Glutamate-induced Exocytosis of Glutamate from Astrocytes*§

Recent studies indicate that astrocytes can play a much more active role in neuronal circuits than previously believed, by releasing neurotransmitters such as glutamate and ATP. Here we report that local application of glutamate or glutamine synthetase inhibitors induces astrocytic release of glutamate, which activates a slowly decaying transient inward current (SIC) in CA1 pyramidal neurons and a transient inward current in astrocytes in hippocampal slices. The occurrence of SICs was accompanied by an appearance of large vesicles around the puffing pipette. The frequency of SICs was positively correlated with [glutamate]o. EM imaging of anti-glial fibrillary acid protein-labeled astrocytes showed glutamate-induced large astrocytic vesicles. Imaging of FM 1-43 fluorescence using two-photon laser scanning microscopy detected glutamate-induced formation and fusion of large vesicles identified as FM 1-43-negative structures. Fusion of large vesicles, monitored by collapse of vesicles with a high intensity FM 1-43 stain in the vesicular membrane, coincided with SICs. Glutamate induced two types of large vesicles with high and low intravesicular [Ca2+]i. The high [Ca2+]i vesicle plays a major role in astrocytic release of glutamate. Vesicular fusion was blocked by infusing the Ca2+ chelator, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′′-tetraacetic acid, or the SNARE blocker, tetanus toxin, suggesting Ca2+- and SNARE-dependent fusion. Infusion of the vesicular glutamate transport inhibitor, Rose Bengal, reduced astrocytic glutamate release, suggesting the involvement of vesicular glutamate transporters in vesicular transport of glutamate. Our results demonstrate that local [glutamate]o increases induce formation and exocytotic fusion of glutamate-containing large astrocytic vesicles. These large vesicles could play important roles in the feedback control of neuronal circuits and epileptic seizures.

In addition to their nutritive and metabolic functions, astrocytes have been recently discovered to actively participate in and modify neuronal activity by releasing neurotransmitters, such as glutamate and ATP (1–6). Astrocytic Ca2+-dependent glutamate release modulates neuronal activity in hippocampal slices (7–9) and cell cultures (10, 11). However, there is still controversy about the mechanism by which astrocytes release glutamate. Several hypotheses have been proposed, including exocytosis of vesicles using a protein docking system similar to synaptic vesicles (12–17), swelling-induced anion channels (18), gap-junction hemichannels (19), P2X receptor channels (20, 21), reverse transport (22), cystine/glutamate exchangers (23), and volume-sensitive nonselective channels (24). Recently, several groups have reported that a transient astrocytic glutamate release activates a slowly decaying transient inward current (SIC)2 in hippocampal and thalamic neurons (7, 8, 25, 26). Besides transient astrocytic glutamate release (~1 s), a long lasting release of glutamate (several 10 s) from astrocytes has also been reported (7, 21, 24). Transient astrocytic glutamate release could play important roles in modulating neuronal activities under both physiological and pathological conditions (26, 28, 29). In a previous study, we reported that either bath application of 4-aminopyridine (4-AP) or infusion of inositol 1,4,5-trisphosphate (IP3) together with high [glutamate] into astrocytes induced SICs, which were caused by fusion of a high [Ca2+]i large vesicle in astrocytes (26). In this study, we further demonstrate that local increases in [glutamate]o also induce spontaneous SICs and that SICs are activated by astrocytic glutamate release through fusion of a large vesicle.

EXPERIMENTAL PROCEDURES

Slice Preparation—Brain slices were prepared as described previously (9). Briefly, 14–20-day-old (P14–P20) Sprague-Dawley rats of either sex were anesthetized with sodium pentobarbital (55 mg/kg) and then decapitated. Transverse brain slices of 300 μm thickness were cut with a vibratome (TPL, St. Louis, MO) in a cutting solution containing the following (in mM): 2.5 KCl, 1.25 NaH2PO4, 10 MgSO4, 0.5 CaCl2, 10 glucose,

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‡ To whom correspondence should be addressed: Dept. of Cell Biology and Anatomy, New York Medical College, Basic Science Bldg., Rm. 220, Valhalla, NY 10595. Tel.: 914-594-3156; Fax: 914-594-4653; E-mail: jian_kang@NYMC.edu.

2 The abbreviations used are: SIC, slowly decaying transient inward current; TeNT, tetanus toxin; aTC, astrocytic transient inward current; GFAP, anti-glial fibrillary acid protein; TTX, tetrodotoxin; iGlUR, ionotropic glutamate receptor; vGlUT, vesicular glutamate transporter; SNARE, soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors; MSO, methionine sulfoximine; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′′-tetraacetic acid; IP3, inositol 1,4,5-trisphosphate; 4-AP, 4-aminopyridine; ACSF, artificial cerebrospinal fluid; RMP, resting membrane potential; CNQX, 6-cyano-2,3-dihydroxy-7-nitroquinoxaline; TBOA, 0.1-threo-β-benzylxoyaspartate; APV, DL-2-amino-5-phosphonovaleric acid; DlIC, differential inference contrast; MFI, meghom; AP, action potential.
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26 NaHCO₃, and 230 sucrose. Slices containing the hippocampus were incubated in artificial cerebrospinal fluid (ACSF) gassed with 5% CO₂, 95% O₂ for 1–4 h and then transferred to a recording chamber (1.5 ml) that was perfused continually (3 ml/min) with ACSF gassed with 5% CO₂, 95% O₂ at room temperature (23–24 °C) for recording. ACSF contained the following (in mM): 126 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 2 MgCl₂, 10 CaCl₂, 10 glucose, and 26 NaHCO₃ (pH 7.4 when gassed with 5% CO₂, 95% O₂).

Whole-cell Patch Clamp Recording—Cells were visualized with a ×60/0.90 water immersion lens on an Olympus BX51 upright microscope (Olympus Optical Co., New York) equipped with IR differential inference contrast (DIC) optics. Patch electrodes with resistances of 5–10 MΩ were pulled from KG-33 glass capillaries (inner diameter 1.0 mm, outer diameter 1.5 mm; Garner Glass Co., Claremont, CA) using a P-97 electrode puller (Sutter Instrument Co., Novato, CA). Pyramidal neurons in the CA1 pyramidal layer and astrocytes in stratum radiatum were identified by their distinct DIC morphology and electrophysiological properties as described previously (9). Pyramidal neurons were voltage-clamped at −60 mV or current-clamped without holding currents (for measuring Vₘ), and astrocytes were voltage-clamped at −80 mV. Cells with a seal resistance <5 Gigaohms, a holding current of more than −200 pA, or changes in the series resistance >10% of control were rejected from further analysis. The pipette filling solution for neuronal whole-cell recording contained the following (in mM): 123 potassium gluconate, 10 KCl, 1 MgCl₂, 10 HEPES, 0.1 EGTA, 0.01 CaCl₂, 1 ATP, 0.2 GTP, and 4 glucose (pH adjusted to 7.2 with KOH). The pipette solution for whole-cell recording in astrocytes contained the following (in mM): 123 potassium gluconate, 10 KCl, 1 MgCl₂, 10 HEPES, 5 EGTA, 0.5 CaCl₂, 1 ATP, 0.2 GTP, and 4 glucose (pH adjusted to 7.2 with KOH). Recorded signals were filtered through an 8-pole Bessel low pass filter with 2-kHz cut-off frequency and sampled using the PCLAMP 9.0 acquisition program (Axon Instruments Inc.) with an analog-digital point sample interval of 200 μs.

Fluorescence Imaging—A customized two-photon laser scanning Olympus BX61WI microscope with a ×60/0.90 water immersion lens was used to detect fluorescence signals. A Mai-Tai™ laser (Solid-State Laser Co., Mountain View, CA) tuned to 830 or 890 nm was used for excitation. Image acquisition was controlled by Olympus Fluoview FV300 software (Olympus America INC, Melville, NY). In the transluminescence pathway, a 565 nm dichroic mirror was used to separate green and red fluorescence. HQ525/50 and HQ605/50 or HQ525/50 and 710 nm high pass filters were placed in the “green” and “red” pathways, respectively, to eliminate transmitted or reflected excitation light (Chroma Technology Corp., Rockingham, VT). Fluorescence images were scanned in the X-Y-T or X-Y-Z mode with intervals of 2 or 4 s. Alexa Fluor-594 or FM 1-43 fluorescence was detected via the red pathway with the HQ605/50 filter. FM 4-64 was detected via the red pathway with the 710 nm filter. Fluoro-4 fluorescence was detected using the green pathway. Base-line fluorescence (F₀) was the average of four images during control, and ΔF/F was calculated as (ΔF/F(t)) = (F(t) − F₀)/F₀. The position shift in the X-Y section during 5 min of scanning was 0.5 ± 0.3 μm (mean ± S.D., n = 30 cells).

Electron Microscopy—Slices (300 μm) were moved from normal ACSF into ACSF containing 50 mM [glutamate] five times for 5 s at 2-min intervals, with washes in normal ACSF in between. Slices were then fixed with 2.5% glutaraldehyde in 0.1 mol/liter sodium cacodylate buffer (pH 7.4) overnight, and then re-sliced into 100-μm sections with the vibratome. Sections were pretreated with 10% normal goat serum for 4 h at 4 °C. Slices were incubated in the identical solution containing anti-GFAP antibody (1:2000, purified anti-mouse monoclonal GFAP, 2E1; BD Biosciences) for 24 h at 4 °C, and then incubated with the biotinylated secondary antibody (1:200, Histostain-Plus Bulk kit, mouse IgG; Zymed Laboratories Inc.) for 24 h at 4 °C, and with avidin-biotin complex overnight at 4 °C. Immu-
FIGURE 2. SICs depend on [glutamate]. A, puffing 5 mM [glutamate] induced few SICs in a representative pyramidal neuron. Puffing 2 mM [glutamate] did not induce SICs (2 mM Glut). B, the mean frequency (SIC Freq, □) and amplitude (SIC Amp, □) of SICs were plotted against glutamate concentrations in the puffing pipette ([glutamate]). Data were fitted by the Boltzmann equation, and R² is 0.99 and 0.96 for frequency and amplitude, respectively. Open circles and squares are the frequency (○) and amplitude (□) of SICs during control periods. The number on the top of curves indicates the sample size for each concentration. * and **, p < 0.05 and 0.01 compared with control period or 2 mM, paired t test or Student’s t test. C, whole-cell recording from a representative astrocyte showing that puffing 50 mM [glutamate] evoked an I_{puff} and induced spontaneous aTCs (2). I and 2, traces with enlarged time scale from the indicated control period (1) and puffing period (2). Astrocytic aTCs were fully blocked by combination of glutamate transporter inhibitor, TBOA (200 μM), and iGluR blockers CNQX (20 μM) and APV (50 μM) (TBOA/CNQX/APV). Puffing 2 mM [glutamate] did not induce aTCs (2 mM Glut). D, frequency (aTC Freq, □) and amplitude (aTC Amp, □) of aTCs were plotted against concentrations of puffed glutamate ([glutamate]). E, mean amplitude of aTCs in the absence (Con) or presence of TBOA, CNQX, and APV (TBOA/CNQX/APV). **, p < 0.01 compared with control period, paired t test.

no-5-phosphonovaleric acid (APV), 6-cyano-2,3-dihydroxy-7-nitroquinoxaline (CNQX), and di-threo-β-benzylxypaspartate (TBOA) were purchased from Tocris Cookson Ltd. (Ellisville, MO). Tetanus toxin (TeNT) was purchased from EMD Biosciences, Inc. (La Jolla, CA). Tetrodotoxin (TTX), BAPTA, MSO, Rose Bengal, and other chemicals were purchased from Sigma.

RESULTS

Local Application of Glutamate Induces SICs in CA1 Pyramidal Neurons—To study glutamate-induced astrocytic glutamate release, we locally applied glutamate in stratum radiatum of the CA1 area in hippocampal slices through a puffing pipette. Whole-cell recording in pyramidal neurons was performed to detect SICs that are iGluR-mediated currents activated by transient astrocytic glutamate release (7, 8, 26). Experiments were performed in the presence of TTX (1 μM) to block neuronal action potential (AP)-dependent synaptic release. Glutamate was puffed every 1–2 min into the region near apical dendrites of the recorded pyramidal neuron (Fig. 1B, p2). During a control period before placing puffing pipettes into slices, virtually no spontaneous SICs were observed, and after placing puffing pipettes, SICs were detected occasionally, suggesting little leakage of glutamate from puffing pipettes. Puffing glutamate (50 mM) directly evoked an inward current in CA1 pyramidal neurons (Fig. 1A, I_{puff}). After 3–5 puffs, spontaneous SICs began to appear (Fig. 1A). Puffing glutamate induced an increase in SICs in 64 out of 64 tested pyramidal neurons. SICs were blocked by the N-methyl-

D-glutamate receptor antagonist APV (50 μM) plus the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate/kainate receptor antagonist CNQX (20 μM) (Fig. 1A, APV/CNQX), confirming that glutamate-induced SICs are iGluR-mediated currents. The decay constant (mean ± S.D., 394 ± 21 ms, n = 101 events) and the amplitude (105 ± 19 pA) of spontaneous SICs were similar to SICs reported previously (7, 8, 26). When SICs occurred in pyramidal neurons, we always (64/64 neurons) observed, under DIC optics, an appearance of blebs around the puffing pipette tip (Fig. 1B, panel ii,>). Eight puffs of glutamate did not change neuronal resting membrane potential (RMP, −62.6 ± 1.0 mV, n = 10 cells) and ability to fire overshooting action potentials (Fig. 1C, V_{m}). SICs were not a result of high osmolarity of the glutamate solution, because a glutamate-free puff solution with similar osmolarity (ACSF added with 50 mM NaCl) did not induce SICs (Fig. 1D, ACSF + NaCl). Blebs and

nperoxidase labeling was visualized by incubating slices with chromogen (DAB-H₂O₂) in 1% OsO₄ for 1 h at room temperature. Slices were dehydrated and embedded in Epon 812. Finally, ultra-thin sections (70 nm) were cut and stained with uranyl acetate and lead citrate and then observed under a transmission electron microscope (HT 7100, Hitachi, Japan).

Local Application—Glass pipettes with resistances of 10–14 MΩ were used to rapidly pressure-eject (puff) glutamate or MSO dissolved in ACSF. Puffing pressure (3–5 p.s.i.) and puffing duration (100 ms) were controlled by a Picospritzer III (Parker Hannifin Co., Cleveland, OH), and intervals (60–120 s) were controlled by a Master-8 stimulator (A.M.P.I., Jerusalem, Israel). Puffing pipettes were placed in stratum radiatum of the CA1 area 50–80 μm below the slice surface. To control for osmotic effects, the same concentration of NaCl (50 mM) was added to ACSF as the osmolality-control puff solution.

Data Analysis and Chemicals—Data were analyzed using Clampfit 9.0 (Axon Instruments Inc.), Origin 6.0 (OriginLab Co., Northampton, MA), and CorelDraw 9.0 (Corel Co. Ontario, Canada) programs. Statistical data are presented as means ± S.E. unless otherwise indicated.

Fluo-4-AM, Fluo-4 potassium, FM 1-43, FM 4-64, and Alexa Fluor-594 were purchased from Molecular Probes. di-2-Ami-
SICs induced by puffing glutamate were reversible if five or less puffs had been applied. After removing the puffing pipette from the extracellular space, SICs and blebs gradually disappeared (Fig. 1, E and F, panels iii and iv), suggesting that glutamate-induced SICs and blebs are reversible if there are not too many applied puffs. When stopping puffs but keeping the puff pipette in slices, SICs continued to occur with low frequency, probably resulting from leaking of glutamate from the puff pipette. Repetitively puffing 5 mM [glutamate] induced only a few SICs (Fig. 2A), whereas 2 mM [glutamate] did not induce SICs (Fig. 2A, 2 mM Glut). The frequency of SICs was calculated from a 10-min recording period (SICs during which the \( I_{puff} \) were excluded), starting at the fifth puff, and was positively correlated with the concentration of glutamate in the puffing pipette (Fig. 2B, [glutamate], ○). The concentration of glutamate for the half-maximal frequency of SICs (EC\(_{50}\)) is 17 mM. The frequency of SICs before puffing 50 mM [glutamate] (Fig. 2B, 50 mM, ○) was higher than that before puffing 2 mM [glutamate] (Fig. 2B, 2 mM, ○, \( p < 0.05 \), Student’s \( t \) test). This higher base line was probably because of leaking of high [glutamate] from the puffing pipette.

In a previous study (26), we found that astrocytic glutamate release also activates a transient inward current in astrocytes (aTC), which synchronizes with neuronal SICs. Here we tested whether puffing glutamate also induces aTCs in astrocytes. Astrocytes near the puffing pipette were patched with the pipette solution without glutamate. As before, experiments were performed in the presence of TTX. Puffing 50 mM [glutamate] directly evoked an inward current (Fig. 2C, \( I_{puff} \)) and induced an increase in spontaneous aTCs in astrocytes (Fig. 2C, 2). The decay constant of aTCs (442 ± 93 ms, mean ± S.D., \( n = 60 \) events) was similar to the decay of SICs (394 ± 21 ms). In the passive type of astrocytes (30, 31), aTCs may be composed of glutamate transport currents and iGluR-mediated currents (32). After repeated puffing of glutamate and appearance of aTCs, perfusion of slices with the glutamate transporter inhibitor, TBOA (200 \( \mu \)M), the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate/kainate receptor antagonist CNQX (20 \( \mu \)M), and the N-methyl-D-aspartic acid receptor antagonist APV (50 \( \mu \)M) blocked aTCs (Fig. 2, C and E, panels ii and iv).

FIGURE 3. Glutamate induces large vesicles in astrocytes. A–E, EM pictures showing large vesicles (v) in glutamate-treated astrocytes. Slices were exposed to 50 mM [glutamate] and then fixed. Anti-GFAP antibody was used to label astrocytes. A, astrocyte; m, mitochondria. F, astrocytes from control slices showing small vesicles. Scale bars, 1 \( \mu \)m. G, left panel, percentage of astrocytes that showed vesicles in control (Con) and glutamate-treated slices (Glut). Middle panel, the mean diameter (Size) of vesicles in control (Con) or glutamate-treated astrocytes (Glut). \(* * * , p < 0.001 \) compared with control, Student’s \( t \) test. Right panel, relative frequency of vesicular diameters in control (dashed line, \( n = 259 \) vesicles) or glutamate-treated astrocytes (solid line, \( n = 201 \) vesicles). H, glutamate did not induce enlargement in apical dendrites of pyramidal neurons. Pyramidal neurons were loaded with Alexa Fluor-594 by electroporation. A puffing pipette containing glutamate (50 mM) was placed among labeled dendrites. Con, X-Y-Z projection of multiple dendrites before puffing glutamate. Puff, two X-Y scanning sections with 2 \( \mu \)m distance (panels i and ii) and X-Y-Z projection (panel iii) after puffing glutamate showing formation of Alexa Fluor-594-negative large vesicles (v in the circled area) without changes in apical dendrites of pyramidal neurons. Data are representative chosen from five experiments. J, glutamate induced swollen processes of GFAP-eGFP-labeled astrocytes. A puff pipette containing 50 mM glutamate was placed near a GFAP-eGFP-labeled astrocyte. Con, the astrocyte (Ast) before puffing. Puff, two scanning sections with 2 \( \mu \)m distance (panels i and ii) and X-Y-Z projection (panel iii) after five puffs of glutamate. Data are representative chosen from five experiments. Scale bars in H and I, 10 \( \mu \)m.

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FIGURE 4. Glutamate induces formation and collapse of large vesicles. A, FM 1-43 fluorescence images before (0 s) and after one (120 s) and five puffs (600 s), showing glutamate-induced FM 1-43-negative large vesicles (v) around the pipette tip (p). FM 1-43 stains with the high intensity of fluorescence appeared after puffing glutamate (600 s). B, puffing FM 1-43 alone did not induce FM 1-43-negative large vesicles and FM 1-43 stains. C, left images, in the presence of CNQX (20 μM) and APV (50 μM), no large vesicles appeared after five puffs of glutamate (600 s). When washing out CNQX/APV (Wash), puffing glutamate induced large vesicles. Right panel, mean number of large vesicles induced by five puffs of glutamate in the absence (Con) or presence of CNQX and APV (CNQX/APV). **, p < 0.01 compared with control, Student’s t test. D, two large vesicles (v1 and v2) were collapsing. A nearby vesicle (v3) was enlarging. An FM 1-43 stain appeared in the membrane of v1. Scale bars, 5 μm.

TBOA/CNQX/APV, supporting the conclusion that aTCs are glutamate-activated currents in astrocytes. Similar puffing of 2 mM glutamate did not induce aTCs (Fig. 2C, 2 mM Glut). Pooled data showed that the aTC-[glutamate]o relationship (Fig. 2D) was similar to the neuronal SIC-[glutamate]o dose-response curve (Fig. 2B; EC50 is 17 and 18 mM for SICs and aTCs, respectively). However, the mean amplitude of neuronal SICs (Fig. 2B, SIC Amp, □) was about four times the amplitude of aTCs (Fig. 2D, aTC Amp, □; 50 mM, p < 0.01, Student’s t test). These results indicate that ≥5 mM glutamate induces transient astrocytic glutamate release and that aTCs can be used as an indicator for astrocytic glutamate release.

Glutamate Induces Large Vesicles in Astrocytes—To understand the nature of glutamate-induced blebs, we used transmission EM to examine the ultrastructure of glutamate-induced blebs. Slices were transiently exposed five times to ACSF containing 50 mM glutamate for 5 s at intervals of 2 min. Astrocytes were identified by anti-glial fibrillary acid protein (GFAP) antibody staining. Large vesicles were observed in GFAP-positive astrocytes (Fig. 3, A–E, v) in glutamate-treated slices. Vesicles were identified by their single layer membrane without internal structures, different from mitochondria that were double layers of membrane with cristae (Fig. 3, A and B, m). Astrocytes in untreated control slices showed small vesicles (Fig. 3F).

Vesicles were present in 80% (20/25) of glutamate-treated astrocytes (Fig. 3G, left panel, Glut) and 58% (23/40) of control astrocytes (Fig. 3G, left panel, Con). The mean size (diameter) of vesicles in glutamate-treated astrocytes (Fig. 3G, Size, Glut) was significantly larger than in control astrocytes (Fig. 3G, Size, Con, p < 0.001, Student’s t test). Cumulative distribution curve of the vesicle diameter (Fig. 3G, right panel) indicated that glutamate treatment (Fig. 3G, right panel, solid line) increased the number of large vesicles in astrocytes. These results suggest that glutamate induces large vesicles in astrocytes.

Major cellular structures around the puffing pipette in CA1 stratum radiatum (Fig. 1B) are apical dendrites of pyramidal neurons, processes of astrocytes, interneurons, and Shaffer collateral fibers. To exclude the occurrence of large vesicles in apical dendrites of pyramidal neurons, we loaded multiple pyramidal neurons with Alexa Fluor-594 by extracellular electroporation through a pipette containing 5 mM Alexa Fluor-594 in the pyramidal layer (33). After five positive pulses of 15 V, 30-ms duration, stimuli at intervals of 2 s, multiple pyramidal neurons and their dendrites were loaded with Alexa Fluor-594 (Fig. 3H, Con). Whole-cell recording in Alexa Fluor-594-loaded neurons showed a normal range of resting membrane potentials (−56 to −62 mV, n = 4 neurons) and were able to fire action potentials. Glutamate (50 mM) was puffed through a puffing pipette placed among labeled dendrites. After 5–10 puffs, Alexa Fluor-594-negative large vesicles appeared (Fig. 3H, Puff, panels i, ii, and v). X-Y-Z projection images showed that dendrites of pyramidal neurons after puffing glutamate (Fig. 3H, Puff, panel iii) were similar to those before puffing (Fig. 3H, Con). No remarkable enlargement of dendrites was found during or after puffing, suggesting that glutamate does not induce the large vesicle in dendrites of pyramidal neurons.

To further confirm that glutamate-induced large vesicles originated from astrocytes, we used hippocampal slices prepared from GFAP-driven GFP-expressing mice (GFAP-eGFP). GFP fluorescence imaging showed that astrocytes were specifically labeled with GFP (Fig. 3I, Con, Ast) in these animals. Puffing glutamate (50 mM) into CA1 stratum radiatum induced discontinuous swollen astrocytic processes in a circled area (Fig. 3I, Puff, panels i–iii, circled area). The enlargement of astrocytic processes was probably because of the formation of large vesicles that occupied the internal space of astrocytic processes and forced internal contents, including GFAP, into the rest space, making processes swollen. Glutamate-induced morphological changes in the process of astrocytes further support that glutamate induces large vesicles in astrocytes.

Fusion of Large Vesicles Coincides with SICs—To test whether astrocytes release glutamate through fusion of large vesicles, we puffed glutamate (50 mM) together with FM 1-43 (20 μM). FM 1-43 has a hydrophilic head with two positive charges that limit its translocation through the bilayer membrane and a hydrophobic tail with a high affinity for lipid membranes (34, 35). This property allows FM 1-43 to bind and stay.
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on the nonpolar region (lipid tail) of the bilayer membrane only when the nonpolar region is exposed to FM 1-43. During vesicular fusion, the fused membrane (broken membrane) near the fusion pore exposes its nonpolar region to the extracellular space. Before puffing, two-photon imaging showed a very small diffusion volume of FM 1-43 fluorescence in the extracellular space was detectable but was washed out quickly. After puffing glutamate once, a few large vesicles were observed (Fig. 4A, 120 s). Repeated puffing glutamate induced more large vesicles around the puffing pipette (Fig. 4A, 400 s, v). An FM 1-43-negative large vesicle was identified as a round structure with low fluorescence that was surrounded by FM 1-43 fluorescence (Fig. 4A, 600 s, v). The FM 1-43 stain, a small structure with the high intensity of FM 1-43 fluorescence, appeared after repeated puffs (Fig. 4A, 600 s, ↑). As a control, in the presence of TTX, puffing glutamate-free ACSF with FM 1-43 induced neither FM 1-43-negative large vesicles nor FM 1-43 stains (Fig. 4B). Perfusion of slices with the iGluR antagonists, CNQX/APV, significantly reduced the number of glutamate-induced large vesicles (Fig. 4C, 600 s), suggesting that iGluRs are involved in the formation of large vesicles. Fig. 4D showed that two vesicles (v1 and v2) were collapsing, whereas a nearby vesicle (v3) was enlarging (supplemental movie 1). When vesicle 1 was collapsing, an FM 1-43 stain appeared in the vesicular membrane (Fig. 4D, v1 and arrow), indicating the formation of the fusion pore.

To demonstrate that fusion of large vesicles resulted in glutamate release, we examined temporal coincidence of vesicular fusion with SICs in pyramidal neurons. Fig. 5A showed that the collapse of a large vesicle (Fig. 5A, panels i–iv, v) was accompanied by a high intensity FM 1-43 stain in the membrane of the collapsing vesicle (Fig. 5A, panels ii–iv, arrow, and supplemental movie 2). Traces of whole-cell recording in the patched pyramidal neuron (Fig. 5B, Pyr) and FM 1-43 fluorescence in the large vesicle (Fig. 5B, FM) showed that the start of the increase in fluorescence coincided with a SIC (Fig. 5B, Pyr, SIC). Coincident SIC was defined as a SIC that occurred within 4 s (imaging sample interval) following the start of vesicular collapse. Fig. 5C shows another experiment in which eight large vesicles underwent collapse (v1–v8; supplemental movie 3). The start of collapse of all eight large vesicles coincided with SICs in the recorded pyramidal neuron (Fig. 5C, Pyr). The coincidence rate of fusion events with SICs in paired recordings (simultaneously recording FM 1-43 fluorescence and SICs) was 89% (49/55 fusion events), significantly different from unpaired recordings (SICs and FM fluorescence were separately recorded at different time, representing the random rate) (Fig. 5D, Coincidence, p < 0.01, χ² test), suggesting that fusion of large vesicles elicits SICs. The mean diameter of large vesicles measured before collapse was 3.8 ± 0.1 μm (Fig. 5D, Size, range 2–7 μm, n = 68). Above results suggest that glutamate induces formation of large vesicles that fuse with cytoplasmic membrane to exocytose glutamate into the extracellular space.

Inhibiting Glutamine Synthetase Induced Large Vesicles and SICs—To test whether endogenous astrocytic glutamate induces large vesicles and SICs, we used glutamine synthetase inhibitor, MSO, to block glutamate metabolism in astrocytes. In the central nervous system, glutamine synthetase is located in astrocytes (36). Blocking the glutamine synthetase by MSO...
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4-AP Induces Ca\(^{2+}\) and Alexa Fluor-594Containing Large Vesicles in Astrocytes—We have previously reported that bath application of the K\(^{+}\)-channel blocker, 4-AP, induces Ca\(^{2+}\) and Alexa Fluor-594-containing large vesicles in astrocytes (26). Here we describe more data on the appearance of Ca\(^{2+}\) and Alexa Fluor-594-containing vesicles in astrocytes induced by 4-AP. Simultaneous whole-cell recordings from pairs of pyramidal neurons and astrocytes showed that in the presence of 4-AP continuous perfusion leads to accumulation of glutamate in astrocytes. To balance the reduction in extracellular glutamate because of inhibition of astrocytic production of glutamate, 4 mM glutamine was added to the perfusion solution that contained 20 mM MSO. Experiments were first performed in the absence of TTX, and 5 mM QX-314 was added to the perfusion solution to block APs internally. Single application of MSO did not have direct effects on membrane currents and sEPSCs in pyramidal neurons (Fig. 6A, whole-cell). Repetitive puffing MSO slightly hyperpolarized neurons from \(-61.6 \pm 0.5\) to \(-63.5 \pm 0.6\) mV \((p < 0.01,\) paired t test, \(n = 10\) cells) but did not change neuronal firing (Fig. 6A, \(V_{m}\)). After 20 puffs of MSO (40 min), synaptic release remained functional (Fig. 6A, eEPSC), MSO significantly increased the frequency and amplitude of SICs (Fig. 6B and H, MSO). Imaging of FM 1-43 fluorescence showed that MSO also induced formation of large vesicles (Fig. 6C, 960 s, v). Fusion of large vesicles was observed and coincided with SICs (Fig. 6D, G and E). The coincidence rate of vesicular fusion with SICs was 82% (Fig. 6I, Coincidence, 18/22 fusion events), further supporting the idea that fusion of large vesicles causes SICs. Perfusion of slices with TTX before puffing MSO significantly reduced the number of MSO-induced large vesicles (Fig. 6F and I, TTX \(\rightarrow\) MSO) and SICs (Fig. 6G and H, TTX \(\rightarrow\) MSO), suggesting that AP-dependent synaptic release of glutamate significantly contributes to MSO-induced increase in endogenous [glutamate]. If first puffing MSO eight times to form large vesicles and then perfusing TTX, TTX did not significantly reduce the frequency and amplitude of SICs (Fig. 6H, MSO \(\rightarrow\) TTX). These results suggest that AP-dependent synaptic release is only involved in the formation of large vesicles and storage of glutamate in vesicles but does not directly activate SICs.
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black trace, aTC) that immediately followed the disappearance of the vesicle within the imaging sample interval (2 s), suggesting that the disappearing vesicle contained glutamate. Three-dimensional images of Ca\(^{2+}\) and Alexa Fluor-594 fluorescence from another representative cell showed that two large vesicles were in the process of the astrocyte before fusion (Fig. 7D, panel i; Fig. 7D, panel ii, ↑) and disappeared 5 min later (Fig. 7D, panel ii, ↑). All fusion events were examined by X-Y-Z scanning, and changes because of slice moving were excluded. The coincidence of vesicular fusion with aTCs was 75% (Fig. 7E, Coincidence). 25% of vesicular fusion did not coincide with aTCs, suggesting that a small number of large vesicles might contain low [glutamate] under these dye-loading conditions. The mean diameter of Alexa Fluor-594-loaded vesicles before fusion was 3.3 ± 0.3 μm (Fig. 7E, size), similar to the size of glutamate-induced large vesicles (Fig. 5D, size, 3.8 ± 0.1 μm).

Glutamate Induces Ca\(^{2+}\)-containing Large Vesicles in Astrocytes—Because Alexa Fluor-594-loaded large vesicles also contain Ca\(^{2+}\) (Fig. 7B, green), we pre-loaded astrocytes with Fluo-4-AM (6 μM) to examine whether glutamate induces astrocytic high [Ca\(^{2+}\)] large vesicles without patching astrocytes. Astrocytes in stratum radiatum were readily loaded by Fluo-4-AM (Fig. 8A, Ast), and FM-1-43 was used to detect morphology of large vesicles and to avoid fluorescence overlap.

Glutamate plus FM 4-64 (20 μM) was locally applied as described earlier. Puffing glutamate induced the formation of Ca\(^{2+}\)-containing vesicles (Figs. 8A, green, panel ii, ↑) and FM 4-64-negative vesicles (Figs. 8A, red, panel ii, ↑) large vesicles in astrocytes (Figs. 8A, Ast). Ca\(^{2+}\) levels in the vesicle decreased suddenly (Figs. 8, A and B, panel iii, green, ↑), whereas FM 4-64-negative morphology of the vesicle just began to collapse (Figs. 8, A and B, panel iii, red, ↑), indicating the occurrence of fusion. High [Ca\(^{2+}\)] vesicles outlined by FM 4-64 continued to collapse with the decay constant of 12.0 ± 2.3 s calculated by measuring changes in vesicular diameters (n = 15 vesicles), whereas Ca\(^{2+}\) levels fully dropped in 4 s (Fig. 8, A and B, panels ii and iii, green, ↑), suggesting that the diffusion of Ca\(^{2+}\) from large vesicles through the fusion pore is faster than changes in the vesicular morphology. The formation of a low [Ca\(^{2+}\)], FM 4-64-negative large vesicle was also observed (Fig. 8A, >). However, the fusion rate for low [Ca\(^{2+}\)] large vesicles was only 11% (Fig. 8C, open bar, 10 of 91 vesicles), significantly lower than high [Ca\(^{2+}\)] large vesicles (Fig. 8C, solid bar, 94%), 34 of 36 vesicles fused, p < 0.001, χ² test). These results suggest that high [Ca\(^{2+}\)] large vesicles play a major role in glutamate-induced astrocytic release of glutamate.

Ca\(^{2+}\) and SNARE-dependent Fusion of Large Vesicles—It has been reported previously that astrocytic Ca\(^{2+}\) signals play an important role in both astrocytic glutamate release and neuronal SICs (7, 8, 37, 38). To determine whether glutamate-induced SICs require astrocytic Ca\(^{2+}\) signals, we recorded glutamate-induced SICs and imaged astrocytic Fluor-594 fluorescence simultaneously. Astrocytes were pre-loaded with Fluo-4-AM, and SICs were induced by puffing glutamate (50 mM) into stratum radiatum in the presence of TTX. A few Ca\(^{2+}\) oscillations could be seen during the control period (Fig. 9A, right bar graph, Con), and puffing glutamate significantly increased the frequency of astrocytic spontaneous Ca\(^{2+}\) oscillations (Fig. 9A, right bar graph, Puff, p < 0.01, paired t test). Many astrocytes surrounding the puffing pipette directly responded to glutamate application with Ca\(^{2+}\) increases (Fig. 9, A and B, a1 and a2, arrow), and some cells also responded to glutamate with spontaneous Ca\(^{2+}\) oscillations (Fig. 9, A and B, a1–a4). In Fig. 9A, a total of 18 SICs (dotted lines and numbers 1–18) were induced in the recorded neuron during a 400-s recording period. SIC1–5, SIC7–11, and SIC15–18 coincided with Ca\(^{2+}\) oscillations in a1–a4 (Fig. 9A, 1–5, 7–11, and 15–18); SIC6 and

FIGURE 7. 4-AP induces high [Ca\(^{2+}\)] large vesicles in astrocytes. A, whole-cell recording from a pair of a pyramidal neuron (Pyr) and an astrocyte (Ast) showed that 4-AP induced SICs in pyramidal neurons and concurrent aTCs in astrocytes. B, fluorescence images of an astrocyte showing increased Alexa Fluor-594 (red) and Ca\(^{2+}\) (green) in a large vesicle (arrow). C, whole-cell recording (black) of an aTC (aTC) and imaging traces of Alexa Fluor-594 (red) and Ca\(^{2+}\) fluorescence (green) in the large vesicle in B. D, X-Y-Z scanning of two large vesicles (arrow) in another astrocyte before (panel i) and after fusion (panel ii). Alexa Fluor-594 and Ca\(^{2+}\) images were merged. E, left panel, the size of large vesicles (right panel), the coincidence rate of fusion events with aTCs (Coincidence). Scale bars in B and D, 10 μm.

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Astrocytic Ca\(^{2+}\) and 30.8% (16/52) of SICs were not related to astrocytic Ca\(^{2+}\). Passing through gap-junction channels may not be high enough to release glutamate that can be detected by BAPTA- chelator, BAPTA versus Control

62.0% (36/58) of Ca\(^{2+}\) increases in the observed field. On the other hand, the total number of SICs (58 increases) was larger than the total number of SICs (52). 62.0% (36/58) of Ca\(^{2+}\) increases were coincident with SICs, and 38% (22/58) of Ca\(^{2+}\) increases were not related to any SICs recorded in the pyramidal neuron. These results suggest that SICs are related to astrocytic Ca\(^{2+}\) increases.

We further examined whether fusion of large vesicles depended on cytoplasmic [Ca\(^{2+}\)], by adding the high affinity Ca\(^{2+}\) chelator, BAPTA (20 mM), together with Flu-4, Alexa Fluor-594, and 50 mM glutamate to the patch pipette filling solution for astrocytes. 4-AP-induced large vesicles were studied because local application of glutamate induces neighboring cells. In the presence of intracellular BAPTA, the appearance of Alexa Fluor-594-loaded vesicles was still induced by 4-AP in 7 of 11 cells (Fig. 9C), but their fusion was blocked (Fig. 9C, red, 1–3), and whole-cell recordings showed no aTCs (Fig. 9D, black trace). Fusion rate (Fig. 9E, fusion rate, BAPTA) and the mean amplitudes of aTCs (Fig. 9E, aTC Amp, BAPTA versus Control) were significantly reduced by infusion of BAPTA, suggesting that astrocytic Ca\(^{2+}\) is necessary for fusion of large vesicles with the plasma membrane and release of their contents, but not for vesicular formation and uptake of Alexa Fluor-594 into large vesicles. In the presence of BAPTA, Ca\(^{2+}\) fluorescence in large vesicles still increased during their formation (Fig. 9, C and D, green). However, the green/red ratio (Fig. 9E, green/red, BAPTA) was significantly reduced, compared with that in the absence of BAPTA (Fig. 9E, green/red, Con), suggesting that the intensity of Ca\(^{2+}\) fluorescence in large vesicles was attenuated by BAPTA (Fig. 9, C and D, green). The increase in vesicular [Ca\(^{2+}\)] in the presence of BAPTA suggests that, even though BAPTA buffered cytoplasmic free [Ca\(^{2+}\)], Ca\(^{2+}\) was still actively transported into large vesicles against [Ca\(^{2+}\)] gradient.

In a previous study (26), we have demonstrated that fusion of IP\(_{3}\)-induced large vesicles depends on SNARE proteins. Here we tested whether fusion of 4-AP-induced Ca\(^{2+}\), and Alexa Fluor-594-containing large vesicles also depends on SNARE proteins, by adding tetanus toxin (TeNT, 15 \(\mu\)g/ml) to the bath before patching astrocytes, to inhibit the diffusion of glutamate from the patched cell into neighboring coupled cells. In the presence of intracellular BAPTA, the appearance of Alexa Fluor-594-loaded vesicles was still induced by 4-AP in 7 of 11 cells (Fig. 9C), but their fusion was blocked (Fig. 9C, red, 1–3), and whole-cell recordings showed no aTCs (Fig. 9D, black trace). Fusion rate (Fig. 9E, fusion rate, BAPTA) and the mean amplitudes of aTCs (Fig. 9E, aTC Amp, BAPTA versus Control) were significantly reduced by infusion of BAPTA, suggesting that astrocytic Ca\(^{2+}\) is necessary for fusion of large vesicles with the plasma membrane and release of their contents, but not for vesicular formation and uptake of Alexa Fluor-594 into large vesicles. In the presence of BAPTA, Ca\(^{2+}\) fluorescence in large vesicles still increased during their formation (Fig. 9, C and D, green). However, the green/red ratio (Fig. 9E, green/red, BAPTA) was significantly reduced, compared with that in the absence of BAPTA (Fig. 9E, green/red, Con), suggesting that the intensity of Ca\(^{2+}\) fluorescence in large vesicles was attenuated by BAPTA (Fig. 9, C and D, green). The increase in vesicular [Ca\(^{2+}\)] in the presence of BAPTA suggests that, even though BAPTA buffered cytoplasmic free [Ca\(^{2+}\)], Ca\(^{2+}\) was still actively transported into large vesicles against [Ca\(^{2+}\)] gradient.

SIC12 coincided with puffing-induced Ca\(^{2+}\) increases (Fig. 9A, 6 and 12). Only SIC13 and SIC14 were not coincident with astrocytic Ca\(^{2+}\) increases in the observed field. 69.2% (36/52 from five experiments) of SICs occurred during Ca\(^{2+}\) increases, and 30.8% (16/52) of SICs were not related to astrocytic Ca\(^{2+}\) increases in the observed field. On the other hand, the total number of Ca\(^{2+}\) increases (58 increases) was larger than the total number of SICs (52). 62.0% (36/58) of Ca\(^{2+}\) increases were coincident with SICs, and 38% (22/58) of Ca\(^{2+}\) increases were not related to any SICs recorded in the pyramidal neuron. These results suggest that SICs are related to astrocytic Ca\(^{2+}\) increases.

We further examined whether fusion of large vesicles depended on cytoplasmic [Ca\(^{2+}\)], by adding the high affinity Ca\(^{2+}\) chelator, BAPTA (20 mM), together with Flu-4, Alexa Fluor-594, and 50 mM glutamate to the bath before patching astrocytes, to inhibit the diffusion of glutamate from the patched cell into neighboring coupled cells. In the presence of intracellular BAPTA, the appearance of Alexa Fluor-594-loaded vesicles was still induced by 4-AP in 7 of 11 cells (Fig. 9C), but their fusion was blocked (Fig. 9C, red, 1–3), and whole-cell recordings showed no aTCs (Fig. 9D, black trace). Fusion rate (Fig. 9E, fusion rate, BAPTA) and the mean amplitudes of aTCs (Fig. 9E, aTC Amp, BAPTA versus Control) were significantly reduced by infusion of BAPTA, suggesting that astrocytic Ca\(^{2+}\) is necessary for fusion of large vesicles with the plasma membrane and release of their contents, but not for vesicular formation and uptake of Alexa Fluor-594 into large vesicles. In the presence of BAPTA, Ca\(^{2+}\) fluorescence in large vesicles still increased during their formation (Fig. 9, C and D, green). However, the green/red ratio (Fig. 9E, green/red, BAPTA) was significantly reduced, compared with that in the absence of BAPTA (Fig. 9E, green/red, Con), suggesting that the intensity of Ca\(^{2+}\) fluorescence in large vesicles was attenuated by BAPTA (Fig. 9, C and D, green). The increase in vesicular [Ca\(^{2+}\)] in the presence of BAPTA suggests that, even though BAPTA buffered cytoplasmic free [Ca\(^{2+}\)], Ca\(^{2+}\) was still actively transported into large vesicles against [Ca\(^{2+}\)] gradient.

Glutamate Is Transported into Large Vesicles by vGluTs—To test whether glutamate is transported into large vesicles by vGluTs, we added the vGluT inhibitor Rose Bengal (0.5 \(\mu\)M) (13, 42) to the patch pipette filling solution. In the presence of Rose Bengal, accumulation of Alexa Fluor-594 into large vesicles was still observed in 4 of 7 cells. Ca\(^{2+}\) fluorescence in large vesicles was reduced by Rose Bengal by unknown mechanisms (Fig. 10, D, green, and F, green/red, RB). Formation (Fig. 10D, red, >) and fusion (Fig. 10D, red, ↑) of large vesicles were still observed,
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FIGURE 9. Fusion of large vesicles depends on astrocytic [Ca\(^{2+}\)]. A, glutamate-induced SICs coincided with astrocytic Ca\(^{2+}\) oscillations. Left traces, simultaneously recording of Ca\(^{2+}\) oscillations from four astrocytes indicated in B (a1–4) and SICs in a pyramidal neuron (Pyr) during application of glutamate (Glutamate). Slices were preincubated with Fluo-4 AM (6 μM) at 37 °C for 40 min. Glutamate (50 mM) was locally applied through puffing pipettes (upper line). Dotted lines (1–18) indicate the start time of SICs. Data are representative of five experiments. Right bar graph, the frequency of spontaneous Ca\(^{2+}\) oscillations per cell during control (Con) and puffing glutamate (Puff). **, p < 0.01, paired t test, n = 5 experiments. B, Ca\(^{2+}\) fluorescence images in astrocytes at times indicated in A (panels i–v). P2, position of the puffing pipette. Scale bar, 50 μm. C, fusion of high [Ca\(^{2+}\)] vesicles was blocked by infusion of BAPTA into astrocytes. Fluorescence images of an astrocyte showing that in the presence of BAPTA, Alexa Fluor-594 fluorescence increased in three large vesicles (1, 2, and 3), but no fusion occurred. Ca\(^{2+}\) fluorescence also increased to a lesser extent. Scale bar, 10 μm. D, Ca\(^{2+}\) (green) and Alexa Fluor-594 (red) fluorescence from three vesicles in C and no aTC in whole-cell recording (black). E, fusion rate, mean amplitude of aTCs (aTC Amp), and green/red ratio (green/red) in the absence (Con) or presence of BAPTA (BAPTA). **, p < 0.01 compared with control, Student’s t test.

and multiple large vesicles in the astrocyte in Fig. 10D disappeared after a 10-min scanning (Fig. 10D, red, 600 s), but they were no longer associated with aTCs (Fig. 10E, black trace). Pooled data showed that vesicular fusion in the presence of Rose Bengal (Fig. 10F, Fusion rate, RB) was not significantly different from control events (Fig. 10F, Fusion rate, Con, p > 0.10, χ\(^2\) test). However, both the coincidence rate of fusion events with aTCs (Fig. 10F, Coincidence, RB) and the amplitude of fusion-associated aTCs (Fig. 10F, aTC Amp, RB) in the presence of Rose Bengal were significantly reduced compared with controls (Fig. 10F, Coincidence and Amp, Con). These data suggest that glutamate is transported into large vesicles by Rose Bengal-sensitive vGluTs.

DISCUSSION

Extracellular [glutamate]\(_o\) plays an important role in mediating the interaction between neurons and astrocytes under both physiological or pathological conditions. Here we report that transient increases in local [glutamate]\(_o\) induce SICs in CA1 pyramidal neurons through glutamate uptake and storage in large glutamate-containing vesicles in astrocytes and fusion and release of their contents. SICs can be induced in a number of ways, including high frequency stimulation of Shaffer collateral fibers (8), perfusion with Mg\(^{2+}\)-free ACSF (7, 8), perfusion with the A-type K\(^{+}\) channel blocker 4-AP (26), mechanical stimulation of astrocytes (7, 38), perfusion with hypotonic solution (43), and glutamate transport inhibitors (7). These stimuli can all increase extracellular [glutamate]\(_o\), either by triggering neuronal release of glutamate or by inhibiting its uptake. Therefore, it is possible that glutamate-induced exocytosis of glutamate in this study could serve as a mechanism for induction of SICs by fiber stimulation, 4-AP, low Mg\(^{2+}\), and glutamate transporter inhibitors. However, it is also possible that SICs induced by mechanic stimulation, hypotonic solution, and other neurotransmitters might be mechanistically different.

Dose-response curves show that the frequency of SICs is positively correlated with [glutamate]\(_o\). Almost no SICs were recorded during application of 2 mM [glutamate]\(_o\), suggesting that a single synaptic vesicular release of glutamate (~2 mM in the synaptic cleft) (44–46) is probably not be sufficient to stimulate astrocytes to release glutamate. The low frequency of SICs evoked by 5–10 mM [glutamate]\(_o\), indicates that high levels of neuronal activity, such as that during stimulation of fibers at high frequency, may induce transient astrocytic release of glutamate with the low frequency. Synaptic vesicles contain high concentrations of glutamate (100–200 mM) (44, 47), and release of a single vesicle increases the glutamate concentration in the synaptic cleft to about 2 mM (44–46). Spatial and/or temporal summation of multivesicular releases (48, 49) can produce a transient peak of synaptic cleft [glutamate] higher than 5 mM that could induce persynaptic astrocytic processes to form large vesicles and release glutamate. In the absence of TTX, we occasionally observed spontaneous SICs during control periods (Fig. 6, B and C, Con), compared with no SICs in the presence of TTX (Fig. 6C, TTX), suggesting that synaptic release of glutamate can induce transient astrocytic glutamate release. The high frequency of SICs induced by [glutamate]\(_o\) > 20 mM probably only occurs under pathological conditions, such as epileptic seizures.

Local applications of 50 mM glutamate (eight puffs) did not change RMPs (~62.6 ± 1.0 mV) or overshooting action potentials of pyramidal neurons (Fig. 1C, V\(_{m0}\)), suggesting that pyramidal neurons can endure transient exposure to high glutamate. Whole-cell recording in astrocytes in the puffing area showed...
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Our results in this study demonstrated that extracellular glutamate can trigger astrocytes to exocytose glutamate through fusion of a large vesicle. Key pieces of evidence for this hypothesis include the following. 1) Local application of glutamate stimulated both the formation of large vesicles and the occurrence of SICs and aTCs. 2) EM studies showed that glutamate induced large vesicles in astrocytes. 3) Glutamate induced large vesicles in GFAP-eGFP-labeled astrocytic processes but not in apical dendrites of pyramidal neurons. 4) Concurrent vesicular collapse with the high intensity FM 1-43 stain on the membrane of collapsing vesicles indicated exocytotic fusion. 5) Fusion of large vesicles coincided with SICs. 6) Local application of the glutamine synthetase inhibitor, MSO, induced formation and fusion of large vesicles, and the latter coincides with SICs. 7) Glutamate induced the formation and fusion of high [Ca\(^{2+}\)] large vesicles in astrocytes. 8) Infusion of BAPTA or TeNT into astrocytes blocked both fusion of large vesicles and aTCs. 9) Infusion of vGlur inhibitor, Rose Bengal, inhibited aTCs but not formation and fusion of large vesicles. These data suggest that local increases in [glutamate] may induce formation of astrocytic glutamate-containing large vesicles that then fuse with the cytoplasmic membrane to exocytose glutamate. Our study also provides a useful method, using two-photon laser scanning microscopy and FM 1-43, to monitor the dynamic fusion process of large vesicles and formation of the fusion pore for research on vesicular release.

Because no large vesicles exist in deep layer astrocytes in control slices (Fig. 1, B and C, i), large vesicles were most likely incorporating from small undetectable vesicles, through enlargement and/or fusion during application of glutamate. Indeed, we observed large vesicles undergoing enlargement, which presumably requires addition of new membrane. The source of new membranes for the growth of vesicles may include fusion of multiple small vesicles (homotypic fusion) (50, 51), and/or incorporation of new membrane into large vesicles by fusing with other types of organelles (heterotypic fusion). In some cases, we did observe fusion between two large vesicles, but in many other cases we did not, even though vesicles did enlarge, suggesting the incorporation of small invisible organelles to large vesicles.

An astrocytic vesicle with a small diameter (∼30 nm) similar to synaptic vesicles was previously reported to exocytose glutamate (12, 17). However, the quantal of SICs is very large (30–700 pA). There is no evidence showing large pools of these small vesicles in astrocytes. Therefore, it is unlikely that SICs are because of synchronized fusion of a great number of these small vesicles. Thus, even though the small astrocytic vesicle releases glutamate, it may not cause SICs. On the other hand, the large astrocytic vesicle (2–7 μm) in this study explains well the large quantal of SICs.

In previous studies, IP3- or 4-AP-induced formation of high [Ca\(^{2+}\)] large vesicles requires high [glutamate] in astrocyte patch pipettes, which is not physiological. Here we show that the appearance of high [Ca\(^{2+}\)] large vesicles could also be induced by local extracellular application of either glutamate or MSO without patching astrocytes, suggesting that formation and fusion of high [Ca\(^{2+}\)] large vesicles are not because of artifacts of patching astrocytes with high intracellular [glutamate]. Moreover, we found that glutamate induced high [Ca\(^{2+}\)] and low [Ca\(^{2+}\)] large vesicles. Glutamate release is predominantly mediated by high [Ca\(^{2+}\)] vesicles.

Transmitter-induced astrocytic release of glutamate has been reported previously to depend on astrocytic Ca\(^{2+}\) signals (7, 8, 12, 13, 37, 38, 52). Here, the observation that fusion of...
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large vesicles was blocked by intracellular BAPTA supports that Ca\(^{2+}\) dependency of large vesicle fusion. The observation that high [Ca\(^{2+}\)] large vesicles are the major vesicle undergoing fusion and releasing glutamate implies that intravesicular Ca\(^{2+}\) may be involved in fusion of these large vesicles, as it is for homotypic fusion between yeast vacuoles (53, 54). By infusing the specific SNARE protein inhibitor, TeNT, into astrocytes, we demonstrated that fusion of high [Ca\(^{2+}\)] large vesicles also depends on astrocytic SNARE proteins. Therefore, glutamate-induced astrocytic release of glutamate is a Ca\(^{2+}\)- and SNARE-dependent process. By internal application of the vGluT inhibitor, Rose Bengal, into astrocytes, we found that Rose Bengal only blockedɑTCs but not formation and fusion of large vesicles (Fig. 10, D and E). These results suggest that transporting glutamate into large vesicles and formation/fusion of large vesicles are separately controlled. Vesicular glutamate transport is controlled by vGluTs, whereas fusion of large vesicles depends on Ca\(^{2+}\) and SNARE proteins; and formation of large vesicles may be controlled by glutamate receptors and other factors. Although we demonstrated that vGluTs were used to transport cytoplasmic glutamate into large vesicles, the driving force for vesicular transport of glutamate is still unknown. SICs induced by low Mg\(^{2+}\) (7) or hypotonic solution (43) have been reported to be insensitive to the V-type H\(^{+}\)/ATPase inhibitor, bafilomycin A, leading to a thought of the channel-mediated mechanism underlying SICs (43). However, large astrocytic vesicles may be different from synaptic vesicles in the driving force for transporting glutamate into vesicles. Large astrocytic vesicles have the Ca\(^{2+}\) pump (Fig. 9C) that can build up an electrical gradient across vesicular membrane for transporting glutamate into large vesicles. In supporting this possibility, in contrast to synaptic vesicles that are acridine orange-positive (55), all FM 1-43-negative large vesicles were acridine orange-negative, implying that large astrocytic vesicles are not acidic.

Our results suggest that glutamate is a key factor for stimulating astrocytes to form glutamate-containing large vesicles, fusion of which causes SICs. However, formation of glutamate-containing large vesicles requires [glutamate]\(_o\) higher than 5 mm. This high [glutamate]\(_o\) may occur in local neuronal circuits when neurons are close to overexcitation. Transient astrocytic release of glutamate by large vesicles may activate GABAergic synaptic inputs (9, 27) and serve as a negative feedback control to balance the overexcitation of local circuits. Recently, Fiacco et al. (43) have reported that stimulation of astrocytic Ca\(^{2+}\) signals by activating a G\(_q\)-coupled receptor that is specifically expressed in astrocytes does not induce SICs. There are two possible mechanisms underlying their observation. One is that formation of glutamate-containing large vesicles in astrocytes is a precondition for inducing SICs, and without stimulating formation of glutamate-containing large vesicles with high [glutamate]\(_o\), astrocytic Ca\(^{2+}\) signals alone cannot induce SICs. Another possibility is that SICs are not directly triggered by astrocytic cytoplasmic Ca\(^{2+}\). However, glutamate-induced astrocytic release of glutamate is a Ca\(^{2+}\)- and SNARE-dependent process.

In this study, we demonstrated a glutamate-stimulated transient astrocytic glutamate release that is through fusion of a glutamate-containing large vesicle. This glutamate-stimulated astrocytic release of glutamate is well controlled by extracellular [glutamate], and may serve as a negative feedback control of neuronal circuits by activating GABAergic synapses, but may, when the GABAergic inhibitory system is impaired, contribute to epileptic seizures.

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