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P2X7 receptors regulate engulfing activity of non-stimulated resting astrocytes

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ABSTRACT

We previously demonstrated that P2X7 receptors (P2X7Rs) expressed by cultured mouse astrocytes were activated without any exogenous stimuli, but its roles in non-stimulated resting astrocytes remained unknown. It has been reported that astrocytes exhibit engulfing activity, and that the basal activity of P2X7Rs regulates the phagocytic activity of macrophages. In this study, therefore, we investigated whether P2X7Rs regulate the engulfing activity of mouse astrocytes. Uptake of non-opsonized beads by resting astrocytes derived from ddY-mouse cortex time-dependently increased, and the uptaken beads were detected in the intracellular space. The bead uptake was inhibited by cytochalasin D (CytD), an F-actin polymerization inhibitor, and agonists and antagonists of P2X7Rs apparently decreased the uptake. Spontaneous YO-PRO-1 uptake by ddY-mouse astrocytes was reduced by the agonists and antagonists of P2X7Rs, but not by CytD. Down-regulation of P2X7Rs using siRNA decreased the bead uptake by ddY-mouse astrocytes. In addition, compared to in the case of ddY-mouse astrocytes, SJL-mouse astrocytes exhibited higher YO-PRO-1 uptake activity, and their bead uptake was significantly greater. These findings suggest that resting astrocytes exhibit engulfing activity and that the activity is regulated, at least in part, by their P2X7Rs.

1. Introduction

Astrocytes are the most abundant brain neuronal cells. Although originally defined as gap filters for the neuronal network, astrocytes have now been found to play a number of active roles in the brain. It was demonstrated that astrocytes are capable of engulfing the corpses and debris of cells [1–5], and Lööv et al. found very recently that astrocytes protect neurons by engulfing apoptotic cells [6]. This accumulating evidence clearly indicates that astrocytes play an important role as an innate immune system to keep the CNS environment clean. However, the mechanism underlying the regulation of engulfing activity has not been clarified so far.

In general, the innate immune response plays a critical role in maintaining homeostasis of the central nervous system (CNS) [7,8]. Microglia are immune resident cells in the CNS [9,10], act as the first line of defense, and are activated by nucleotides/nucleosides [11,12] and zinc [13,14]. Activated microglia migrate to lesion foci, and release matrix metalloproteinases, reactive oxygen species and certain cytokines. This innate immune response augments host defenses under pathological conditions such as infection, but contributes to neuronal death in cerebral ischemia, trauma and neurodegenerative disorders [9,15]. However, the different roles of phagocytosis/engulfment carried by astrocytes and microglia in the innate immune response are little known.

Gu et al. reported that the monocytic phagocytosis of xenobiotics such as non-opsonized particles and heat-killed bacteria is regulated by P2X7 purinergic receptors (P2X7Rs), and ATP stimulation of P2X7Rs attenuates the phagocytic activity via dissociation of the P2X7R from their complexes with nonmuscle myosin heavy chain IIA (MHC-IIA) [16–18]. Thus, they proposed a new role of P2X7Rs as scavenger receptors [19].

In general, P2X7Rs are known to be activated by high concentrations (0.1 or more mM) of ATP, and their prolonged stimulation results in large pore formation, leading to cell death [20–25]. On the other hand, basal activation of P2X7Rs has been found to stimulate cell proliferation and to be involved in wound healing in an in vitro keratinocyte model [26]. Previously, we found that P2X7Rs expressed by cultured mouse astrocytes were activated in the absence of any exogenous stimuli [27,28], but their role in resting astrocytes remains unknown.

Overall, we hypothesized that P2X7Rs functionally expressed by resting astrocytes might regulate their engulfing activity. Here, we showed that in cultured astrocytes, a decrease of P2X7R basal activity resulted in a decrease of their engulfing activity, and therefore it is indicated that P2X7Rs play a critical role in regulation of the engulfing activity of resting astrocytes.

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2. Materials and methods

2.1. Reagents

The chemicals and reagents for experiments were purchased from Wako Pure Chemical Ind. (Osaka, Japan), except where otherwise noted.

2.2. Primary cell cultures

As reported previously, primary astrocyte cultures were prepared from the cortices of 1-day-old ddY mice (Japan SLC, Hamamatsu, Japan) and 1-day-old SJL mice (Japan Charles River, Kanagawa, Japan) [27,28]. 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. Astrocyte cultures were used at 20–40 DIV. At this age, any neurons present on the initial plating have been killed by glutamate in the feeding medium. These cultures comprised >95% astrocytes and <5% microglia (data not shown). For the transfection study, astrocyte cultures at the growth phase were prepared by replating confluent cultures at a reduced density (0.5×10^5 cells/well of 24-well plates), and then cultured for 2 days until they reached 70–80% sub-confluency.

2.3. Assessment of cell death

Astrocyte death was quantified by measuring lactate dehydrogenase(LDH) activity in the supernatants of cells as previously described [28]. Cell viability was expressed as the percentage of LDH activity to control LDH activity, which was defined as the mean value obtained for three or four control wells in the same 24-well plate as the test well.

2.4. Dye uptake

As reported previously [27], experiments were initiated by replacing the culture medium with a balanced salt solution (BSS) comprising 3.1 mM KCl, 134 mM NaCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.25 mM KH_2PO_4, 15.7 mM $NaHCO_3$, and 2 mM glucose. The pH was adjusted to 7.2 although the solution was equilibrated with 5% CO_2 at 37 °C. After 10 min preincubation in BSS, cells were incubated with 1 μM YO-PRO-1 iodide (YP; Invitrogen, Eugene, OR) for the designated times in a 5% CO_2 incubator at 37 °C. Drugs were added at the onset of incubation with YP, from a concentrated stock prepared in BSS immediately before use and adjusted to pH 7.2 when necessary. YP incubation was terminated by washing three times with warmed BSS, and the fluorescence photomicrographs were taken (three random fields/well) within 10 min using a fluorescence microscope (IX51; Olympus, Tokyo, Japan) equipped with a filter system of 470–490 nm for excitation and 510–550 nm for emission, an LCAch 40X object lens, and a digital camera (coolSNAP; Nippon Roper, Tokyo, Japan).

YP dye uptake was evaluated in three to five randomly selected fields of 3–4 wells in each experiment. The fluorescence intensity of all nuclei present in each field (more than 20) was measured after background subtraction using the histogram program of the Photoshop software (Adobe). The values were averaged to generate a single value for each experiment.

2.5. Bead uptake

Almost the same protocol as for the YP uptake experiments was used for the bead uptake ones. After replacing the culture medium with BSS, astrocytes cultured on glass cover slips were preincubated in BSS for the designated times, and then incubated with 1 μL/well (24 well-plates) of yellow-green carboxylate latex beads (1 μm; Polysciences, Warrington, PA) for the designated times in a CO_2 incubator. Then, the cells were fixed with 4% paraformaldehyde (PFA) for 20 or more min, and the nuclei were counterstained with 5 μM propidium iodide (PI). Thereafter, photomicrographs were taken using a Laser confocal microscope (LSM510META; Carl Zwiss, Jena, Germany). The numbers of beads taken up by astrocytes were determined using the ColonyCounter software (Microtec Co., Ltd., Chiba, Japan). Bead uptake was evaluated in three randomly selected fields of 3–4 wells in each experiment.

2.6. Western blots

Western blots were performed by the protocol described previously [27]. The following primary antibodies were used: a rabbit anti-P2X7R antibody (1:500, #APR-008; Alomone Labs, Jerusalem, Israel) or a mouse anti-GAPDH antibody (1:5000, #sc-32233; Santa Cruz, CA), and the second antibodies used were: anti-rabbit or anti-mouse IgG HRP-linked antibodies at 1:10,000 dilution. ECL reagent (Perkin Elmer, Boston, MA) was used for detection of the signals.

2.7. Immunocytochemistry

As described previously [27], immunostaining was performed for cells grown on glass cover slips, and the primary antibodies used were as follows: a rabbit anti-GFAP antibody (1:200, #AB5804; Millipore, Temecula, CA) and a rat anti-MHC class II (I-A/I-E) antibody (1:25, #14-5321; eBioscience, San Diego, CA). Immunoreactivity was visualized using Alexa Fluor 546-labeled secondary antibodies (Invitrogen) at 1:500 dilution, and the nuclei were conterstained with Hoechst33258 (2 μg/mL). Photomicrographs of them were obtained with a Laser confocal microscope. The intracellular space of astrocytes was visualized using CellTraker® (Invitrogen). Astrocytes incubated with beads for the designated times were further incubated with 5 μM CellTraker for 30 min, followed by washing out, and then they were fixed with 4% PFA. Photomicrographs of them were taken using a Laser confocal microscope.

2.8. siRNA transfection

siRNA transfection into astrocytes was performed with RNAiFect™ (QIAGEN, Valencia, CA, USA) as described previously [27,28]. A cocktail of three siRNAs (siTrio Full Set) for P2X7Rs was purchased from B-Bridge International Inc., (Sunnyvale, CA) and used for P2X7R down-regulation. The antisense sequences of the siRNAs used were as follows: UUGCUCUGUGGGUCCAUCCTT, UUCUCCUUAUAGUACUUGGTT and UUGGCCUUUGACAACUUGCTT. For transfection, siRNAs (42 pmol of RNA/cm^2) were transfected into astrocyte cultures using OPTI-MEM reduced serum medium (Invitrogen). The cells were incubated with siRNAs for 6 h and the medium was replaced with EMEM supplemented with 10% FBS. Uptake experiments and evaluation of P2X7R protein expression levels were performed 2 days after siRNA transfection.

2.9. Statistical analysis

The data are expressed as means ± SD. Comparisons between two or more groups were performed by means of analysis of variance (ANOVA, followed by Fisher's PLSD), differences with a p-value of 0.05 or less being considered statistically significant.

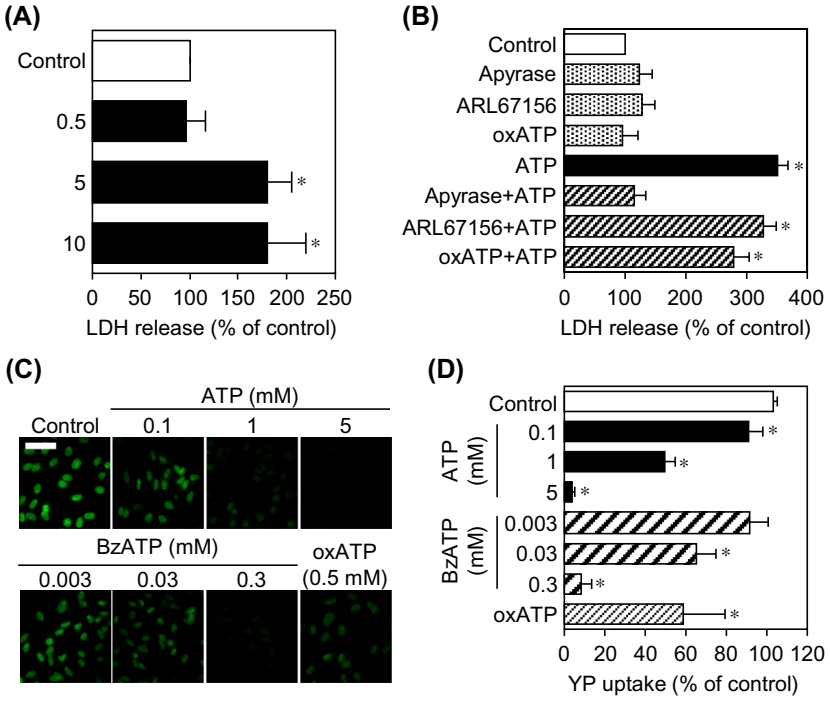

Fig. 1. The agonists and antagonists of P2X7Rs decrease cell viability and YP uptake in ddY-mouse astrocytes. (A, B) After cells had been treated with the indicated concentrations of ATP (A) or 5 mM ATP (B) in a 5% CO_2 incubator for 2 h, followed by twice washes with warmed BSS, they were incubated in a fresh BSS for 3 h, and then the LDH activity in the supernatant was measured by the LDH assay. In panel (B), 25 U/mL apyrase was co-treated with ATP, while 0.1 mM ARL67156, an ecto-ATPase inhibitor, and 0.5 mM oxATP was pretreated for 1 h, and then the former was co-treated with ATP for 2 h and the latter was removed by twice washes, followed by the 2 h ATP treatment of the cells. Under this condition, oxATP gives selective full antagonism for P2X7R [27]. Each column represents the mean ± SD ($N = 3$). $^*p < 0.01$ (*vs.* control). (C, D) After 10 min pre-incubation in BSS, ddY-mouse astrocytes were incubated with 1 μM YP in the presence or absence of the indicated concentrations of ATP or BzATP in 5% CO_2 incubator for 30 min at 37 °C. Representative photomicrographs and its quantitative results were shown in panel (C) and (D), respectively. As for oxATP, cells were pre-treated with 0.5 mM of it, followed by twice washes, and then used for experiments to obtain the P2X7R selectivity [27,36]. Each column represents the mean ± SD ($N = 3$). $^*p < 0.01$ (*vs.* control).

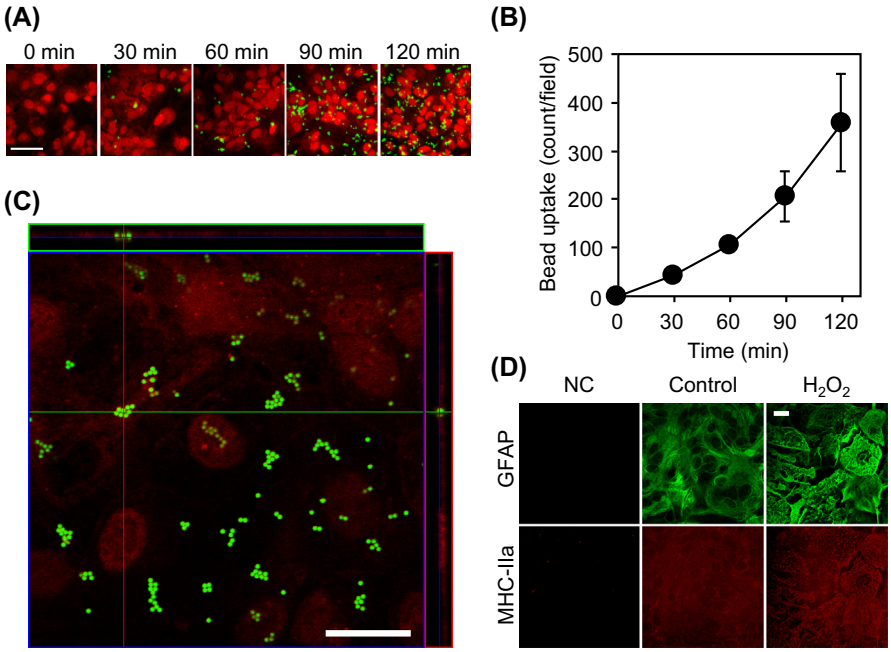

Fig. 2. ddY-Mouse astrocytes exhibit engulfing activity. (A, B) After 10 min preincubation in BSS, astrocytes were incubated with 1 μL/well of beads (green) for the designated times in a 5% CO_2 incubator. Representative photomicrographs and their quantification results from 5 independent experiments were shown in panel (A) and (B), respectively. Each point represents the mean ± SD. The nuclei were counterstained with PI (red). Scale bar = 25 μm. (C, D) After 10 min preincubation in BSS, astrocytes were incubated with 1 μL/well of beads (green) for 60 min in a 5% CO_2 incubator, and then they were stained with CellTracker (red) (C). Representative photomicrographs from four independent experiments were shown. Scale bar = 20 μm. (D) Astrocytes were immunostained with anti-MHC-IIa (red) and anti-GFAP (green) antibodies. Astrocytes treated with 100 μM H_2O_2 for 24 h work as a positive control for MHC-IIa expression. NC means the negative control. Representative photomicrographs were from three independent experiments. Scale bar = 20 μm. (For interpretation of the reference to color in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Effects of agonists and antagonists of P2X7R on astrocytic viability and P2X7R pore activity

Treatment of astrocytes with 5 or more mM ATP increased their LDH release, which was not due to degradation products such as AMP and adenosine. However, the ATP-induced decreased viability of astrocytes was not abolished by oxATP, a selective P2X7R antagonist (Fig. 1A and B). To confirm the activity of P2X7Rs in cultured astrocytes, their YP uptake was measured. As shown in Fig. 1C and D, astrocytes took up YP without ATP treatment, which was decreased by oxATP as reported previously [27], while the uptake was decreased by the administration of ATP and BzATP dose-dependently and almost completely disappeared at the concentrations of 5 and 0.3 mM, respectively, at which P2X7Rs are known to be activated. These results suggested that P2X7Rs expressed by cultured astrocytes had no or only a negligible contribution to ATP-induced astrocytic death, and that the agonists and antagonists inactivated P2X7Rs.

3.1. Characteristics of bead uptake

The engulfing activity of astrocytes was assessed as the uptake of beads as a xenobiotic [16]. On the administration of beads to astrocytes, they incorporated the beads time-dependently (Fig. 2A and B), and the uptaken beads were present in the intracellular space visualized with CellTracker (Fig. 2C). Gu et al. reported that exhibition of engulfing activity by non-professional phagocytes required coexpression of nonmuscle MHC-IIa [16,18], and thus its expression was examined. Fig. 2D shows that MHC-IIa immunoreactivity was detected in GFAP-positive astrocytes, but its expression showed a diffuse pattern differing from a dotted one in the positive control cells, which were treated with 100 μM H_2O_2 for 24 h and exhibited intense expression of GFAP, an indicator of activated astrocytes.

An F-actin polymerization inhibitor, CytD, by which phagocytosis is blocked, clearly decreased the uptake of beads, but not that of YP. In addition, the astrocytic bead uptake was decreased by the agonists, ATP and BzATP, and antagonists, KN-62 and A438079, the same as in the case of YP uptake (Fig. 3A and B). Thus, it was indicated that the bead uptake was decreased by inactivation of P2X7Rs and required rearrangement of actin filaments, differing from the case of YP uptake.

3.2. Effect of P2X7R activity on engulfing activity

In our previous studies, knock-down of P2X7Rs resulted in their decreased pore activity [27,28], and thus the effect of P2X7R knocked-down on the engulfing activity of astrocytes was examined. Transfection of siRNAs into cultured astrocytes successfully decreased the expression of P2X7Rs (Fig. 4A), and bead uptake by the P2X7R-knocked-down astrocytes was less than that by the scrambled siRNA-transfected ones (Fig. 4B). In addition, in astrocytes derived from SJL-mouse cortex, in which YP uptake is higher than that in ddY-mouse cortex (Fig. 4C), the bead uptake was apparently greater than in the case in ddY-mouse ones (Fig. 4D), the engulfing activity of astrocytes being correlated with their P2X7R activity.

4. Discussion

In this study, we demonstrated that P2X7Rs expressed by astrocytes do not act as death receptors, while their basal activity regulates astrocytic engulfing activity. Our cultured astrocytes expressed MHC-IIa and GFAP, but the expression levels were less than those in H_2O_2-treated activated astrocytes (Fig. 2D), and similar alteration of the expression pattern of MHC-IIa was reported for interferon-gamma-stimulated astrocytes [29]. Thus, the astrocytes used in this study are considered to be non-stimulated "resting" astrocytes, and that they are capable of engulfment.

In the innate immune response, professional phagocytes, i.e., microglia, play primary roles. It is well-known that the phagocytic activity of microglia is expressed after their activation by exposure to danger signals such as ATP [11–14]. Then, as the first line of defense in the CNS, activated microglia must react quickly to increased inflammatory signals and destroy the infectious xenobiotics before they damage sensitive neural tissues. In contrast, the astrocytic response is usually delayed, and activation of astrocytes, astrogliosis, is initiated by factors/mediators derived from activated microglia [30]. Thus, astrocytes and microglia are considered to play different roles in the innate immune response. Our findings that P2X7Rs functionally expressed by resting astrocytes play a key role in regulation of their engulfing activity, suggested that the contributing stages of the immune response should be different between astrocytes and microglia. That is to say, the engulfing activity exhibited by resting astrocytes is thought to be important before and/or after pathological stages, because their engulfing activity is decreased in the presence of ATP (Fig. 3A), while ATP can trigger microglial activation [11,13]. Therefore, it is speculated that a danger signal, ATP, might switch the immune response from the resting mode to the active one, in which astrocytes and microglia, respectively, play major roles. More detailed investigations are needed to confirm this.

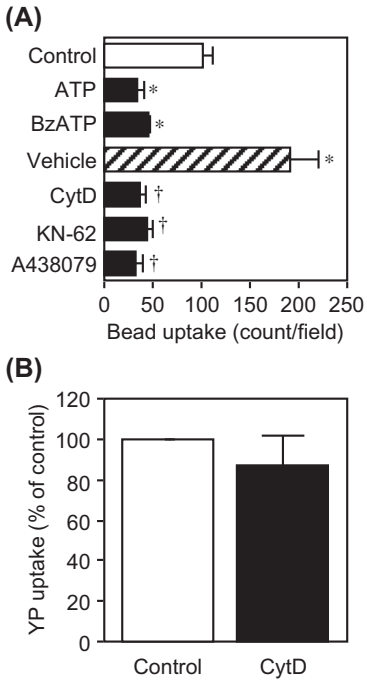

Fig. 3. The agonists and antagonists of P2X7Rs, and an F-actin polymerization inhibitor decrease engulfing activity in ddY-mouse astrocytes, but the latter had no effect on YP uptake in ddY-mouse astrocytes. (A) After 10 min preincubation in BSS, astrocytes were incubated with 1 μL/well of beads in the presence or absence of 5 mM ATP, 0.3 mM BzATP, 10 μM KN-62 or 10 μM A438079 for 60 min in a 5% CO_2 incubator. In the case of CytD, after astrocytes had been preincubated with 20 μM CytD in BSS for 20 min, they were incubated with 1 μL/well of beads for 60 min in a 5% CO_2 incubator. The vehicle group was treated with 0.1% DMSO as the solvent for KN-62, A438079 and CytD. Each column represents the mean ± SD ($N = 3$). *$p < 0.05$ (*vs.* control). †$p < 0.05$ (*vs.* vehicle). (B) After astrocytes had been preincubated with 20 μM CytD in BSS for 20 min, they were incubated with 1 μM YP for 30 min in a 5% CO_2 incubator. Each column represents the mean ± SD ($N = 3$).

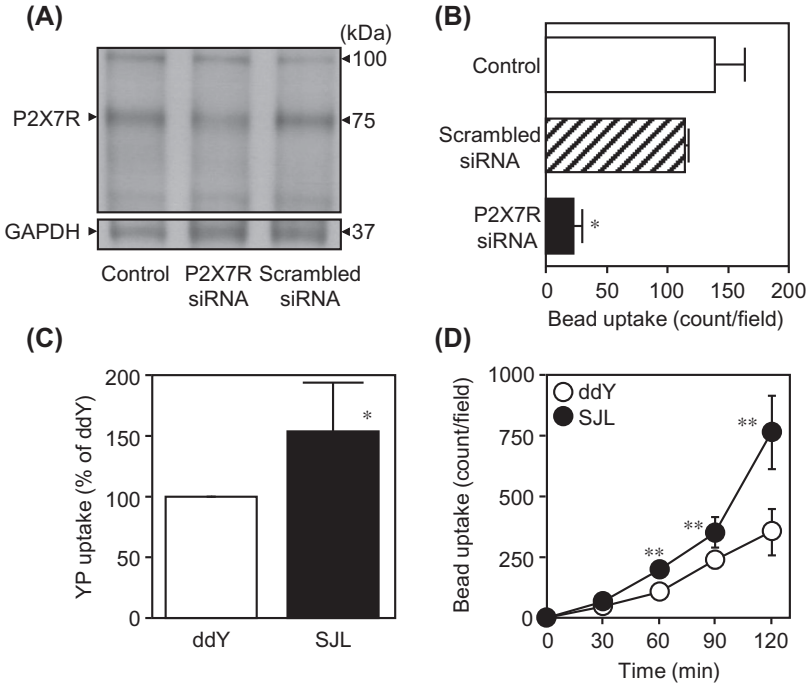

Fig. 4. Engulfing activity of mouse astrocytes depends on their P2X7R activity. (A, B) Astrocytes were transfected with scrambled or P2X7R-specific siRNA and then cultured for 2 days. Thereafter, western blot (A) and bead uptake (B) experiments were performed. Representative Western blots from three independent experiments are shown in panel (A). As for bead uptake, the astrocytes were preincubated for 10 min in BSS, and then incubated with 1 μM beads for 60 min in a 5% CO_2 incubator. Each column represents the mean ± SD ($N = 3$). $^{*}p < 0.01$ (*vs.* scrambled siRNA). (C) After 10 min preincubation in BSS, astrocytes obtained from ddY- and SJL-mouse cortices were incubated with 1 μM YP for 30 min in a 5% CO_2 incubator. Each column represents the mean ± SD ($N = 3$). $^{*}p < 0.01$ (*vs.* ddY-mouse astrocytes). (D) After 10 min preincubation in BSS, astrocytes were incubated with 1 μL/well of beads for the designated times in a 5% CO_2 incubator. Each column represents the mean ± SD ($N = 3$). $^{**}p < 0.05$ (*vs.* ddY-mouse astrocytes at the corresponding time points).

There is an apparent difference in YP uptake activity between ddY- and SJL-mouse astrocytes (Fig. 4C), meaning different pore activities of P2X7Rs. Although the molecular mechanism underlying this has been investigated in our laboratory (under submission), this difference is not due to the differences in the protein expression levels of full-length P2X7Rs between these astrocytes (data not shown), and thus it is indicated that the activation levels of P2X7Rs are important as to the engulfing activity of astrocytes.

Both the agonists and antagonists of P2X7Rs attenuated the astrocytic uptake of beads and YP. Gu et al. clearly demonstrated that ATP stimulation of P2X7Rs caused dissociation of MHC-IIa from its complex with P2X7Rs, resulting in decreased engulfing activity [16,18,31,32]. Although it is not clear why not only the agonists but also the antagonists decrease the activity of P2X7Rs and engulfment, it might be due to that both of them bind to P2X7Rs, and then alter the conformation of their complexes.

A difference in the characteristics of bead and YP uptake was the dependency of the former, but not the latter, on rearrangement of actin filaments (Fig. 3). Translocation of MHC-IIa to plasma membranes is dependent on the cytoskeletal network consisting of intermediate filaments [33], and cytoskeletal components also are involved in the pore formation by P2X7Rs [34]. In this study, we measured YP uptake without any stimuli, and so the pores of P2X7Rs were considered to be constitutively formed. Therefore, CytD inhibited astrocytic bead uptake but it had no effect on YP uptake. In addition, this difference in the involvement of cytoskeleton components might also explain the increased bead uptake by 0.1% DMSO-treated astrocytes (Fig. 3A). In the YP uptake experiments, the reaction solution contained 0.1% DMSO derived from the solvent for YP (YP is provided as a DMSO solution), and additional administration of DMSO, to give a final DMSO concentration of 0.2%, did not influence YP uptake (data not shown). As DMSO is known to have an effect on actin filaments [35], DMSO might enhance bead uptake, but not YP uptake, by astrocytes.

Treatment of astrocytes with high concentrations of ATP caused their death, but P2X7Rs were not involved in this (Fig. 1A and B). We have no explanation for the mechanism of ATP-induced astrocytic death, and so detailed investigations are in progress in our laboratory.

5. Conclusion

The findings obtained here clearly demonstrate that P2X7Rs expressed by astocytes do not act as death receptors, while their basal activity is critical for astrocytic engulfing activity, and this is considered to be a novel role of P2X7Rs expressed by resting astrocytes in the innate immune system of the CNS.

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