

Ca²⁺ Entry is Required for Mechanical Stimulation-induced ATP Release from Astrocyte

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Astrocytes and neurons are inseparable partners in the brain. Neurotransmitters released from neurons activate corresponding G protein-coupled receptors (GPCR) expressed in astrocytes, resulting in release of gliotransmitters such as glutamate, D-serine, and ATP. These gliotransmitters in turn influence neuronal excitability and synaptic activities. Among these gliotransmitters, ATP regulates the level of network excitability and is critically involved in sleep homeostasis and astrocytic Ca²⁺ oscillations. ATP is known to be released from astrocytes by Ca²⁺-dependent manner. However, the precise source of Ca²⁺, whether it is Ca²⁺ entry from outside of cell or from the intracellular store, is still not clear yet. Here, we performed sniffer patch to detect ATP release from astrocyte by using various stimulation. We found that ATP was not released from astrocyte when Ca²⁺ was released from intracellular stores by activation of G_{α_q}-coupled GPCR including PAR1, P2YR, and B2R. More importantly, mechanical stimulation (MS)-induced ATP release from astrocyte was eliminated when external Ca²⁺ was omitted. Our results suggest that Ca²⁺ entry, but not release from intracellular Ca²⁺ store, is critical for MS-induced ATP release from astrocyte.

Key words: Astrocytes, ATP, Mechanical stimulation, Ca²⁺

INTRODUCTION

Astrocytes communicate with neurons by forming a tripartite synapse [1, 2]. Astrocytes express a multitude of neurotransmitter receptors and can respond to neuronal activity with elevated intracellular Ca²⁺ [2-4]. In turn, astrocytes release gliotransmitters to regulate neuronal activity [3]. It has been reported that ATP is a major astrocytic gliotransmitter, and is a source of extracellular

adenosine in the brain [5-7]. ATP regulates synaptic transmission and plasticity [8], and is important for Ca²⁺-based intercellular communications between astrocytes and other cell types in the central nervous system [9-11].

Up to date, even if the mechanism of ATP release from astrocytes has been extensively investigated, the precise release mechanism is not completely understood. It has been suggested that ATP is released through non-vesicular pathway including gap junction hemichannels [12-14], volume regulated anion channels [15], cystic fibrosis transmembrane conductance regulator (CFTR) [16], and P2X7 receptors [17]. Other studies have reported exocytotic, vesicular release of ATP from astrocytes [18-20]. All of these mechanisms appear to require Ca²⁺ increase in the cytoplasm, but it is still not clear whether the source of Ca²⁺ is Ca²⁺ entry or Ca²⁺

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release.

To address this, we performed the sniffer patch technique to detect ATP release from astrocytes using HEK293T cells expressing a mutant form of the P2X2 receptor, P2X2-V343Q, with enhanced ATP sensitivity. This receptor has high affinity, with an EC₅₀ of 0.7 μ M, for ATP, which can detect submicromolar level of ATP. Very recently, it has been reported that astrocytic Ca²⁺ increase by Ca²⁺ uncaging, TFLLR, or NMDA causes ATP release from astrocytes [18]. To induce Ca²⁺ increase from astrocytes, we used various agonists for GPCR such as PAR1, P2Y receptor, and B2 receptor. We also utilized mechanical stimulation that is known to cause Ca²⁺ increase in astrocytes [21].

MATERIALS AND METHODS

Primary astrocytes culture

Cultured astrocytes were prepared from P0–P3 of C57BL/6 mice. The cerebral cortex was dissected free of adherent meninges, minced and dissociated into single cell suspension by trituration through a Pasteur pipette. Dissociated cells were plated onto either 12 mm glass coverslips or six-well plates coated with 0.1 mg/ml poly d-lysine. Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 25 mM glucose, 10% heat-inactivated horse serum, 10% heat-inactivated fetal

bovine serum, 2 mM glutamine and 1000 units/ml penicillin–streptomycin. After three days later, cells were vigorously washed with repeated pipetting using medium and the media was replaced to get rid of debris and other floating cell types.

Ca²⁺ imaging and Sniffer Patch

For Ca²⁺ imaging, astrocytes with HEK293T cells transfected with P2X2-V343Q were incubated with 5 μ M Fura-2 AM (mixed with 5 μ l of 20% Pluronic acid) (Invitrogen, Grand Island, NY, USA) for 30 min and washed at room temperature, and subsequently transferred to a microscope stage for imaging. External solution contained (in mM): 150 NaCl, 10 HEPES, 3 KCl, 2 CaCl₂, 2 MgCl₂, 5.5 glucose, 20 Sucrose, pH adjusted to pH 7.3. Intensity images of 510 nm wavelength were taken at 340 nm and 380 nm excitation wavelengths using either iXon EMCCD (DV887 DCS-BV, ANDOR technology, UK). Two resulting images were used for ratio calculations in Axon Imaging Workbench version 6.2 (Indec System, CA, USA). P2X2-V343Q-mediated currents were recorded from HEK293T cells expressing P2X2-V343Q under voltage clamp (V_h = -70 mV) using Multiclamp 700B amplifier (Molecular Devices), acquired with pClamp 9.2. Recording pipettes were filled with (mM): 110 Cs-Gluconate, 30 CsCl, 0.5 CaCl₂, 10 HEPES, 4 Mg-ATP, 0.3 Na₃-GTP and 10 BAPTA (pH adjusted to 7.3 with CsOH). For simultaneous recording, Imaging

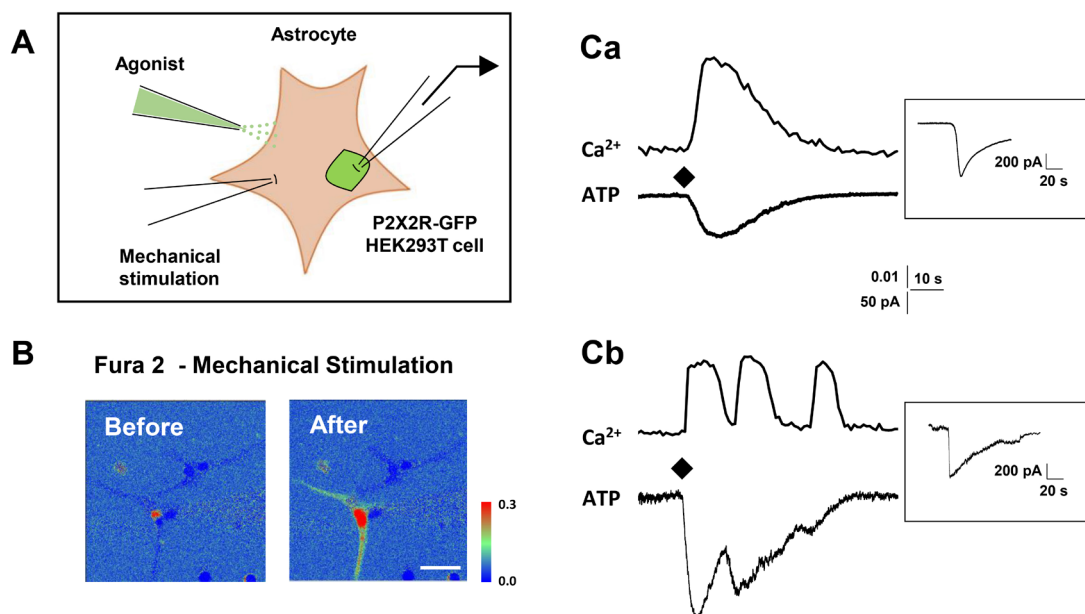


Fig. 1. ATP release from astrocyte by mechanical stimulation. (A) Schematic illustration of sniffer-patch technique stimulated by pressure application of GPCR agonists and mechanical stimulation. Right pipette, recording from HEK293T cell expressing P2X2-V343Q. (B) Pseudo color images from Fura2-loaded astrocyte before and after mechanical stimulation. (Ca, Cb) Representative traces recorded from sniffer-patch experiment. Upper trace, Ca²⁺ transient by mechanical stimulation recorded from astrocyte. Lower trace, whole-cell current by mechanical stimulation recorded from HEK293T cell expressing P2X2-V343Q. Diamond, mechanical stimulation. Inset box, Full activation current recorded from HEK293T cell by bath application of 100 μ M ATP.

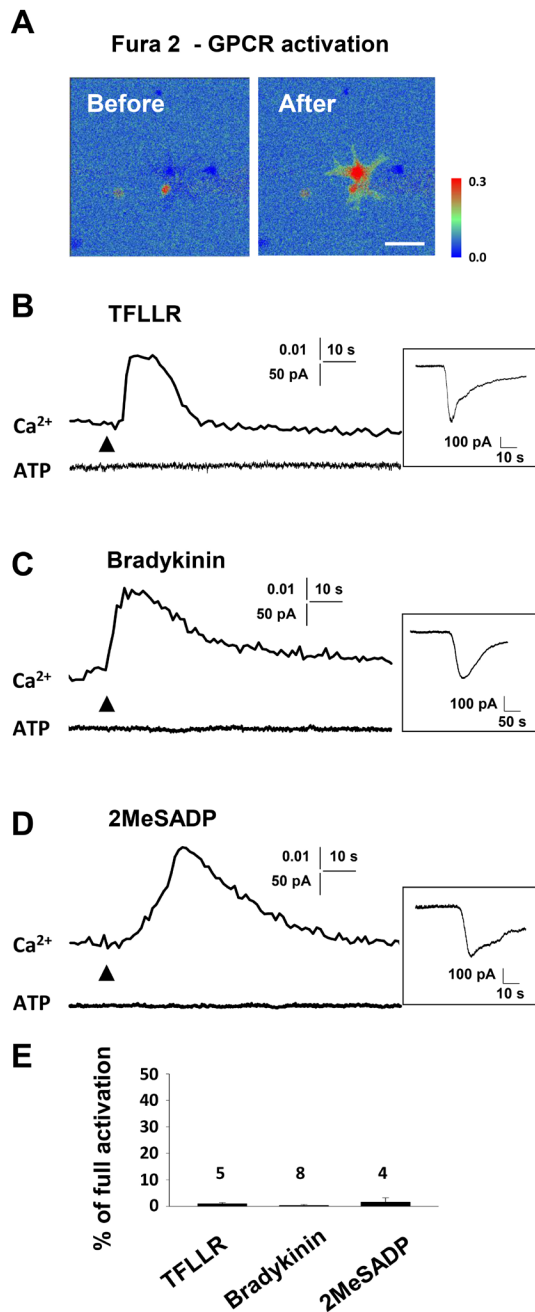


Fig. 2. Agonists for GqPCRs did not induce ATP release from astrocyte. (A) Pseudocolor images from Fura2-loaded astrocyte before and after pressure application. (B–D) Representative traces recorded from sniffer-patch experiment. Upper trace, Ca²⁺ transient by 500 μM TFLLR (B; n=10), 500 μM Bradykinin (C; n=8), and 500 μM 2MeSADP (D; n=4) recorded from astrocyte. Lower trace, whole-cell current by TFLLR (B), Bradykinin (C), and 2MeSADP (D) recorded from HEK293T cell expressing P2X2-V343Q. Arrow head, pressure application for 100 ms. Inset box, Full activation current recorded from HEK293T cell by bath application of 100 μM ATP to measure total expression of P2X2-V343Q. (E) Summary bar graph showing percent of full activation.

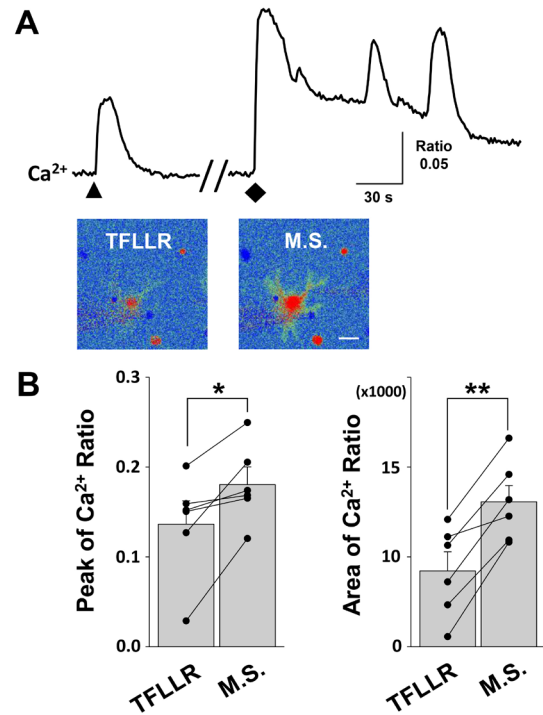


Fig. 3. Comparison of the Ca²⁺ response evoked by TFLLR and mechanical stimulation. (A) Representative trace of Ca²⁺ increase induced by TFLLR and mechanical stimulation. Below image indicates Fura2 ratio image at the peak of pixel intensity (B) Summary bar graph showing peak and area-under-the-curve of Ca²⁺ ratio induced by TFLLR and mechanical stimulation respectively. Paired two-tailed t-test (*p<0.05, **p<0.01).

Workbench was synchronized with pClamp 9.2.

Mechanical stimulation

To make mechanical stimulation glass pipette was placed right above the membrane of cultured astrocyte. With monitoring its resistance, pipette generally pressed cell membrane until the value of membrane resistance reaches to approximately as double. When the pressure by pipette evoked Fura2 signals, the position of pipette was returned to original place.

All data are given as mean±SEM; n denotes the number of individual cells assessed in sniffer patch-clamp studies. Statistical differences were determined by two-tailed unpaired Student's t test and considered significant at p<0.05.

RESULTS

Mechanical stimulation induces ATP release from astrocyte

We performed sniffer patch to detect ATP release from astrocyte. On the experimental date, HEK293T cells transfected with P2X2-V343Q, known as a non-selective cation permeable

channel [22], and GFP (sensor cells) were mixed with cultured astrocytes, allowing sensor cells to be located nearby astrocytes. For monitoring of the astrocytic Ca^{2+} signal, Fura-2 intensity from the astrocyte was recorded from the beginning of the experiment. To make mechanical stimulation, glass pipette placed above a cultured single astrocyte and monitored pipette resistance to determine when the pipette touched the cell membrane. Pipette was slowly lowered until the value of resistance doubled. Once the Fura-2 signal started to change, the pipette was immediately

returned to original height. Whole cell patch recording from HEK 293T cell expressing P2X2-V343Q nearby astrocyte was performed to sense the ATP release from astrocyte (Fig. 1A). Fig. 1B shows a representative Fura-2 intensity images of before and after mechanical stimulation on cultured astrocyte reflecting the increase of Ca^{2+} signals in cytoplasm of astrocyte evoked by mechanical stimulation.

MS-induced ATP release and Ca^{2+} signals were simultaneously monitored while performing whole-cell patch clamp recording

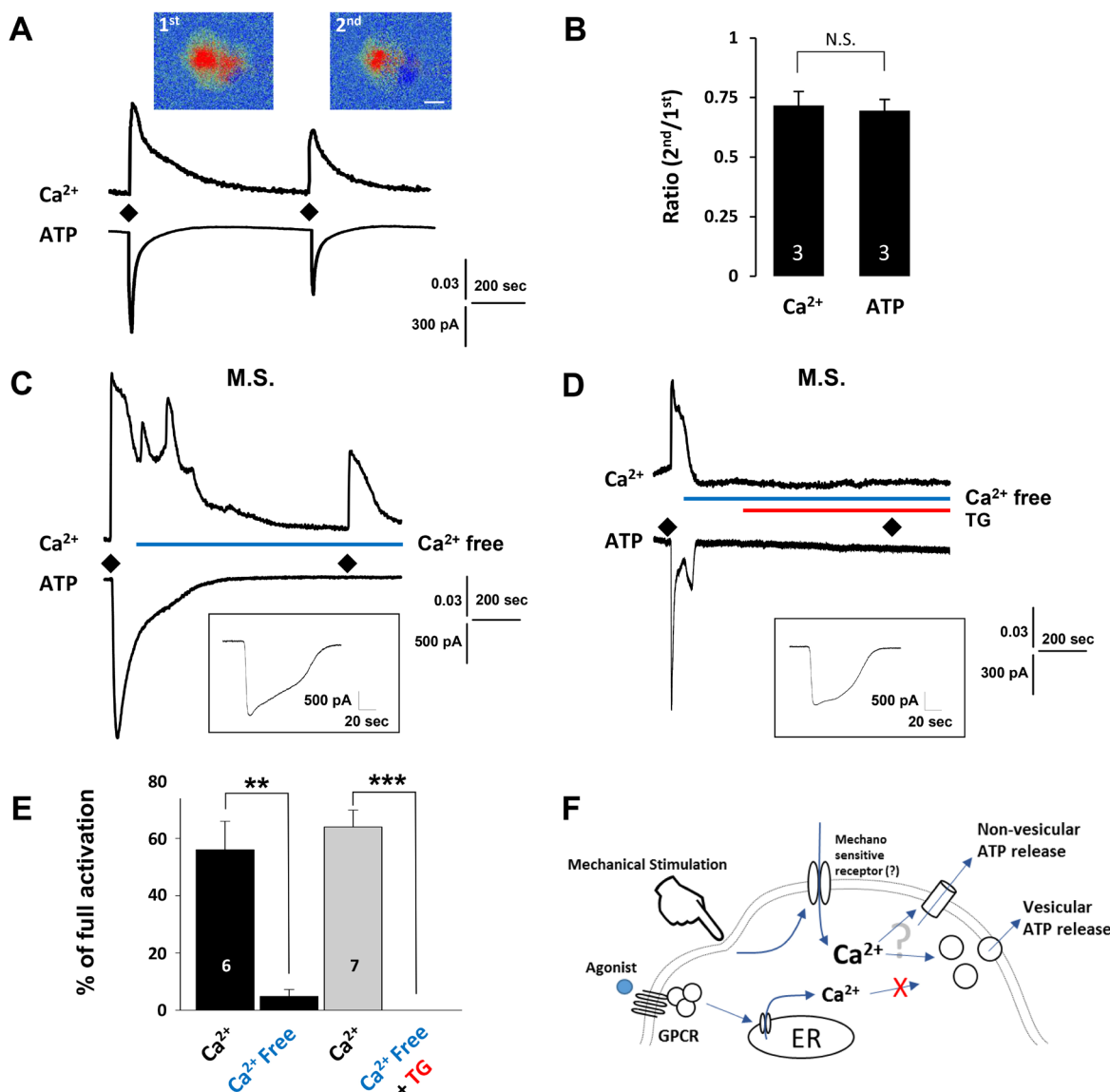


Fig. 4. Ca^{2+} entry is critical for MS-induced ATP release from astrocyte. (A, B) Representative trace of sequential recording of Ca^{2+} and ATP release in same astrocyte and summary bar graph of 1st/2nd ratio for the response of Ca^{2+} and ATP amplitude. (C, D) Representative traces recorded from Fura2 imaging and sniffer-patch experiment in Ca^{2+} free condition (C) and Ca^{2+} free with TG condition (D). Diamond indicates the timing of mechanical stimulation. (E) Summary bar graph showing the ratio of Ca^{2+} response with Ca^{2+} , Ca^{2+} free and Ca^{2+} free + TC in external solution induced by mechanical stimulation. Paired two-tailed t-test (** $p < 0.01$, *** $p < 0.001$). (F) Schematic diagram of pathway of ATP release compared with mechanical stimulation v.s. GPCR activation.

from sensor cell nearby a single astrocyte and Fura-2 ratiometric imaging (Fig. 1Ca and 1Cb). At the end of each experiment, we bath-applied 100 μ M ATP to measure the full extent of expression of biosensor, P2X2-V343Q. The amount of ATP release was expressed by measuring the peak amplitude of MS-induced ATP release and taking the percent of full activation. We found that mechanical stimulation elevates Ca²⁺ in astrocytes and triggers a release of ATP from astrocyte that was detected in sensor cell as an inward current (Fig. 1Ca). Some astrocytes showed Ca²⁺ oscillation which caused ATP release from astrocytes with a similar time course (Fig. 1Cb).

Agonists of GqPCR did not induce ATP release from astrocyte

It has been recently shown that acutely dissociated astrocytes release ATP by Ca²⁺ elevation via metabotropic and ionotropic receptors or Ca²⁺-uncaging [18]. To increase astrocytic Ca²⁺, we applied various agonists of GqPCRs by pressure-mediated agonist application (puffing) onto cultured astrocyte. TFLLR, an agonist for Protease Activated Receptor 1 (PAR1), increased Ca²⁺ in cultured astrocyte (Fig. 2A). Contrary to previous report [18], TFLLR did not induce ATP release in our experimental system (Fig. 2B). We also found that bradykinin, an agonist for B2 receptor, and 2MeSADP, an agonist for P2Y receptor, did not induce ATP release from cultured astrocytes (Figs. 2C and 2D). Bath application of 100 μ M ATP was used for the full activation of P2X2-V343Q channels expressed in the sensor cell again (Fig. 2B, 2C, and 2E). Fig. E shows summary bar graph of Ca²⁺ increase induced by GPCR agonists (% of full activation, TFLLR: 1.02 \pm 0.43, n=10 cells; Bradykinin: 0.38 \pm 0.33, n=8 cells; 2-(Methylthio) adenosine 5'-diphosphate trisodium salt hydrate (2MeSADP): 1.62 \pm 1.58, n=4 cells). This result indicates that, unlike MS-induced Ca²⁺ increase, GPCR-induced Ca²⁺ increase might not be sufficient to cause ATP release from cultured astrocyte.

Since Ca²⁺ increase by mechanical stimulation, but not by activation of GPCRs, causes ATP release, we hypothesized that the concentration of intracellular Ca²⁺ is different between mechanical stimulation and activation of GPCRs. To test our hypothesis, we measured the ratio of the Ca²⁺ transient by application of TFLLR and mechanical stimulation from Fura-2 loaded astrocytes (Fig. 3). The ratio of Ca²⁺ increase by mechanical stimulation was significantly greater than by application of TFLLR (ratio of Ca²⁺ response, peak: TFLLR, 0.14 \pm 0.03, M.S., 0.18 \pm 0.02, n=5 cells; area: TFLLR, 8.40 \pm 2.15, M.S., 16.09 \pm 1.83, n=6 cells; Fig. 3B), suggesting the ATP release from astrocyte might be regulated by concentration of intracellular Ca²⁺.

Internal Ca²⁺ regulates ATP release from astrocyte

To determine the source of Ca²⁺ increase required for MS-induced ATP release from astrocyte, we tested whether MS-induced ATP release can be detected in the absence of extracellular Ca²⁺. Under normal condition, MS-induced Ca²⁺ increase and ATP release were evoked by two sequential mechanical stimulations (ratio of 2nd/1st, Ca²⁺, 0.72 \pm 0.06, n=3 cells; ATP, 0.70 \pm 0.05, n=3 cells) (Fig. 4A and B). However, when the extracellular Ca²⁺ was omitted, ATP release was dramatically reduced, but a small portion of Ca²⁺ increase was still detected in astrocyte (Fig. 4C). Because this residual Ca²⁺ increase might come from endoplasmic reticulum (ER) store, we used 1 μ M thapsigargin (TG) to deplete intracellular Ca²⁺ from ER in Ca²⁺ free extracellular bath solution and performed sniffer patch. Under this condition, mechanical stimulation did not induce Ca²⁺ elevation or ATP release (Fig. 4D). Fig. 4E shows summary of these experiments with bar graph (% of full activation, Ca²⁺, 55.97 \pm 9.83 and Ca²⁺ free, 9.83 \pm 4.92, n=6 cells; Ca²⁺, 63.92 \pm 5.94 and Ca²⁺ free + TG, 0.001 \pm 0.01, n=7 cells). These results strongly indicate that accumulated cytoplasmic Ca²⁺ is required for ATP release and external Ca²⁺ is critical source for initiating ATP release from astrocyte.

DISCUSSION

Our study demonstrates that mechanical stimulation induces ATP release from cultured astrocytes using sniffer-patch technique, a highly sensitive detection method. We found that MS-induced Ca²⁺ entry allows a release of ATP. Our results indicate that mechanical stimulation leads to influx of Ca²⁺ that is required for release of ATP. In addition, the concentration of intracellular Ca²⁺ seems to be a critical factor regulating ATP release from astrocytes (Fig. 4F).

Astrocytic Ca²⁺ signaling is considered as a critical component for release of gliotransmitters. Astrocytes express many ion channels and receptors that allow increases in cytosolic Ca²⁺ concentration. These include voltage-gated calcium channels (VGCCs), GPCR mediated Ca²⁺ release from ER, transient receptor potential (TRP) channels [23-25]. Among them, GPCR-mediated Ca²⁺ increase is the most accepted mechanism for astrocytic Ca²⁺ signaling. The bath application of TFLLR, an agonist for PAR1, has been reported to trigger release of ATP and elevation of Ca²⁺ from acutely dissociated cortical astrocyte [18]. On the contrary, we did not observe GPCR-induced ATP release (Fig. 2). This discrepancy might be caused by different experimental conditions of recorded cells, or by differences in acutely dissociated versus cultured astrocyte. Nevertheless, it should be noted that MS-induced ATP release from cultured astrocyte is critically dependent on Ca²⁺

entry, not Ca^{2+} release (Fig. 1 and 4).

The molecular identity of MS-induced Ca^{2+} entry is unknown and it needs to be examined in the future. Astrocytes have several kinds of mechano-sensitive ion channels. Among the mechano-sensitive channels, astrocytes are known to express TRP family such as TRPC, TRPV, and TRPA, which are non-selective cationic channels [25]. Recently ATP was shown to be released by TRPV4-mediated calcium entry that leads to a cytoplasmic Ca^{2+} increase in hypotonically induced swollen porcine lens epithelium [26]. Therefore, mechano-sensitive receptors / stretch-activated channels of the TRP family could be potential candidates for the molecular identity of Ca^{2+} entry to trigger MS-induced ATP release.

In summary, mechanical stimulation seems to be enough to allow Ca^{2+} entry in astrocyte for ATP release. Current study demonstrates that MS-induced Ca^{2+} entry causes ATP release from astrocytes. The results and conclusions of our study should help understand the release mechanism of ATP in astrocyte and physiological role of ATP in the brain.

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