Astrocytic Glutamate Release-Induced Transient Depolarization and Epileptiform Discharges in Hippocampal CA1 Pyramidal Neurons

Ning Kang, Jun Xu, Qiwu Xu, Maiken Nedergaard, and Jian Kang

Department of Cell Biology and Anatomy, New York Medical College, Valhalla; and Center for Aging and Developmental Biology, Rochester University Medical Center, Rochester, New York

Submitted 2 May 2005; accepted in final form 25 August 2005

N. Kang, J. Xu, Q. Xu, M. Nedergaard, and J. Kang. Astrocytic glutamate release-induced transient depolarization and epileptiform discharges in hippocampal CA1 pyramidal neurons. J Neurophysiol 94: 4121–4130, 2005; doi:10.1152/jn.00448.2005. A paroxysmal depolarization shift (PDS) has been suggested to be a hallmark for epileptic activity in partial-onset seizures. By monitoring membrane potentials and currents in pairs of pyramidal neurons and astrocytes with dual patch-clamp recording and exocytosis of vesicles from astrocytes with two-photon laser scanning microscopy in hippocampal slices, we found that infusion of inositol 1,4,5-trisphosphate (IP₃) into astrocytes with patch pipettes induced astrocytic glutamate release that triggered a transient depolarization (TD) and epileptiform discharges in CA1 pyramidal neurons. The TD is due to a tetrodotoxin (TTX)-insensitive slowly decaying transient inward current (STC). Astrocytic glutamate release simultaneously triggers both the STC in pyramidal neurons and a transport current (TC) in astrocytes. The neuronal STC is mediated by ionotropic glutamate receptors leading to the TD and epileptiform discharges; while the astrocytic TC is a glutamate reuptake current resulting from transporting released glutamate into the patched astrocyte. Fusion of a large vesicle in astrocytes was immediately followed by an astrocytic TC, suggesting that the fused vesicle contains glutamate. Both fusion of large vesicles and astrocytic TCs were blocked by tetanus toxin (TeNT), suggesting that astrocytic glutamate release is via SNARE-dependent exocytosis of glutamate-containing vesicles. In the presence of TTX, the epileptogenic agent, 4-AP, also induced similar neuronal STCs and astrocytic TCs in the absence of action potential (AP)-driven synaptic activities.

METHODS

Slice preparation

Brain slices were prepared as described previously (Kang et al. 1998). Briefly, 14- to 20-day-old (P14-P20) Sprague-Dawley rats were anesthetized with pentobarbitone sodium (55 mg/kg) and decapitated. Brains were removed rapidly and glued with the posterior border of the corpus callosum and the olfactory bulb removed. The brain was cut into two hemispheres and a 350-µm thick slice containing the hippocampus was cut with a vibratome (Sturk, St Louis, MO) in a cutting solution containing (mM): 2.5 KCl, 1.25 NaH₂PO₄, 10 MgSO₄, 0.5 CaCl₂, 10 glucose, 26 NaHCO₃, and 230 sucrose. Slices were incubated on a gravity perfusion chamber to allow 40 min for the solution to equilibrate to room temperature (23–24°C) before recording. The standard slice solution contained (mM): 126 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 2 MgCl₂, 2 CaCl₂, 10 glucose, and 26 NaHCO₃ (pH 7.4 when gassed with 95% O₂-5% CO₂).

Whole cell patch-clamp recording

Cells were visualized with a 63× water immersion lens on an Olympus BX51 upright microscope (Olympus Optical Co., NY) equipped with differential interference contrast (DIC) optics. Patch electrodes with a resistance of 4–7 MΩ were pulled from KG-33 glass capillaries (inner diameter 1.0 mm, outer diameter 1.5 mm, Garner Scientific, Oxnard, CA).}

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: J. Kang, Dept. of Cell Biology and Anatomy, New York Medical College, Basic Science Bldg., Rm. 220, Valhalla, NY 10595.
Glass, Claremont, CA) using a P-97 electrode puller (Sutter Instrument, Novato, CA). Pyramidal neurons in the CA1 pyramidal layer and astrocytes in the stratum radiatum were identified by their DIC morphology and electrophysiological properties as described previously (Kang et al. 1998). The whole cell voltage-clamp configuration was used in astrocytes, and the whole cell voltage-clamp and current-clamp configurations (Hamill et al. 1981) were alternatively performed in pyramidal neurons. Pyramidal neurons with the seal resistance < 5 GΩ and a holding current > −200 pA were rejected. The pipette solution for neuronal whole cell recordings contained (in mM): 123 K-glucolate, 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 astrocyte whole cell recordings contained (in mM): 20 or 50 mM K-glutamate (L), 113, 103, or 73 K-glucolate, 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). Astrocytes were recorded with a holding potential of −80 mV. The Ca²⁺-free slice solution contained (in mM): 126 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 2 MgCl₂, 10 glucose and 26 NaHCO₃ (pH at 7.4 when gassed with 95% O₂, 5% CO₂). Signals were amplified by an Axoclamp 2A with low-pass filter of 2 kHz cut-off frequency and sampled with 200 ms intervals.

Field recording

A glass pipette with a tip of 10−15 μm diameter was placed in the CA1 pyramidal layer 30−40 μm from the patched astrocyte in the stratum radiatum as the electrode for field recording. The pipette filling solution for field recording electrode was the standard slice solution. Signals were amplified by an Axoclamp 2A with low-pass of 2 kHz cut-off and sampled with 200 ms intervals.

Calcium image

A customized 2-photon laser scanning Olympus BX61WI microscope with a 60x objective lens was used to detect astrocytic Ca²⁺ signals. A Mai/Tai™ laser (the solid-state laser company, Mountain America, Melville, NY) was used to excite the specimen. In the transfluorescence pathway, a 565 nm diode laser was used to excite the specimen. HQ525/50 and HQ605/50 filters were placed in the “green” and “red” dichroic mirror was used to separate green and red fluorescence. A Mai/Tai™ laser (the solid-state laser company, Mountain America, Melville, NY). In the transfluorescence pathway, a 565 nm diode laser was used to excite the specimen. HQ525/50 and HQ605/50 filters were placed in the “green” and “red” dichroic mirror was used to separate green and red fluorescence. The Ca²⁺ signals were collected and averaged from the specified structures to obtain F(t). Baseline fluorescence (F₀) was the average of four images during control and F/F was calculated as (F/F(t))/F₀ = (F(t)−F₀)/F₀. The position shift in the X-Y section during 5 min scanning was 0.5 ± 0.3 μm (mean ± SD, 30 cells, by measuring the movement of somatic edges indicated by Alexa Fluor-594).

DATA ANALYSIS

Recoding signals were filtered through an 8-pole Bessel low-pass filter with a 2 kHz cut-off frequency and sampled by a PCLAMP 8.2 program (Axon Instruments) with an interval of 50 μs or 200 μs. Data were further analyzed by Clampfit 8.2 program (Axon Instruments). Analyzed data were further processed with Origin 5.1 (Microcal Software, Northampton, MA) and CorelDraw 9.0 (Corel Ontario, Canada) programs. Statistical data are presented as means ± SE if not indicated.

RESULTS

Astrocytic IP₃ induces the TD and epileptiform discharges in pyramidal neurons

To test whether astrocytic glutamate release induces epileptiform discharges, we applied IP₃ (2 μM) into astrocytes through whole cell patch pipettes when dual patch-clamp recording was performed in pairs of pyramidal neurons and astrocytes. Identification of pyramidal neurons and astrocytes in the CA1 area of the hippocampus in slices was described in our previous study (Kang et al. 1998). A pyramidal neuron was patched in an alteration of the whole cell voltage-clamp configuration with a holding potential of −60 mV and the current-clamp configuration with a membrane potential of −60 mV (controlled by injecting currents). Current recordings showed spontaneous excitatory postsynaptic currents (sEPSCs) (τ = 15.3 ± 0.9 ms) in the voltage-clamp (Fig. 1A, Con) and spontaneous excitatory postsynaptic potentials (sEPSPs) in the

![FIG. 1. Astrocytic IP₃ induces neuronal STCs and astrocytic TCs. A: whole cell voltage-clamp recording from a representative pyramidal neuron (Pyr) before (Con) and 10 min after patching the astrocyte (IP₃). The pipette solution for the astrocyte contained 2 μM IP₃ and 20 mM K-glutamate. Recordings from the pyramidal neuron (Pyr) and the astrocyte (Ast) showed neuronal STCs and astrocytic TCs. The traces with enlarged time scale from the indicated periods showed that large STCs (STC) triggered action potential sodium currents (INa). B: mean frequency (Freq) and amplitude (Amp) of neuronal STCs before (Con) and after patching astrocytes with the IP₃ glutamate pipette solution (IP₃), after patching astrocytes with the glutamate pipette solution without IP₃ (IP₃−, n = 20 pairs), and with the IP₃− pipette solution without glutamate (Glut−, n = 10 pairs). ***, P < 0.001, compared with control, paired t-test, and **, P < 0.01, compared with the IP₃− glutamate pipette solution (IP₃−). C: mean frequency (Freq) and amplitude (Amp) of astrocytic TCs obtained with the IP₃ glutamate pipette solution (Glut) or with the IP₃ pipette solution without glutamate (Glut−).](/jn.physiology.org/lookup/doi/10.1152/jn.00216.2005/-/media/jn/jn002162005/jn002162005.jpg)
current-clamp configuration. An astrocyte in the stratum radiatum near the apical dendrite of the recorded pyramidal neuron was patched with the pipette solution containing 2 µM IP$_3$, and 20 mM L-glutamate. Five to ten minutes after forming the whole cell configuration in the patched astrocytes, voltage-clamp recording in the pyramidal neuron showed a slowly decaying inward current (STC) with a decay constant of 454 ± 34 ms (Fig. 1A, STC). The STC was observed in 15 of total 24 tested pairs. Placing the patch pipette on astrocytes without suction for 20 min did not induce STCs in pyramidal neurons (n = 5 cells), indicating that the STC is not due to diffusion of IP$_3$ or glutamate into the extracellular space. The traces with enlarged time scale showed that large STCs caused multiple voltage-dependent sodium currents (Fig. 1A, $I_{Na}$) even though the cell soma was held at −60 mV, suggesting that STCs significantly depolarize cells to activate voltage-gated sodium channels due to the limitation of spatial clamping in slices. Patching astrocytes with the patch pipette solution containing 20 mM glutamate without IP$_3$ did not induce any STCs (Fig. 1B, $I_{Na}$, n = 20 cells), suggesting that the STC is due to the diffusion of IP$_3$ into the patched astrocytes. Patching astrocytes with the patch pipette solution containing 2 µM IP$_3$ without glutamate induced STCs with a significantly smaller amplitude (Fig. 1B, Glut$, P < 0.01$, compared with 20 mM glutamate, t-test), suggesting that intracellular glutamate concentrations in patched astrocytes are important for the amplitude of neuronal STCs. The frequency of astrocytic IP$_3$-induced STCs was 1.62 ± 0.35/min and the mean amplitude (baseline to negative peak) was 145 ± 38 pA (Fig. 1B, IP$_3$, n = 15 cells). In 7 of 15 pairs, we found that an inward current occurred in the patched astrocyte simultaneously with the STC in the patched pyramidal neuron (Fig. 1A, Ast). We term this astrocytic inward current as astrocytic TC because it is a transport current in the patched astrocyte (Fig. 4). Astrocytic IP$_3$-induced neuronal STCs occurred in 15 of 24 pairs that is more than double of the occurrence of astrocytic TCs (7/24), suggesting that neuronal STCs are not only triggered by glutamate release from the patched astrocyte, but also triggered by glutamate release from the gap-junction coupled astrocytes because intracellular applied IP$_3$ could pass through gap-junction channels (Boitano et al. 1992; Christ et al. 1994) to stimulate glutamate release from coupled cells. Recording with the glutamate-free pipette solution obtained almost none of astrocytic TCs (Fig. 1C, Glut$^-$), further suggesting that intracellular glutamate is important for astrocytic glutamate release. The mechanisms underlying this intracellular glutamate-dependency of astrocytic glutamate release are not known yet. However, fusion events of large vesicles (Fig. 5) could still be observed in the absence of intracellular glutamate when using two-photon laser scanning microscopy to monitor Alexa Fluor-594 and Fluo-4 fluorescence (data not shown), suggesting that vesicular fusion might not be affected by low intracellular glutamate, but vesicular glutamate concentrations are influenced by cytoplasmic glutamate concentrations. The results also imply that major astrocytic TCs are due to glutamate release from the patched astrocyte, which was abolished in the absence of intracellular glutamate (Fig. 1C). Whereas attenuated amplitude of neuronal STCs observed in the absence of astrocytic intracellular glutamate (Fig. 1B) might result from glutamate release predominantly from astrocytes that were coupled to the patched astrocyte.

When the recording mode in pyramidal neurons was changed from the voltage-clamp to the current-clamp configuration, the TD and spontaneous spikes occurred (Fig. 2A and B, TD). During control, spontaneous spikes were very few (Fig. 2A, Con). The traces with enlarged time scale showed that spontaneous firing occurred at the top of each TD (Fig. 2B, arrows) and during the inter-TD period (Fig. 2A and B, arrowheads). The mean frequency of spontaneous spikes significantly increased (Fig. 2D, IP$_3$, $P < 0.01$, paired t-test, n = 7 cells). Each TD was followed by an afterhyperpolarization (Fig. 2B, AHP), which is similar to the PDS found in the epilepsy animal models (Johnston and Brown 1984). To study the synchronized neuronal activities induced by astrocytic intracellular IP$_3$, we simultaneously performed field recording with an extracellular electrode placed at the pyramidal layer and patch-clamp recording in one astrocyte in the stratum radiatum with the IP$_3$ pipette solution. Intracellular IP$_3$-induced astrocytic TCs (Fig. 2D, Ast) were accompanied by a negative shift in field potential in 8/14 experiments (Fig. 2D, Field). Neither astrocytic TCs nor negative shifts in field potential were blocked by TTX (Fig. 2D, F, TTX), suggesting that both astrocytic TCs and negative shifts in field potential are not induced by AP-driven synaptic activity. Moreover, the time course of negative shifts in field potential was similar to that of astrocytic TCs, and no following field potential fluctuation was observed (Fig. 2D). The results suggest that although astrocytic glutamate release leads to a synchronized NMDA/AMPA-mediated depolarization in a group of pyramidal neurons, epileptic seizures may not occur under physiological conditions due to the inhibition system of neuronal circuits. If inhibition of circuits is impaired under some pathological conditions, astrocytic glutamate release may initiate epileptic seizures.

Astrocytic glutamate release has been reported to cause slow inward currents in cultured neurons (Araque et al. 2000) and in thalamic neurons (Parri et al. 2001) and hippocampal pyramidal neurons in slices (Angulo et al. 2004; Fellin et al. 2004). To test whether AP-driven synaptic activities are involved in astrocytic IP$_3$-induced STCs, we used TTX (1 µM) to perfuse slices. TTX altered neither the frequency nor the amplitude of neuronal STCs (Fig. 3, A-C, TTX), suggesting that STCs are due to direct activation of neuronal postsynaptic receptors by astrocytic release of neurotransmitters. To test whether STCs are mediated by ionotropic glutamate receptors, we used the NMDA receptor antagonist APV (50 µM) and AMPA/Kainate receptor antagonist CNQX (20 µM). Neuronal STCs were fully blocked by APV and CNQX (Fig. 3, A-C, APV/CNQX, $P < 0.001$, paired t-test, n = 7 pairs), suggesting that astrocytic IP$_3$-induced STCs are due to the activation of ionotropic glutamate receptors by astrocytic glutamate release.

Spontaneous spikes occurred during the inter-TD period (Fig. 2A and B, arrowheads) that may be due to increased sEPSCs. Therefore we examined astrocytic IP$_3$-induced changes in sEPSCs. Spontaneous EPSCs increased after patching astrocytes with the IP$_3$ pipette solution (Fig. 3D). The amplitude distribution of sEPSCs indicated that astrocytic IP$_3$ induced an increase in the frequency of large sEPSCs (Fig. 3E). Both the mean frequency and amplitude of sEPSCs were increased by astrocytic IP$_3$ (Fig. 3F, IP$_3$, $P < 0.05$ for both, paired t-test, n = 7 cells). The increased sEPSCs and basal sEPSCs were blocked by APV/CNQX (Fig. 3, D and F, APV/CNQX, $P < 0.001$, paired t-test, n = 7 pairs), suggesting that astrocytic IP$_3$-induced STCs are due to the activation of ionotropic glutamate receptors by astrocytic glutamate release.
APV/CNQX), indicating that the increased sEPSCs are ionotropic glutamate receptor-mediated events. The results are in accordance with previous observations by other groups (Fiacco and McCarthy, 2004).

The recorded astrocytic inward current could be a Glut/H efflux current through channels (Basarsky et al. 1999; Duan et al. 2003; Kimelberg 2004; Ye et al. 2003), a glutamate transport current that is caused by transporting two Na⁺, one H⁺ and one Glu⁻ in and one K⁺ out (Diamond et al. 1998), or an astrocytic AMPA receptor-mediated current in response to astrocytic glutamate release (Clasen et al. 1995; Meucci et al. 1996; Seifert et al. 1997; Shelton and McCarthy 1999). To characterize astrocytic inward currents, we analyzed the kinetics of IP₃-induced astrocytic TCs and neuronal STCs. Neuronal STCs showed a large variation in their rising time (Fig. 4A, Pyr, 1–5), while the rising time for astrocytic TCs showed a linear relationship with their amplitude (Fig. 4A, Ast). The distribution for rising slopes illustrated a relative constant rising slope for astrocytic TCs (Fig. 4B, top left), but a broad range of the rising slope for neuronal STCs (Fig. 4B, left bottom). The half-width of astrocytic TCs was linearly correlated with the amplitude (Fig. 4B, W₁/₂, closed circle, solid line, r = 0.91, P < 0.0001) compared with more dispersed distribution of neuronal STCs (Fig. 4B, W₁/₂, open circle, dashed line, r = 0.37, P < 0.05). The homogeneity of the rising slope and the linear relationship between the half-width and amplitude of astrocytic TCs are in accordance with the linear relationship between the transport time and the quantity of released glutamate (Marie and Attwell 1999), implying that they are transport currents. Neuronal STCs are influenced by kinetics of NMDA/AMPA receptors and thereby exhibited more dispersed distributions for their rising slope and half-width. To further identify astrocytic TCs, we perfused slices with the specific glutamate transport inhibitor, d,l-threo-beta-benzyloxyaspartate (TBOA, 250 μM). TBOA attenuated

**FIG. 2.** Astrocytic IP₃ induces the TD and epileptiform discharges. A: current-clamp recording from the same pyramidal neuron in A. TDs and spontaneous firing occurred after patching the astrocyte with the IP₃ pipette solution. B: traces with enlarged time scale from the indicated period. Spontaneously firing occurred on the top of each TD. C: mean frequency of spontaneous spikes (Freq of sAP) before (Con) and after patching astrocytes (IP₃). ***, P < 0.01 compared with control, n = 7 cells. D: whole cell recording from an astrocyte (Ast) and field recording (Field) in the absence (Con) and presence of TTX. E: time course of astrocytic TCs (closed circle) and negative shifts in field potential (open circle). F: mean amplitude of astrocytic TCs (left panel) and negative shifts in field potential (right panel) in the absence (open bar) and presence of TTX (solid bar).
the amplitude and broadened the width of astrocytic TCs (Fig. 4C, IP$_3$/TBOA). Pooled data showed that TBOA significantly reduced the amplitude of astrocytic TCs (Fig. 4D, Amp, P < 0.01, paired t-test, n = 35 TCs from 5 cells) and increased the half-width of astrocytic TCs (Fig. 4D, W$_{1/2}$, P < 0.001). The results suggest that astrocytic TCs are transport currents. Although TBOA attenuated astrocytic TCs, it did not inhibit neuronal STCs. On the contrary, we observed an increase in the frequency of neuronal STCs in the presence of TBOA (data not shown), which is similar to the observation previously reported by other groups (Angulo et al. 2004). Since glutamate AMPA receptors have been reported to mediate an inward current in some astrocytes (Clasen et al. 1995; Shelton and McCarthy 1999), we next tested whether the astrocytic TC is blocked by the AMPA receptor blocker NBQX (20 μM). The mean amplitude of astrocytic TCs was not significantly decreased by NBQX (Fig. 4D, NBQX, P = 0.85, paired t-test, n = 5 cells), suggesting that the AMPA receptor was not involved in astrocytic TCs. To exclude anion channel-mediated Glu$^-$ efflux in astrocytic TCs, we used the anion channel inhibitor, NPPB (5-nitro-2-(3-phenylpropylamino)-benzoic acid) to perfuse slices. NPPB (100 μM) did not significantly reduce the amplitude of astrocytic TCs (Fig. 4E, P = 0.56, n = 5 cells), suggesting that the astrocytic TC is not due to anion channel-mediated glutamate efflux.

**Fusion of a large vesicle precedes the astrocytic TC**

To further demonstrate the astrocytic origin of glutamate release for neuronal STCs and astrocytic TCs, we performed whole cell recording in astrocytes with the pipette solution containing Ca$^{2+}$ indicator Fluor-594 (100 μM), structure dye Alexa Fluor-594 (100 μM), glutamate (50 μM), and IP$_3$ (2 μM). Fifty millimolar glutamate was used in the pipette solution to obtain more remarked astrocytic TCs because the amplitude of sEPSCs increased with its amplitude, but some neuronal STCs, respectively.

**FIG. 4. Astrocytic inward current is a transport current.** A: representative traces showing that the rising time and the width of astrocytic TCs (Ast) increased with its amplitude, but some neuronal STCs (Pyr) had a large amplitude with a narrow width (2 and 3). B: left panel, distribution for the rising slope of astrocytic TCs (top) or neuronal STCs (bottom) panel. Right panel, the half-width (W$_{1/2}$) of astrocytic TCs (closed circle) or neuronal STCs (open circle) was plotted against its amplitude. Solid and dashed lines, linear regression for astrocytic TCs and neuronal STCs, respectively. C: top, sample traces of IP$_3$-induced astrocytic TCs in the absence (Ast IP$_3$) or presence of 250 μM TBOA (IP$_3$/TBOA). D: mean amplitude (Amp) and decay (r) of astrocytic TCs in the absence (open bars) or presence of TBOA (solid bars), ***, and ***, P < 0.05 and 0.001, respectively, paired t-test, n = 35 TCs from 5 cells. E: mean amplitude of astrocytic TCs in the absence (IP$_3$) or presence of NBQX (NBQX, 20 μM). n = 5 cells. F: mean amplitude of astrocytic TCs in the absence (IP$_3$) or presence of NBQX (NBQX, 20 μM), n = 5 cells.
in pancreatic β cells (Mitchell et al. 2001) and catecholamine storage vesicles in rat pheochromocytoma PC12 cells (Mahapatra et al. 2004), our finding that vesicles can be loaded by membrane-impermeable Fluo-4 (potassium salt) and structure dye Fluo-594 (potassium salt) is novel. This finding allows us to monitor the dynamics of vesicles including priming and fusion. By combining whole cell recording of astrocytic TCs that indicate glutamate release into the extracellular space, we can test whether a quick drop of fluorescence is immediately followed by an astrocytic TC, which suggests that the fused vesicle most likely contains glutamate. Intracellular application of IP3 induced an increase in the intensity of Ca2+ (Fig. 5A, green, arrow) and Alexa Fluor-594 (Fig. 5A, red, arrow) fluorescence in a large vesicle. The size of the vesicle was enlarging with time (Fig. 5A, 2–4). Ca2+ and structure dye fluorescence suddenly decreased (Fig. 5A, 4–5 and C, arrowhead), indicating the occurrence of vesicular fusion (supplementary movie 1 and 2).1 Fusion is defined as a sudden decrease (>0.5 DF/F) in Alexa Fluor-594 fluorescence within 2 s (the imaging sample interval). Whole cell recording (Fig. 5C, black) showed an astrocytic TC (Fig. 5C, TC) immediately following the fusion event of the large vesicle (Fig. 5C, arrowhead). The intensity of Ca2+ and Alexa Fluor-594 fluorescence in the large vesicle increased before fusion (Fig. 5C, Ca2+ and Alexa) implies a priming process. Pooled data indicate the coincidence of vesicular fusion (3/4 fused vesicles) with astrocytic TCs (Fig. 5D). The results suggest that astrocytic exocytosis of a large glutamate-containing vesicle induces the neuronal STC and astrocytic TC. To further demonstrate astrocytic TCs are due to vesicular release, we added the specific SNARE protein inhibitor, tetanus toxin (TeNT, 15 mg/ml), to the patch pipette filling solution. In the presence of TeNT, eight large vesicles were observed in 4 of 8 cells (Fig. 5F, % cells). Alexa Fluor-594 fluorescence accumulated in large vesicles (Fig. 5E, arrowheads), but no vesicular fusion was observed during recording (Fig. 5F, Fusion, TeNT). Whole cell recording showed no astrocytic TCs in TeNT-infused astrocytes, and both the mean frequency and amplitude of TCs were significantly reduced (Fig. 5F, Freq of TCs and Amp of TCs). The results support that SNARE-dependent astrocytic exocytosis causes astrocytic TCs and neuronal STCs.

**Epileptogenic reagent, 4-AP, induces the STC**

4-aminopyridine (4-AP) is an A-type K+ channel blocker, and 50 or 100 μM 4-AP induces epileptiform discharges that are used as an model of epileptic seizures (Colom and Saggau 1994; Rutecki et al. 1987). Effects of 4-AP include increased spontaneous spikes and long-lasting depolarization (Johnston and Brown 1986; Wheeler et al. 1996) that are related to activation of glutamatergic and GABAergic receptors (Konerth et al. 1986; Perreault and Avoli 1992; Rutecki et al. 1987; Scanziani et al. 1994; Taylor and Dudek 1982). To study roles of astrocytic glutamate release in epileptiform discharges in slice models of epileptic seizures, we tested the occurrence of neuronal STCs and astrocytic TCs during perfusion of slices with 4-AP (100 μM). To eliminate effects of 4-AP on neuronal synaptic activity, TTX (1 μM) was used to block AP-driven synaptic activity. Dual whole cell recording was performed in pairs of pyramidal neurons and astrocytes. The pipette solution for astrocytes contained 20 mM K-glutamate. During control, very few neuronal STCs could be observed (Fig. 6A and B, Con), and perfusion of slices with 4-AP increased the frequency of neuronal STCs (Fig. 6A and B, 4-AP). 4-AP induced STCs in pyramidal neurons in 15 of 28 pairs, in which 9 pairs showed simultaneous neuronal STCs and astrocytic TCs (Fig. 6A). The frequency of 4-AP-induced STCs (Fig. 6B, 4-AP; range: 0.05 to 6 events/min) is significantly different from that during control (P < 0.01, paired t-test). Similar to astrocytic IP3-induced neuronal STCs, 4-AP-induced neuronal STCs were also blocked by APV/CNQX (Fig. 6B, APV/CNQX). The rising time (Fig. 6C, Rise) and decay (Fig. 6C, t) of 4-AP-induced astrocytic TCs (Fig. 6C, solid bars) are similar to those of 4-AP-induced astrocytic TCs (Fig. 6C, open bars), suggesting the same nature of 4-AP- and IP3-induced astrocytic TCs.

It has been previously reported that astrocytic Ca2+ signaling plays a key role in astrocytic glutamate release and neuronal STCs (Angulo et al. 2004; Bezzi et al. 2004; Fellin and Carmignoto 2004; Fellin et al. 2004; Montana et al. 2004; Parpura and Hayden 2000; Parri et al. 2001). We examined whether 4-AP-induced STCs are coincident with astrocytic Ca2+ signals and vesicular fusion. We first performed whole cell recording in pyramidal neurons in slices that were preloaded with fluorescent dye Fluo-4 AM (5 μM). Astrocytic Ca2+ signals were detected by a two-photon laser scanning microscopy. Ca2+ oscillations occurred spontaneously during control conditions even in the presence of TTX (Fig. 7A, TTX), which is in accordance with previous observations by other groups (Nett et al. 2002). Perfusion of slices with 4-AP induced an increase in the frequency of Ca2+ oscillations (Fig. 7A, 4-AP/TTX, and 7B, TTX: 0.07 ± 0.32, 4-AP: 4.39 ± 0.84, P < 0.05, paired t-test, n = 7 slices). Then we simultaneously recorded neuronal STCs and astrocytic Ca2+ oscillations to examine the coincidence of two events. The coincidence was found in 5 of 15 experiments (Fig. 7C2). Neuronal STCs always occurred behind the astrocytic Ca2+. The mean latency from the Ca2+ peak to the STC peak is 2.3 ± 1.3 s (mean ± SEM).
astrocyte, 4-AP first induced an increase in the intensity of cytic TCs in 4 of 12 patched astrocytes. In a representative observed the coincidence between vesicular fusion and astro-Alexa Fluo-594 (100 \mu M). M), and K-glutamate (50 mM). We showed that 4-AP induced both neuronal STCs and astrocytic TCs. B: mean frequency of neuronal STCs during control (Con), perfusion of 4-AP (4-AP), and 4-AP/APV/CNQX (APV/CNQX). *, $P < 0.05$ compared with control (Con), paired t-test, $n = 15$ cells. C: The mean rising time (Rise) and decay ($\tau$) of IP$_F$ (open bars) and 4-AP-induced astrocytic TCs. SD). We further tested whether 4-AP-induced astrocytic TC is coincident with fusion of a large vesicle in astrocytes that were patched with the pipette solution containing Fluo-4 (50 \mu M), Alexa Fluo-594 (100 \mu M), and K-glutamate (50 mM). We observed the coincidence between vesicular fusion and astrocytic TCs in 4 of 12 patched astrocytes. In a representative astrocyte, 4-AP first induced an increase in the intensity of Ca$^{2+}$ and Alexa Fluor-594 fluorescence in a large vesicle (Fig. 7D, 2 and 3, arrow). Then a sudden decrease in the intensity of Ca$^{2+}$ and Alexa Fluor-594 fluorescence (Fig. 7D, bottom, arrowhead) occurred (fusion) and was immediately followed by an astrocytic TC (Fig. 7D, I, TC). The results suggest that 4-AP-induced TCs result from astrocytic exocytosis of single large glutamate-containing vesicles.

**DISCUSSION**

In this study, we demonstrated that astrocytic glutamate release induces a transient depolarization (TD) and epileptiform discharges in CA1 pyramidal neurons (Fig. 2). The results in our study support that the astrocytic IP$_3$-induced TD and epileptiform discharges are due to astrocytic glutamate release because: 1) the onset of astrocytic TCs is earlier than the onset of neuronal STCs; 2) both astrocytic TCs and neuronal STCs are TTX-insensitive, suggesting they are not due to AP-dependent synaptic activities (Fig. 2, E-G and Fig. 3, A-C, TTX); and 3) astrocytic TCs are glutamate transport currents (Fig. 4) and coincident with fusion of large astrocytic vesicles (Figs. 5), which provides direct evidence for the hypothesis that astrocytic exocytosis of glutamate-containing vesicles results in neuronal STC and astrocytic TC.

Astrocytic glutamate release induces a negative shift in field potential (Fig. 2, D-F), suggesting that astrocytic glutamate release causes synchronized neuronal activities. The intracellular glutamate concentration used in these experiments (Fig. 2) is 20 mM that is higher than the resting level of astrocytic glutamate (millimolar levels) (Hertz et al. 1988; Ottersen 1989; Levi and Patrizio 1992; Ye et al. 2001). However, under epileptic conditions, astrocyte intracellular glutamate levels may be elevated to such high levels due to increased glutamate uptake and decreased activities of glutamine synthetase (Eid et
al. 2004). The quantal size of STCs and TCs is positively correlated with intracellular glutamate concentrations (Fig. 1B and C, Amp), suggesting that intracellular glutamate levels are important for the filling of vesicles with glutamate. Under physiological conditions, the dynamic balance between glutamate uptake and glutamate metabolism results in millimolar levels of astrocyte intracellular glutamate (Hertz et al. 1988; Ottersen 1989; Levi and Patrizio 1992; Ye et al. 2001), and glutamate release with such intracellular glutamate levels may keep in low levels of quantum. Under epileptic conditions, the large quantum of astrocytic glutamate release resulting from elevated intracellular glutamate levels may initiate epileptic seizures.

Epileptogenic reagent, 4-AP, induces a similar STC in pyramidal neurons (Fig. 6), suggesting that in 4-AP slice models of epileptic seizures, astrocytic glutamate release occurs and may contribute to 4-AP-induced epileptic seizures. Astrocytic glutamate release occurs spontaneously and each release causes activation of ionotropic glutamate receptors for 1–2 s (Fig. 2B). The characteristics of the TD induced by astrocytic glutamate release in individual neurons are similar to the PDS observed in animal models of epileptic seizures (Lee and Hablitz 1991; Mody et al. 1987; Schiller 2002; Segal 1991). Therefore the astrocytic glutamate release-induced TD may serve as a mechanism for initiation of epileptic seizures under pathological conditions. Although our results demonstrated that astrocytic glutamate release-induced TD and epileptiform discharges occurred in 4-AP slice seizure models, how many epileptiform discharges during perfusion of 4-AP are attributable to astrocytic glutamate release has not yet been determined in this study due to the lack of pharmacological tools to selectively block astrocytic glutamate release. The mechanisms by which 4-AP induces astrocytic release of glutamate are not known yet. One possible mechanism is that 4-AP directly stimulates astrocytic Ca\(^{2+}\) signals by refilling internal Ca\(^{2+}\) stores (Grimaldi et al. 2001). Another possibility is that 4-AP depolarizes glutamatergic neurons to release glutamate and indirectly activates astrocytic glutamate receptors.

Using dual recording in pairs of pyramidal neurons and astrocytes, we have found that the astrocytic TC is paired with the neuronal STC. The results in our study suggest that the astrocytic TC is a glutamate transport current because: 1) the astrocytic TC is inhibited and broadened by specific transport inhibitor TBOA (Fig. 4, C and D); 2) the constant rising slope of astrocytic TCs (Fig. 4B, top) supports the homogenous transporter-mediated current; and 3) the astrocytic TC is inhibited by neither the AMPA receptor antagonist NBQX (Fig. 4E) nor the anion channel inhibitor NPPB (Fig. 4F). When an astrocyte releases glutamate, glutamate acts on ionotropic glutamate receptors in neurons leading to a neuronal STC. Meanwhile, the releasing astrocyte and neighbor astrocytes reuptake released glutamate leading to an astrocytic TC in these astrocytes.

The results that astrocytic Ca\(^{2+}\) oscillations preceded neuronal STCs (Figs. 7C) suggest that astrocytic Ca\(^{2+}\) oscillations may be required for STCs. However, astrocytic Ca\(^{2+}\) oscillations occur spontaneously during control conditions even in the presence of TTX (Fig. 7, A and B, TTX) (Nett et al. 2002), while very few STCs occurred during control conditions (Fig. 7B, Con). These results imply that although the Ca\(^{2+}\) oscillation is required for glutamate release, the Ca\(^{2+}\) oscillation alone may not be sufficient to trigger astrocytic glutamate release. There may be other factors that regulate astrocytic glutamate release. One possible mechanism that regulates astrocytic exocytosis of glutamate release is the priming process that makes vesicles release-ready. During control conditions, there are very few vesicles in the release-ready status, and thereby astrocytic Ca\(^{2+}\) oscillations could not trigger many vesicles to fuse. IP\(_3\) or 4-AP not only increases Ca\(^{2+}\) oscillations but also promotes the priming process of vesicles (Figs. 5C and 7D), and thereby induces exocytosis of vesicular glutamate.

In this study, we report that astrocytic release of glutamate induces the TD and epileptiform discharges in pyramidal neurons by activating ionotropic glutamate receptors. Astrocytic glutamate release can be monitored by recording glutamate transport currents in astrocytes and by Ca\(^{2+}\) and Alexa Fluor-594 fluorescence in large vesicles in astrocytes. Our results indicated a novel mechanism for initiation of epileptic seizures and provide a potential target for developing drugs to control epileptic seizures.

ACKNOWLEDGMENTS

The work was supported by National Institute of Neurological Disorders and Stroke Grants IR29NS-37349 and ROINS-39997.

REFERENCES


