of P2X7R channel/pore activity, was inhibited by representative antagonists of P2X7R, but they had no effect on the uptake by astrocytes in membrane-permeabilized fixed slices. These findings indicate that astrocytes, but not neurons, in acute cortical slices exhibit constitutive activation of P2X7Rs under non-stimulated resting conditions as in the case of cell culture systems.

Key words  P2X7 receptor; astrocyte; mouse cortical slice; neuron

Among purine receptors, in particular, P2X7 receptors (P2X7Rs) have unique characteristics, and are activated by high concentrations (ca. m M) of ATP, resulting in the formation of a non-selective cationic channel/pore, through which large molecules of up to 900 Da can pass. Through activation of P2X7Rs in neurons, cell death is induced via calcium influx, followed by related cellular events, while activation of P2X7Rs expressed by microglia, brain-resident immune cells, causes generation and/or release of reactive oxygen species (ROS) and pro-inflammatory cytokines. These responses help keep the brain environment clean, while under severe pathological conditions, neuronal injury is exacerbated. 1–8 Astrocytes, a major cell population in the brain, also express a P2X7R, and its activation leads to the release of gliotransmitters, such as glutamate, 9 ATP, 10 and zinc, 11 which are considered to play critical roles in the neuron–glia network system in the central nervous system (CNS), but the details have not been fully clarified.

Previously, we demonstrated that in primary cultured mouse astrocytes, P2X7Rs were activated without any exogenous ligands, and that this constitutive activation was due, at least in part, to autocrine/paracrine stimulation by ATP released from astrocytes themselves into restricted intercellular space, 12 regulated by P2X7R splice variants, 13 and involved in regulation of astrocytic engulfing activity. 14 These findings suggest that under non-stimulated resting conditions, P2X7Rs expressed by astrocytes play roles in the maintenance of brain neuronal homeostasis.

It is well known that astrocytic morphology differs between in vitro cultured and in vivo tissue situations, and thus, in many studies concerned with astrocytic functionality, astrocytes have been pre-treated with cAMP to alter their morphological property from a polygonal to process-bearing activated one. 15–18 When astrocytes are activated under pathological conditions, they exhibit different functionalities such as neuro-/gliotransmitter clearance systems. 18–22 These findings may imply the possibility that there are differences in the functional expression of P2X7Rs between in vitro cultured and in vivo tissue astrocytes. Brain acute slice cultures are a widely accepted in vitro experimental system that reflects in vivo cell conditions better than in vitro cell culture ones. In this study, to clarify whether or not constitutive activation of P2X7Rs occurs in astrocytes in an in vivo situation, we examined their functionalities using mouse acute brain cortical slices.

MATERIALS AND METHODS

Animals  In this study, we used female ddY- (12–15 week-old; Japan SLC, Hamamatsu, Japan) and SJL- (9–12 week-old, Japan Charles River, Kanagawa, Japan) strain mice. All experiments were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University and were performed according to the Guidelines for Animal Experimentation of Kyoto Pharmaceutical University.

Mouse Cortical Slice Preparations  Under deep anesthesia with pentobarbital sodium (50 mg/kg, intraperitoneally (i.p.)), the brains were removed from mice, and immediately placed in ice-cold artificial cerebrospinal fluid (aCSF) comprising 130 mM NaCl, 3.5 mM KCl, 1.25 mM NaH2PO4, 2 mM MgSO4, 2 mM CaCl2, 20 mM NaHCO3 and 10 mM glucose, pH 7.2, with equilibration with 95% O2 and 5% CO2. In ice-cold aCSF having 95% O2 and 5% CO2 bubbled through it, the brains were vibratome-sectioned to yield 300 µm slices of cerebral cortices using a MicroSlicer® (DTK-1000 ZERO-1, DSK, Japan). The slices were transferred to a bath of aCSF at 30°C that had 95% O2 and 5% CO2 continuously bubbled

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through it, and incubated for 30–60 min before initiating experiments. To obtain membrane-permeabilized fixed slices, we exposed freshly prepared slices to ice-cold 4% paraformaldehyde (PFA) in ACSF for 2 h, and thereafter, propidium iodide (PI) uptake experiments were performed.

**Immunostaining** Slices were immunostained by the protocol reported previously.23 After the slices had been fixed with 4% PFA for 2 h at 4°C, followed by three rinses with ice-cold phosphate buffered saline (PBS) (0.1 M PBS), they were incubated overnight at 4°C in a blocking buffer (2% goat serum, 0.4% Triton X-100, 0.3% bovine serum albumin (BSA), and 0.05% sodium azide in PBS). Thereafter, the slices were incubated with primary antibodies diluted in the blocking buffer for 3 d at 4°C. The following primary antibodies were used: rabbit anti-P2X7R (1 : 50, #APR004; Alomone Labs, Jerusalem, Israel), rabbit anti-glial fibrally acidic protein (GFAP) (1 : 500, #AB5804; Millipore, Temecula, CA, U.S.A.), mouse anti-GFAP (1 : 500, #G3893; SIGMA, St. Louis, MO, U.S.A.) and mouse anti-MAP2 (1 : 500, #M4403; SIGMA) antibodies. After three rinses in ice-cold PBS, the slices were incubated with Alexa Fluor® 488-conjugated goat anti-rabbit immunoglobulin G (IgG) antibodies, or Alexa Fluor® 546-conjugated goat anti-rabbit or -mouse IgG antibodies (1 : 1000; Life Technologies, Tokyo, Japan) for a day at 4°C. The slices were three-times with ice-cold PBS, and then were mounted to glass slides and enclosed using a Prolong® antifade kit (Life Technologies). Photomicrographs were obtained under a confocal laser microscope (LSM 510 META; Carl Zeiss, Germany).

**Dye Uptake** The functionalities of P2X7Rs were evaluated as the uptake of PI, a marker for channel/pore formation.12,24 After slices had been pre-incubated with or without the designated concentration of a P2X7R antagonist, brilliant blue G (BBG),25 KN62,26 or A438079,27 for 10 min in ACSF equilibrated with 95% O2 and 5% CO2 at 30°C, they were incubated with 5 μM PI for 30 min, this time point being determined based on time-dependent PI uptake profiles, in which PI uptake increased linearly from 15 to 60 min (data not shown). The uptake was terminated by washing three times with warmed ACSF and then the slices were subjected to the aforementioned immunostaining procedure. Photomicrographs of three random fields/slice were taken within 4 d using the confocal laser microscope. The fluorescence intensity of all nuclei of GFAP-immunopositive cells in a region of interest was quantified using the histogram program of the Photoshop® software (Adobe Systems Inc., San Jose, CA, U.S.A.), the values being averaged to obtain a single value for each experiment.12

**Statistical Analysis** The data are expressed as means ± S.E.M. Comparisons were performed by means of one-way ANOVA, followed by the Fisher’s protected least significant differences (PLSD) or Bonferroni/Dunn (control) test. A *p*-value of 0.05 or less was considered statistically significant.

**RESULTS**

**Functionalities of P2X7Rs in Astrocytes and Neurons**

First, we confirmed the expression of P2X7Rs in astrocytes and neurons in ddY-mouse acute cortical slices. As shown in Fig. 1, clear immunoreactivity of P2X7Rs was detected in both GFAP-positive and MAP2-positive cells. In the same cortical slices, double staining for GFAP and MAP2 showed their immunoreactivity in the same field. For both GFAP- and MAP2-immunopositive cells, PI was taken up into the nuclei (Fig. 2a).

Figures 2b and c show the results of quantitative assessment of PI uptake by the two types of cells. The GFAP-immunopositive cells showed significantly greater PI uptake than the MAP2-immunopositive ones did, and the PI uptake by the former, but not the latter, was significantly decreased by BBG, a P2X7R antagonist.

The P2X7R-selective antagonists, KN62 and A438079, as well as BBG, decreased the PI uptake by the GFAP-immunopositive cells, implying functional expression of P2X7Rs in astrocytes in acute cortical slices (Fig. 3). On the other hand, GFAP-immunopositive cells in fixed slices, of which the cell membranes had been permeabilized, took up greater amounts of PI than normal ones, while none of the P2X7R antagonists used had any effects on PI uptake by the fixed ones (Fig. 3), demonstrating that PI was incorporated into astrocytes via P2X7R channels/pores in the normal slices, differing from in the case of the fixed slices, in which PI seemed to be taken up into cells non-specifically. Therefore, these findings indicate that P2X7Rs are expressed by both astrocytes and neurons in acute cortical slices, and those in the former, but not the latter, exhibited constitutive channel/pore activity under non-stimulated resting conditions, the same as in the case of in vitro cultures of them.

**Comparison of P2X7R Channel/Pore Activity between ddY- and SJL-Strain Mouse Acute Cortical Slices**

In our previous study, cultured astrocytes obtained from both ddY- and SJL-mouse cortices exhibited constitutive activation of P2X7Rs, but the activity of the former was less than that of the latter.13 To determine whether or not this difference is also observed in acute slice preparations, we compared the PI uptake by the two types of astrocytes. As shown in Fig. 4a, PI uptake by the GFAP-immunopositive cells in ddY- and SJL-mouse cortices decreased in the presence of BBG concentration-dependently, but quantification revealed that the uptake by the GFAP-immunopositive cells in the slices obtained from ddY-mice was significantly less than in ones from SJL-ones.
Fig. 2. Characteristics of PI Uptake by Astrocytes and Neurons in ddY-Mouse Acute Cortical Slices

After incubation of slices with or without the indicated concentrations of BBG, they were incubated with 5 µM PI for 30 min in aCSF equilibrated with 95% O₂ and 5% CO₂ at 30°C. (a) After PI uptake experiments, the slices were incubated with rabbit anti-GFAP and mouse anti-MAP2 antibodies, followed by incubation with Alexa Fluor® 405-conjugated goat anti-rabbit and Alexa Fluor® 488-conjugated goat anti-mouse secondary antibodies. Representative photomicrographs are shown (N=3), and blue and green signals were derived from the immunoreactivity of GFAP and MAP2, respectively. (Color images were converted into gray scale.) Arrowheads and arrows indicate representative PI uptake by astrocytes and neurons, respectively. (b, c) After PI uptake experiments, the slices were incubated with mouse anti-GFAP or anti-MAP2 antibodies, followed by incubation with Alexa Fluor® 488-conjugated goat anti-mouse secondary antibodies. Representative photomicrographs (N=3), and the quantitative results are shown in panels b and c, respectively. Arrowheads indicate representative PI uptake by astrocytes and neurons. Scale bar=20 µm. *p<0.01 (vs. GFAP-control).

Fig. 3. Effects of P2X7R Antagonists on Astrocytic PI Uptake in ddY-Mouse Acute Cortical Slices

As for fixed slices, freshly prepared slices were treated with 4% PFA and then subjected to PI uptake experiments. After incubation of slices with or without 250 nM BBG, 10 µM KN62 or 10 µM A438079 for 10 min, they were incubated with 5 µM PI for 30 min in aCSF equilibrated with 95% O₂ and 5% CO₂ at 30°C. Dimethylsulfoxide (DMSO), as a vehicle for KN62 and A438079, was added to the reaction mixture to the final concentration of 0.1%. After PI uptake experiments, the slices were incubated with mouse anti-GFAP antibodies, followed by incubation with Alexa Fluor® 488-conjugated goat anti-mouse secondary antibodies. Representative photomicrographs (N=3) and the quantitative results are shown in panels a and b, respectively. Arrowheads indicate representative astrocytic PI uptake. Scale bar=20 µm. *p<0.01 (vs. normal-control). **p<0.01 (vs. normal-DMSO).


Fig. 4. Characteristics of Astrocytic PI Uptake in ddY- and SJL-Mouse Acute Cortical Slices

After incubation of slices with or without the indicated concentrations of BBG, they were incubated with 5 µM PI for 30 min in aCSF equilibrated with 95% O2 and 5% CO2 at 30°C. After PI uptake experiments, the slices were incubated with mouse anti-GFAP antibodies, followed by incubation with Alexa Fluor® 488-conjugated goat anti-mouse secondary antibodies. Representative photomicrographs (N=3) and the quantitative results are shown in panels a and b, respectively. Arrowheads indicate representative astrocytic PI uptake. Scale bar=20 µm. *p<0.01 (vs. ddY-control). **p<0.01 (vs. SJL-control).

(Fig. 4b). Thus, the P2X7R channel/pore activity differed between ddY- and SJL-mouse astrocytes in the acute slices, as found in the primary culture ones.13

DISCUSSION

In acute cortical slices, astrocytes took up PI via P2X7R-related channels/pores, while there was no or only negligible such activity in neurons. In addition, the astrocytic P2X7R channel/pore activity was lower in ddY-strain mouse acute cortical slices than in SJL-strain ones. The finding that P2X7Rs in in vivo tissue astrocytes, but not neurons, exhibit constitutive activation, is identical to that in in vitro cell culture systems of astrocytes and neurons.5,12,13) Therefore, it is suggested that under in vivo non-stimulated resting conditions, astrocytic P2X7Rs play roles in the maintenance of brain homeostasis.

In vitro cultured astrocytes exhibit a flat, fibroblast-like polygonal morphology, which is different from the stellate, process-bearing morphology of in vivo tissue astrocytes. It is well-known that treatment of in vitro cultured astrocytes with cAMP can alter their morphological characteristics, as observed for in vivo tissue ones.15–17) Together with this morphological change, the astrocytic functionality, by which cellular signaling mediated by neuro-/glio-transmitters and degradation of glycogen as the main energy reserve in the brain are tightly regulated, is also altered.18–20) ATP is a cellular energy source and also plays a critical role as a neuro-/glio-transmitter,20) and thus the functionality of its receptors involved in the cellular signaling might be different between in vitro cultured and in vivo tissue astrocytes. In this study, however, we demonstrated that in vivo tissue astrocytes express constitutively activated P2X7Rs the same as in vitro cultured ones do. Therefore, we propose that the finding as to P2X7R functionality for in vitro cultured astrocytes under non-stimulated resting conditions, together with that for acute cortical slices, should provide valuable information for a better understanding of the roles of astrocytic P2X7Rs under physiological, but not pathological, conditions.

As for the P2X7R functionality, SJL-mouse astrocytes exhibited greater PI uptake activity than ddY-mouse ones did, and this was considered to be due to the different expression profiles of P2X7R splice variants, by which P2X7R channel/pore activity in primary cultured astrocytes was regulated.13 In contrast to the case of astrocytes, neurons in acute slices were not constitutively activated under non-stimulated resting conditions, and this was the same with their primary cultures.5,13) Although detail mechanism underlying this is unclear, this difference is considered to be due to the difference in cell types, implying different roles of P2X7Rs in astrocytes and neurons. SJL mice are known to exhibit extreme aggression,29) differing from ddY ones. In addition to differences in astrocytic P2X7R functionality between ddY- and SJL mice,13) very recently, we found that cultured neurons derived from ddY-strain mouse cortices exhibit lower ATP sensitivity than those from SJL-strain ones (data not shown). As for microglia as immune cells in the CNS, ATP can induce their chemotaxis via P2X7R activation,21) while astrocytic engulfing activity, which involves in an innate immune response, was regulated by P2X7Rs under non-stimulated resting conditions.14) On the other hand, alteration of P2X7R-related channel/pore activity caused by single nucleotide polymorphisms of the P2rx7 gene is reported to be associated with human psychiatric diseases such as affective mood disorders.30–32) Recently, it was found that ATP appears to be a key factor involved in astrocytic modulation of depressive-like behavior in adult mice.33) Together, functional alteration of P2X7R-mediated signal transduction in brain neuronal cells might cause dysfunction of the neuron-glia network system in the CNS, and thus systematic investigation of functional expression of P2X7Rs in neuronal and glial cells under resting conditions might provide novel insights as to therapeutic approaches for psychiatric disorders.

Overall, we revealed that astrocytes in acute cortical slices exhibit constitutive activation of P2X7Rs the same as in vitro cultured ones do under non-stimulated resting conditions.

Acknowledgment A part of this study was financially supported by a Grant-in-Aid for Scientific Research (C) (24590128) from the Japan Society for the Promotion of Science (JSPS).

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