Astrocytic group I mGluR-dependent potentiation of astrocytic glutamate and potassium uptake

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Devaraju P, Sun MY, Myers TL, Lauderdale K, Fiacco TA. Astrocytic group I mGluR-dependent potentiation of astrocytic glutamate and potassium uptake. J Neurophysiol 109: 2404–2414, 2013. First published February 20, 2013; doi:10.1152/jn.00517.2012.—One of the most important functions of astrocytes is removal of glutamate released during synaptic transmission. Surprisingly, the mechanisms by which astrocyte glutamate uptake is acutely modulated remain to be clarified. Astrocytes express metabotropic glutamate receptors (mGluRs) and other G protein-coupled receptors (GPCRs), which are activated during neuronal activity. Here, we test the hypothesis that astrocytic group I mGluRs acutely regulate glutamate uptake by astrocytes in situ. This hypothesis was tested in acute mouse hippocampal slices. Activation of astrocytic mGluRs, using a tetanic high-frequency stimulus (HFS) applied to Schaffer collaterals, led to potentiation of the amplitude of the synaptically evoked glutamate transporter currents (STCs) and associated charge transfer without changes in kinetics. Similar potentiation of STCs was not observed in the presence of group I mGluR antagonists or the PKC inhibitor, PKC 19–36, suggesting that HFS-induced potentiation of astrocyte glutamate uptake is astrocytic group I mGluR and PKC dependent. Pharmacological stimulation of a transgenic GPCR (MrgA1R), expressed exclusively in astrocytes, also potentiated STC amplitude and charge transfer, albeit quicker and shorter lasting compared with HFS-induced potentiation. The amplitude of the slow, inward astrocytic current due to potassium (K⁺) influx was also enhanced following activation of the endogenous mGluRs or the astrocyte-specific MrgA1 Gq GPCRs. Taken together, these findings suggest that astrocytic group I mGluR activation has a synergistic, modulatory effect on the uptake of glutamate and K⁺.

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Materials and Methods

Hippocampal slices. All animal experiments were performed at the University of California, Riverside (UCR), using protocols that had centered through the act of binding to the high-affinity transporters alone (Tzingounis and Wadiche 2007). These properties suggest the importance of mechanisms modulating the location and expression levels of transporters in shaping the spatial extent of glutamatergic signaling. PKC-dependent phosphorylation, leading to potentiation of glutamate uptake, was first demonstrated in HeLa cells transfected with glutamate transporter GLT-1 (Casado et al. 1993). Following this study, a large body of work on various cultured cell types, expressing different subtypes of glutamate transporters, has provided conflicting results on the modulation of astrocyte glutamate transport by G protein-coupled receptors (GPCRs) or their signaling molecules [reviewed in Grover et al. (2006)].

Metabotropic glutamate receptor (mGluR)- and PKC-dependent long-term potentiation (LTP) of postsynaptic neuronal glutamate uptake has been demonstrated in the climbing fiber-Purkinje cell synapse following tetanic stimulation of climbing fibers (Shen and Linden 2005). The hypothesis that activation of astrocytic mGluRs, or GPCRs in general, might be involved in regulating glutamate uptake has not been reported in a slice preparation or in vivo. Synaptically evoked inward currents in astrocytes representing glutamate and potassium (K⁺) uptake have been reported in hippocampal slices (Bergles and Jahr 1997). Two studies in hippocampal slices aimed at deciphering the locus of neuronal LTP to the presynaptic or postsynaptic site measured astrocytic transporter currents and reported no change after a 100-Hz stimulation of Schaffer collaterals (SCs) (Diamond et al. 1998; Luscher et al. 1998). However, another study reported protein transcription and N-methyl-d-aspartate receptor (NMDAR)-dependent late but not early LTP of astrocytic glutamate transporter GLT-1 (Pita-Almenar et al. 2006). Here, we used a protocol to isolate the glutamate transporter component from K⁺ influx (Diamond 2005) and demonstrate that glutamate uptake is potentiated as early as 15 min following tetanic high-frequency stimulation (HFS; 200 Hz) of SCs by a group I mGluR-PKC-dependent mechanism. As group I mGluRs are Gq coupled, we tested the sufficiency of an analogous Gq GPCR for the potentiation of glutamate uptake using a transgenic MrgA1R mouse model (Fiacco et al. 2007). It was unexpected that the slow, inward K⁺ component of the astrocytic current was also enhanced following both HFS and MrgA1R activation, suggesting a synergistic, modulatory effect of these astrocytic Gq GPCRs on the uptake of glutamate and K⁺.
been approved in advance by the Institutional Animal Care and Use Committee of UCR. All experiments were performed using hippocampal slices from 12- to 18-day-old C57BL/6J (The Jackson Laboratory, Bar Harbor, ME) or transgenic MrgA1 mice, backcrossed a minimum of 10 generations to the C57BL/6J background (Fiacco et al. 2007). Brains were isolated following isoflurane anesthesia and decapitation. Parasagittal hippocampal slices were then prepared in ice-cold, nominally calcium (Ca\(^{2+}\))-free saline containing (in mM): 125 NaCl, 2.5 KCl, 3.8 MgCl\(_2\), 1 NaH\(_2\)PO\(_4\), 26.6 NaHCO\(_3\), and 25 glucose, bubbled with 5% CO\(_2\)-95% O\(_2\). Subsequently, slices were incubated at 35°C in oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM): 125 NaCl, 2.5 KCl, 2.5 CaCl\(_2\), 1.3 MgCl\(_2\), 1 NaH\(_2\)PO\(_4\), 26.6 NaHCO\(_3\), and 15 glucose, bubbled with 5% CO\(_2\)-95% O\(_2\). Following the incubation period at physiological temperature, slices were given 15 min to equilibrate to room temperature and then transferred to a recording chamber and superfused continuously with oxygenated, room-temperature ACSF.

**Electrophysiology.** Either a Multiclamp 700B + Digidata 1440 or Axopatch 200B + Digidata 1320 amplifier-digitizer combination (Axon Instruments, Molecular Devices, Sunnyvale, CA) was used for patch-clamp experiments. Individual astrocytes in CA1 stratum radiatum were identified initially by their location, size, and morphological characteristics (Fiacco and McCarthy 2004) and patch-clamped using a borosilicate patch pipette (7–8 MΩ) filled with (in mM): 130 K-gluconate, 4 MgCl\(_2\), 10 HEPES, 10 glucose, 1.185 Mg-ATP, 10.55 phosphocreatine, and 0.1315 mg/ml creatine phosphokinase, pH 7.3, by KOH (standard astrocyte internal solution). In a few cases during initial experiments to isolate astrocyte glutamate transporters pharmacologically, 150 μM Alexa 488 was included in the internal pipette solution to verify astrocyte morphology (Fig. 1A). Current signals from the patch electrode were low-pass filtered at 2 kHz and digitized at 100 kHz. During recordings, drugs were bath applied using an electronic valve controller (Warner Instruments, Hamden, CT).

Astrocytes in the CA1 stratum radiatum were identified initially by their morphology using differential interference contrast optics. After visually locating an astrocyte for patch clamp, a glass monopolar-stimulating electrode was placed ~150 μm toward the CA3 side from the astrocyte. The stimulating electrode was constructed as follows: two chlorided silver wires—one inside a glass micropipette of resistance, ~3 MΩ in contact with ACSF and the other outside of the glass pipette, fixed using soft clay and touching the bath ACSF—were connected to an stimulii isolator (Warner Instruments). Pulse duration was set to 200 μs. The stimulating electrode was placed slightly below (20–30 μm) the optical plane of the chosen astrocyte. After the stimulating electrode was in place, the astrocyte was patch clamped, and the current profile was obtained using 20 mV steps, ranging from ~180 mV to +80 mV (14 steps total; Fig. 1B). Evoked currents were passive, exhibiting no voltage-dependent changes or rectification with a clear, linear I–V relationship. Only cells exhibiting this passive electrophysiological phenotype were used for further study, as these are the astrocyte subtypes known to take up glutamate (Matthias et al. 2003). A test pulse of ~5 mV was included after each voltage step to monitor changes in access resistance (R\(_a\)), a standard procedure in whole-cell patch-clamp studies. Recordings in which R\(_a\) changed by 20% or more were rejected. It was difficult to record transporter currents in astrocytes beyond 30 min, due to changes in R\(_a\) that have been described elsewhere for reasons contributing to "rundown" of astrocyte glutamate transporter currents over time (Luscher et al. 1998).

We used a protocol developed by Diamond (2005) to isolate the glutamate transporter component without resorting to bath-three-benzoyloxyaspartic acid (TBOA) application in experiments to determine the effects of stimulation of astrocytic Gq GPCRs on glutamate uptake (Fig. 1, C–F). In this protocol, the K\(^+\) currents are acquired in separate experiments by blocking transporter currents using TBOA. The kinetic parameters of these currents are then used to estimate and subtract the K\(^+\) current from the total current in later experiments.

**Fig. 1.** Glutamate transporter and potassium (K\(^+\)) currents were recorded in passive hippocampal astrocytes. A: example of a patch-clamped stratum radiatum astrocyte filled with Alexa 488 to demonstrate the typical morphology of a passive astrocyte. B: voltage steps (20 mV) from ~180 mV to +80 mV did not produce any voltage-dependent currents in astrocytes. C: bath application of 25 μM of the nonselective glutamate transport blocker bath-three-benzoyloxyaspartic acid (TBOA) for 5 min inhibited almost totally the fast component of the current evoked using a 75-μA pulse. D: example of a pharmacologically isolated glutamate transporter current. E and F: example of nonpharmacological isolation of glutamate transporter and K\(^+\) components from the total evoked current. Astrocytic currents evoked using a single 75-μA pulse (black trace: 1) and a pair of pulses (20 Hz; gray trace: 2) to the Schaffer collaterals (SCs). Current evoked by the single pulse (trace 1 in E) was subtracted from that evoked by the paired-pulse protocol (trace 2 in E) to isolate the total facilitated current, which has a greater glutamate transport component due to presynaptic facilitation of glutamate release (gray trace in F: 3). Subtraction of the K\(^+\) current component obtained in separate experiments (trace 4 in F) from trace 3 produced the isolated, facilitated astrocytic synaptically evoked glutamate transporter current (STC; trace 5 in F).

The sequence of steps in this protocol is as follows: a 10-s electrophysiological recording (sweep) containing a single-pulse stimulus was followed by another 10-s sweep with paired pulses. Stimulation pulses were 75 μA in amplitude and 200 μs in duration. This stimulation sequence was repeated continuously using the sequencing tool in the Clampex acquisition software (Molecular Devices). Sequential stimulation was stopped for brief moments to apply train stimulation or for monitoring cell health. Two such single pulse-paired pulse combinations were chosen at specific time intervals for isolating transporter currents from the total evoked currents. As the TBOA-insensitive component (referred to as "K\(^+\) current" hereafter, as it is mainly due to K\(^+\) uptake) had a slower τ\(_{dec}\) and reached steady state ~70 ms after the stimulation, it was fitted using a single exponential fit. The steady state of the total evoked current, ~100 ms after the stimulation, is entirely due to the K\(^+\) current, because it matches exactly the amplitude in the absence of TBOA. Hence, the
amplitude of this K⁺ current can be determined easily by measuring the steady-state current, ~100 ms after stimulation. An estimate of the τrise of this slowly rising K⁺ current was obtained from a set of slices following a 5-min TBOA (25 μM) application. A pilot experiment suggested that 25 μM TBOA was sufficient to block the fast transporter component completely, as has been reported for 100 μM TBOA (Diamond 2005). We then generated the slow-rising K⁺ current using Clampfit software by a standard, single exponential fit using the τrise estimate and the steady-state amplitude (measured ~100 ms after the stimulation). This enabled us to isolate nonpharmacologically the synaptically activated astrocyte glutamate transporter currents (STCs) in subsequent experiments. Charge transfer, a measure of total glutamate uptake, was calculated by measuring the area encompassed by the isolated transporter current trace and baseline for a duration of 75 ms. This area, with units of femtomolons (pA × ms), was normalized to before-treatment levels and is expressed as a percentage of before-treatment levels.

Kynurenic acid (KYN) or other ionotropic GluR (iGluR) antagonists were not used during recordings of transporter currents. iGluR antagonists have been used in pioneering experiments to define nonpharmacologically the synaptically activated astrocyte glutamate transporter currents (STCs) in subsequent experiments. Charge transfer, a measure of total glutamate uptake, was calculated by measuring the area encompassed by the isolated transporter current trace and baseline for a duration of 75 ms. This area, with units of femtomolons (pA × ms), was normalized to before-treatment levels and is expressed as a percentage of before-treatment levels.

Kynurenic acid (KYN) or other ionotropic GluR (iGluR) antagonists were not used during recordings of transporter currents. iGluR antagonists have been used in pioneering experiments to define astrocyte glutamate transporter currents (Bergles and Jahr 1997; Diamond et al. 1998). The main effects of KYN are twofold: elimination of a fast outward current, due to the extracellular field potential, and reduction of K⁺ uptake, due to postsynaptic release of K⁺ from synaptically active astrocytes. These were considered Ca²⁺/H⁺-transporter component completely, as has been reported for 100 μM TBOA (Honsek et al. 2010; Porter and McCarthy 1996). The same test-pulse procedure consisted of lowering a pressure pipette of low resistance to the slice to an ~50-μm depth; leaving it in place for ~1 min; and repeating it at two more neighboring locations, such that approximately four to 10 astrocytes are filled with the dye (Garaschuk et al. 2006; Nimmerjahn et al. 2004; Sullivan et al. 2005). Astrocytes in the slice were labeled with Sulforhodamine 101 (SR-101) prior to bolus loading (Nimmerjahn et al. 2004). Prelabeling was done in a 12-well culture plate by submerging the slices in oxygenated ACSF (650 μM SR-101. Trolox (0.1 mM) dissolved in DMSO (final 5.4 M) was avoided, as this would increase the ambient glutamate concentration within the slice with unpredictable confounds. This concern also prevented the use of TBOA or other transport blockers to isolate transporter currents, which was blocked by 25 μM of the nonselective glutamate transport blocker TBOA (Fig. 1C). There was also a TBOA-insensitive current with a slower τrise and very long decay τ, thought mainly to be due to K⁺ influx (Bergles and Jahr 1997). Application of TBOA for longer times or at concentrations >25 μM was avoided, as this would increase the ambient glutamate concentration within the slice with unpredictable confounds. This concern also prevented the use of TBOA or other transport blockers to isolate transporter currents in subsequent experiments, which required recording transporter currents repeatedly over a period of 30 min. We therefore used a protocol developed by Diamond (2005), which enabled isolation of the STCs without resorting to TBOA application. In this protocol, the K⁺ currents were acquired in separate recordings, and these were subtracted from the total current acquired in later experiments, thus isolating the glutamate transporter currents nonpharmacologically (Fig. 1, E and F; see MATERIALS AND METHODS).

Results

Isolation of glutamate transporter currents from the total inward astrocytic current evoked by SC stimulation. Stimulation of SCs using a 75-μA pulse reliably evoked inward currents in astrocytes. The amplitude of the current evoked using this stimulation intensity was in the 50- to 100-pA range. This evoked current, although smaller in amplitude relative to using larger stimulation intensities, had a fast component due to glutamate transport, which was blocked by 25 μM of the nonselective glutamate transport blocker TBOA (Fig. 1C). There was also a TBOA-insensitive current with a slower τrise and very long decay τ, thought mainly to be due to K⁺ influx (Bergles and Jahr 1997). Application of TBOA for longer times or at concentrations >25 μM was avoided, as this would increase the ambient glutamate concentration within the slice with unpredictable confounds. This concern also prevented the use of TBOA or other transport blockers to isolate transporter currents in subsequent experiments, which required recording transporter currents repeatedly over a period of 30 min. We therefore used a protocol developed by Diamond (2005), which enabled isolation of the STCs without resorting to TBOA application. In this protocol, the K⁺ currents were acquired in separate recordings, and these were subtracted from the total current acquired in later experiments, thus isolating the glutamate transporter currents nonpharmacologically (Fig. 1, E and F; see MATERIALS AND METHODS). Single and double pulses were applied with a 10-s interval to allow for the slow K⁺ current to

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Minimal stimuli used to evoke astrocyte glutamate transporter currents could not reliably evoke astrocyte Ca\(^{2+}\) elevations. A 50-µA pulse stimulation of SCs in our preparation evoked STCs with some incidences of failures. Hence, we chose a stimulation intensity of 75 µA for our test pulses, which evoked transporter currents reliably (with negligible incidences of failures). We next tested whether this 75-µA stimulation was sufficient to evoke astrocyte Gq GPCR-driven Ca\(^{2+}\) elevations. This was important to know, as test-pulse stimulation could possibly then play a role in potentiating glutamate uptake in addition to HFS. Ca\(^{2+}\) elevations were recorded in astrocyte cell bodies, bolus loaded with an OGB-1 Ca\(^{2+}\) indicator and SR-101 (Fig. 2A; see MATERIALS AND METHODS), and test-pulse stimuli were applied in triplicate to mimic stimuli used to evoke astrocyte transporter currents. The percentage of astrocytes responding with a Ca\(^{2+}\) increase was calculated following stimulation intensities ranging from 25 to 200 µA to determine a threshold for evoking an astrocyte Ca\(^{2+}\) response (Fig. 2B). The 75-µA stimulation intensity used for our test pulses did not significantly increase the percentage of astrocytes exhibiting a Ca\(^{2+}\) elevation compared with basal, spontaneous Ca\(^{2+}\) elevations (22.4% after 75 µA stimulation vs. 15.7% under basal conditions; Fig. 2C). A significant increase in the percentage of astrocytes with a Ca\(^{2+}\) elevation was observed, on average, following 150 µA test-pulse stimuli (28.3%; \(P < 0.05\)).

**Fig. 2.** Astrocytic calcium (Ca\(^{2+}\)) responses following various stimulation intensities measured in slices, bolus loaded with the Ca\(^{2+}\) indicator dye Oregon Green BAPTA-1 (OGB-1). A: identity of the stratum radiatum cells loaded with OGB-1 was verified using the astrocyte specific marker Sulforhodamine 101 (SR-101). Numbered regions of interest (ROIs) match fluorescence over time traces in B. B: representative fluorescence over time traces from astrocytes shown in A. In general, the percentage of astrocytes responding to stimulation increased as stimulation intensities increased. In this particular recording, astrocytes began responding to 150 µA test-pulse stimulation but not to 75 µA test pulses. Six out of the 7 astrocytes in the field responded to the 200 Hz, 50 µA high-frequency train, which was used to stimulate endogenous astrocytic metabotropic glutamate receptors (mGluRs) in experiments measuring potentiation of astrocyte glutamate uptake. \(\Delta F/F_0\), change in fluorescence intensity over baseline fluorescence. C: the percentage of astrocytes exhibiting a somatic Ca\(^{2+}\) elevation is plotted against the amplitude of the current used to stimulate SCs. The 75-µA test-pulse stimulation was not sufficient, on average, to significantly increase the percentage of astrocytes exhibiting Ca\(^{2+}\) elevations compared with baseline levels (n = 27 cells; 4 slices). The percentage of astrocytes exhibiting a Ca\(^{2+}\) elevation increased significantly over baseline spontaneous activity following 150 µA test pulses (\(^* P < 0.05\)). High-frequency stimulation (HFS; 200 Hz, 1 s, 50 µA) further increased the percentage of responsive astrocytes (\(^* P < 0.05\)). D: astrocyte Ca\(^{2+}\) elevations evoked by HFS were significantly longer lasting compared with spontaneous Ca\(^{2+}\) elevations. Representative spontaneous astrocyte Ca\(^{2+}\) activity and HFS-evoked responses are shown in the traces (middle) that correspond to individual astrocytes marked with boxes in the image; \(^* P < 0.05\).
compared with baseline). The percentage of responding astrocytes increased further to 56% following HFS (200 Hz for 1 s at 50 μA). Furthermore, astrocyte Ca\(^{2+}\) responses evoked by the high-frequency train were more robust compared with spontaneous Ca\(^{2+}\) transients, as indicated by significantly longer response durations (Fig. 2D). Evoked astrocyte Ca\(^{2+}\) responses to test-pulse stimulation or tetanic stimulation were also often synchronized among multiple astrocytes in the recording field, which is atypical of spontaneously occurring astrocyte Ca\(^{2+}\) activity (Nett et al. 2002; Tian et al. 2006).

We observed in these recordings that there was variability in the stimulation intensity sufficient to evoke a Ca\(^{2+}\) elevation among multiple astrocytes in a given slice. For example, in the representative traces in Fig. 2B, astrocytes 5 and 7 responded to 150 and 200 μA test-pulse stimuli, whereas astrocytes 2 and 3 only responded to the 200-Hz train. Cell 1 only responded to an agonist cocktail consisting of 10 μM each of histamine, carbacol, and ATP. We suspect that this variability stems from a combination of stimulating electrode distance from each cell in the field and the density and affinity of the Gq GPCRs on a given astrocyte. Glutamate transporter currents were very reliable at 75 μA at this same distance from the stimulating electrode (150 μm), suggesting that relative to astrocytic Gq GPCRs, the density and/or affinity of glutamate transporters are greater and more consistent across astrocytes. With regard to astrocyte Ca\(^{2+}\) elevations, as only activity at the level of the astrocyte soma was recorded, it also remained a possibility that Ca\(^{2+}\) responses to SC stimulation occurred in smaller astrocytic compartments that were not visualized. In experiments using astrocytic MrgA1R transgenic mice, antagonists for group I mGluRs were included in the ACSF to prevent possible activation of endogenous astrocytic group I mGluRs by test-pulse stimulation.

**HFS of SCs potentiates astrocyte glutamate transporter current amplitude and charge transfer in a mGluR-PKC-dependent manner.** Based on the percentage of astrocytes responding to test-pulse stimulation vs. HFS of SCs described above and previous work demonstrating that only ~50% of the synapses in the hippocampus have an adjacent astrocytic process (Ventura and Harris 1999), we used HFS (200 Hz, 1 s) to stimulate astrocytic metabotropic receptors. HFS-induced late but not early LTP of astrocytic GLT-1 had been reported earlier (Pita-Almenar et al. 2006), but this potentiation was dependent on stimulation of NMDARs, which are not thought to be expressed by passive hippocampal astrocytes (Agulhon et al. 2010; Petrvavicz et al. 2008). Astrocytic STCs, recorded 15 and 30 min after the tetanic train, had significantly higher amplitudes compared with before the train (130% at 15 min and 161% at 30 min after vs. before HFS; Fig. 3, A, B, and E). Charge transfer, a measure of the total glutamate uptake, also showed a significant increase at 15 and 30 min after the tetanic train (127% at 15 min and 149% at 30 min after vs. before HFS; Fig. 3F). An essential requirement for the nonpharmacological transporter current isolation protocol is that the \(\tau_{\text{rise}}\) of the K\(^+\) current should not exhibit significant differences due to HFS. We verified that this requirement is satisfied by measuring the \(\tau_{\text{rise}}\) of the K\(^+\) current, 30 min after HFS, in a different set of slices (to avoid residual effects of TBOA application). The 30-min time point was chosen for these measurements, because the greatest potentiation (~60%) of the STC was observed at this time point. The \(\tau_{\text{rise}}\) of the K\(^+\) current, measured 30 min after HFS, did not differ significantly from that measured before HFS (5.74 ± 0.49 ms before vs. 7.29 ± 0.81 ms after HFS; \(P = 0.126; n = 8\) cells). We reasoned that glutamate diffusing out of the cleft was leading to astrocyte STC amplitude potentiation by activating astrocytic mGluRs. Activation of group I mGluRs of CA1 astrocytes following tetanic stimulation of SCs has been observed previously (Bowser and Khakh 2004; Porter and McCarthy 1996). LTP of neuronal glutamate transport in Purkinje neurons of the cerebellum following tetanic stimulation of climbing fibers has also been shown to be mGluR dependent (Shen and Linden 2005). To test if the observed potentiation of STCs in our model was group I mGluR mediated, we repeated the experiment in the presence of the group I mGluR antagonists MPEP (10 μM) and LY 367385 (100 μM) in the ACSF. There was no effect of the mGluR antagonists on test STCs recorded prior to HFS (63.3 ± 7.84 pA without mGluR antagonists; 52.6 ± 8.01 pA + mGluR antagonists; \(P = 0.348\)). There was also no significant change in the STC amplitude or charge transfer following HFS in the presence of the group I mGluR antagonists (Fig. 3, C, E, and F). These data suggest that group I mGluR activation is necessary for potentiation of astrocytic STC amplitude following HFS of SCs.

Activation of astrocytic Gq GPCRs can trigger multiple signaling molecules within the cell (Waldo et al. 2010). Of these, PKC might be a likely downstream candidate involved in the potentiation of transporter currents. PKC-dependent phosphorylation of the neuronal glutamate transporters leading to an increase in the amplitude of the transporter current has been observed earlier (Shen and Linden 2005). We sought to determine if inhibiting PKC intracellularly would prevent the HFS-mediated potentiation of astrocytic STCs. The PKC inhibitor PKC 19-36 (10 μM) was included in the patch-pipette solution and allowed to diffuse into the astrocyte for at least 15 min prior to recording STCs. PKC 19-36 has a molecular weight of 2.15 kDa, and so, this additional 15-min duration was provided to help fill the smaller astrocytic compartments with the inhibitor. The PKC inhibitor had no effect on basal astrocytic STCs recorded just prior to HFS (63.3 ± 7.84 pA without PKC 19-36; 74.6 ± 10.47 pA + PKC 19-36; \(P = 0.417\)). However, after HFS, there was no increase in the amplitude or charge transfer of the STCs in the presence of PKC 19-36 (Fig. 3, D–F), suggesting that PKC is necessary for HFS-induced potentiation of astrocyte glutamate uptake. Unexpectedly, we also observed a highly significant decrease in the amplitude of the STCs in the presence of the PKC inhibitor at 2 and 5 min after HFS (79% at 2 min and 82% at 5 min after HFS vs. before; Fig. 3, D–F). The charge transfer associated with STCs also decreased significantly in the presence of the PKC inhibitor at 2 min after HFS (73% after HFS vs. before). There was no significant difference in the amplitude of the astrocytic STCs or charge transfer in the presence of the PKC inhibitor at other time points following HFS (Fig. 3, D–F). These data suggest that PKC is necessary, not only for the potentiation of glutamate uptake by astrocytes but also for its maintenance after HFS. It appears that when PKC is inhibited, glutamate uptake is diminished rapidly in astrocytes following HFS, perhaps due to activation of other astrocytic signaling molecules. These findings open the possibility that astrocyte glutamate uptake is regulated bidirectionally depending on the ratio-signaling molecules activated within the astrocyte. Future experiments are planned to try to identify these signaling molecules, but one possibility is that stimulation of hippocampal afferents also activates astrocytic inhibitory-regulative G-protein
(Gi) or stimulative-regulative G-protein (Gs) GPCRs to acutely inhibit astrocyte glutamate uptake.

The kinetics of the normalized STCs remained unaffected after HFS in the absence and presence of mGluR antagonists. The normalized, negative rise slope had a mean of 0.38 (n = 16; SD = 0.13). This decay τ was similar to that observed in adult slices but approximately 4–5 ms longer than that of postnatal ages 12–14 (P12–P14) slices observed by Diamond (2005). It is possible that this difference arises from the inclusion of a wider postnatal age group (P12–P18) in our study. Inhibition of PKC led to a faster rise of the glutamate transporter current at 5 min, following the HFS, as indicated by a significant decrease in τrise (mean of 1.48 ms and 1.27 ms before vs. 5 min after the HFS, respectively; P < 0.01), and a steeper, normalized, negative slope (mean of 0.43 “before” vs. 0.50 ms, 5 min after the HFS; P < 0.01). Similar to the significant reduction in STC amplitude and charge transfer, these effects of PKC inhibition on the rise kinetics of STCs may occur as a consequence of an imbalance between astrocytic Gq and Gi/Gs signaling pathways, wherein the initial inward transport of glutamate and sodium occurs faster, with a net decrease in the total amount of glutamate taken up.

Stimulation of astrocytic MrgA1Rs is sufficient to potentiate astrocyte glutamate transporter current amplitude and charge transfer. HFS of hippocampal afferent fibers can lead to activation of receptors other than group I mGluRs in astrocytes, including purinergic and adenosine receptors (Cunha 2008; Pankratov et al. 2006), GABA receptors (Kang et al. 1998; Serrano et al. 2006), cannabinoid receptors (Navarrete and Araque 2008), and possibly many others, in addition to stimulation of a variety of neuronal pre- and postsynaptic receptors. It is also not feasible to selectively activate astrocytic group I mGluRs in a slice preparation by washing in a ligand. In an effort to exclude mechanisms extrinsic to astrocytic Gq receptor activation, we took advantage of the...
recently developed MrgA1R transgenic mice (Fiacco et al. 2007). These mice express a transgenic Gq GPCR selectively in astrocytes. With these mice, it is possible to stimulate intracellular Gq signaling cascades and Ca$^{2+}$ elevations that mimic activation of group I mGluRs selectively in astrocytes, using a ligand that does not activate endogenous receptors on other cell types. Bath application of the small peptide MrgA1R agonist phenylalanine-methionine-arginine-phenylalanine (FMRF) evoked strong astrocyte Ca$^{2+}$ elevations (Fig. 4, A and B), as observed previously (Fiacco et al. 2007). To prevent possible activation of group I mGluRs by glutamate released during test pulses, the group I mGluR antagonists MPEP and LY 367385 were added to the ACSF in these experiments. After a 90-s bath application of FMRF, we observed an increase in the amplitude of the isolated STC and charge transfer in the presence of group I mGluR antagonists (Fig. 4, C–F). The increase in amplitude was smaller than that observed after HFS but initiated more rapidly (111.3%; *P < 0.05 at 5 min after FMRF in MrgA1$^+$ mice vs. 160.7%; *P < 0.05 at 30 min post-HFS). Increases in charge transfer were also of lower magnitude compared with HFS but observable out to 15 min after FMRF application (109% at 5 min and 114% at 15 min after FMRF in MrgA1 mice; *P < 0.05 vs. 127% at 15 min and 149% at 30 min after vs. before HFS; *P < 0.05). These results suggest that selective stimulation of astrocytic Gq GPCR signaling cascades is sufficient to potentiate glutamate uptake, although the effect was more rapid and shorter lasting compared with HFS. Similar to the results from HFS, we did not observe any significant differences in the kinetics of the STCs after stimulation of astrocytic MrgA1Rs (data not shown).

HFS of SCs or stimulation of astrocytic MrgA1Rs leads to significant increases in the amplitude of the slow, inward K$^+$ current. To determine the effect of HFS or selective stimulation of astrocytic MrgA1Rs on the slow, inward current mediated by K$^+$ uptake, we measured the steady-state current amplitude, 500 ms after the test pulse. This time point was chosen to eliminate the possible inclusion of any residual transporter current and to assess the “steady-state” K$^+$ current. There was a significant increase in the K$^+$ current amplitude at all measured time points (118%, 124%, 120%, and 137% at 2, 5, 15, and 30 min, respectively) after HFS (Fig. 5A). In the presence of group I mGluR antagonists, the

Fig. 4. Selective stimulation of astrocytic MrgA1Rs led to significant increases in astrocyte glutamate uptake. A: MrgA1R-expressing astrocytes were patch clamped with an OGB-1 Ca$^{2+}$ indicator and ROIs placed over astrocyte compartments, including fine processes. B: stimulation of MrgA1Rs with phenylalanine-methionine-arginine-phenylalanine (FMRF) resulted in robust astrocyte Ca$^{2+}$ elevations, including in the small processes (for example, boxes 4, 9, 11, 15, and 16 but not box 17, which was placed off of the astrocyte). C and D: representative STC traces from MrgA1$^+$ mice (C) and littermates (D) before and after bath application of the MrgA1R agonist FMRF. E and F: summary histograms of changes in astrocytic STC amplitude and charge transfer after FMRF application in MrgA1$^+$ and littermate control mice. The amplitude of the STC at 5 min and charge transfer at 5 and 15 min were significantly larger following a 90-s application of FMRF (10 µM) in MrgA1$^+$ but not in littermate control mice (*P < 0.05).
K⁺ current in relation to HFS

K⁺ current in relation to MrgA1R stimulation

**DISCUSSION**

The main finding in this study is that astrocytic group I mGluRs acutely potentiate glutamate transporter currents and K⁺ influx in a PKC-dependent manner in stratum radiatum astrocytes in the acute mouse hippocampal slice preparation. Astrocytic mGluRs were stimulated using HFS of SCs and resulted in increases in the amplitude of the isolated glutamate transporter currents (STCs) and the associated charge transfer. These increases occurred within minutes, without accompanying changes in kinetics. The HFS-induced potentiation of astrocytic glutamate uptake required the activation of group I mGluRs and PKC. Selective pharmacological stimulation of a transgenic Gq GPCR, targeted specifically to astrocytes (Fiacco et al. 2007), also potentiated astrocytic glutamate uptake, further validating the Gq-coupled group I mGluR-mediated potentiation. Our results are similar to the mechanism described for potentiation of the neuronal glutamate transporter excitation amino acid transporter 4 in Purkinje neurons of the cerebellum, following tectonic stimulation of the climbing fibers (Shen and Linden 2005).

One of the seminal papers on modulation of the astrocyte glutamate transporter GLT-1 suggested that phosphorylation of GLT-1 transfected in HeLa cells increases glutamate uptake (Casado et al. 1993). However, a more recent report from a C6 glioma cell line transiently transfected with GLT-1 came to the opposite conclusion—that phosphorylation of GLT-1 decreases its cell-surface expression (Susarla and Robinson 2008). Another study from a reactive gliosis, such as culture preparation (Aronica et al. 2003), reported that activation of groups I and II mGluRs down- and upregulated the expression of astrocytic glutamate transporters, respectively—the opposite of our findings in the present study. As different culture-based studies have provided confounding results regarding the regulatory mechanisms involved in glutamate uptake (Adolph et al. 2007; Aronica et al. 2003; Gegelashvili et al. 2000; Kalandadze et al. 2002; Schlag et al. 1998), we used an acute slice preparation, which is more relevant than culture preparations for studying synaptic physiology. An earlier slice-based study (Pita-Almenar et al. 2006) reported that astrocytic glutamate transporter GLT-1 is involved in late but not early LTP of glutamate uptake in hippocampal CA1. Early LTP was defined as changes occurring within minutes after HFS, whereas late LTP was defined as changes occurring after 1–2 h and dependent on new protein synthesis. In the same study, early LTP of glutamate uptake was attributed to increases in neuronal, not astrocytic, glutamate uptake (Pita-Almenar et al. 2006), and both early and late LTP of glutamate uptake was dependent on activation of NMDARs. Our data suggest that astrocytic mGluRs rapidly potentiate astrocyte glutamate uptake in a PKC-dependent manner. Conserved serine- and threonine-rich motifs serving as phosphorylation loci have been reported on glutamate transporters (Slotboom et al. 1999), supporting a role for PKC in early LTP of astrocytic glutamate uptake. As transporter currents have a higher temporal resolution than expression studies, it is possible that the early LTP of astrocytic glutamate uptake was unrevealed in the previous study (Pita-Almenar et al. 2006), which reported only late LTP of astrocytic glutamate uptake.

Because the STC provides a sensitive assay for measuring changes in the amount of glutamate released presynaptically,
Diamond et al. (1998) and Luscher et al. (1998) recorded astrocyte transporter currents to assay for presynaptic mechanisms of LTP. These authors found no changes in the STC amplitude in response to HFS, suggesting that LTP was not due to changes in release probability. It is difficult to reconcile their findings with those of the present study; however, four possibilities will be discussed. First, due to long, whole-cell recording times (40–60 min) of astrocytes, the previous studies reported significant rundown of the astrocytic STCs. In the present study, we found it very difficult to record past 30 min, due to changes in R,

approximately one-half of the recordings at the 30-min time point were rejected because of this. It is possible that upregulation of STCs was obscured by rundown in the previous studies. This explanation seems unlikely, however, as the previous work accounted for rundown by normalizing the STCs in the test pathway to those recorded following stimulation of a control pathway. A second explanation is that the astrocytic STCs were recorded to those recorded following stimulation of a control pathway. A third explanation is that the astrocytic STCs were recorded to controls in place to address changes in Ra, rapid changes in astrocytes. Last, a 200-Hz HFS train was used in the present study, including nitric oxide, which was previously reported to affect glutamate uptake in hippocampal synaptosomes (Pogun et al. 1994). Furthermore, even small changes in inflammatory mediators can dramatically affect astrocyte signaling capabilities (Agulhon et al. 2012; Santello et al. 2011). Together, the effects of KYN may have hindered upregulation of glutamate uptake by astrocytes. Last, a 200-Hz HFS train was used in the present study, whereas the earlier experiments used a 100-Hz HFS train. We observed a plateauing of the fast astrocytic current during the 200-Hz HFS train, indicative of either transporter saturation or steady-state replenishment of presynaptic vesicles. Previous work does suggest that astrocytic glutamate transporters saturate at room temperature during HFS (Diamond and Jahr 2000). Transporter saturation combined with stronger stimulation of astrocytic GPCRs by a 200-Hz train may be favorable to potentiation of glutamate uptake by astrocytes.

A direct assessment of functional changes in astrocyte K+ and glutamate uptake would seem incomplete without directly recording these currents in astrocytes. This approach does have its limitations, however. Astrocytes are notoriously difficult to space clamp, due to very low input resistances. This means that whole-cell measurement of astrocyte glutamate and K+ uptake is limited spatially to an area near the somatic recording electrode (Bergles and Jahr 1997). Furthermore, although controls were in place to address changes in R, rapid changes in glutamate and K+ uptake may affect input resistance to some degree. For example, one mechanism proposed for changes in glutamate and K+ uptake is rapid insertion of proteins into the membrane from local pools (Robinson 2006). Because astrocytic inwardly rectifying K+ channels have a high, open probability at resting potentials (Ransom and Sontheimer 1995), input resistance may decrease slightly following rapid upregulation of glutamate and K+ uptake. A decreasing input resistance would further reduce space clamp of the astrocyte, creating an artifact in the recorded STCs. However, measured astrocyte glutamate transporter currents become larger as the membrane resistance increases, and space clamp improves (Djukic et al. 2007). Together, this suggests that: 1) the currents recorded underestimate the actual amount of glutamate and K+ uptake by astrocytes, and 2) the amount by which glutamate and K+ uptake is potentiated may be underestimated in our experiments.

Inhibition of PKC was restricted to the recorded astrocyte by including the PKC inhibitor PKC 19–36 in the internal pipette solution used to record astrocytic STCs. The inhibitor PKC 19–36 prevented HFS-induced potentiation of astrocyte glutamate uptake. Because PKC inhibition was restricted to astrocytes, the finding suggests that the group I mGluR antagonists prevent acute upregulation of astrocyte glutamate uptake by blocking astrocytic group I mGluRs. In addition to preventing potentiation of glutamate uptake, inhibition of astrocytic PKC unexpectedly produced a decrease in STC amplitude and charge transfer and slightly modified kinetics of STCs, 2 and 5 min following HFS. This PKC effect required HFS, as the STCs occurring after HFS were normalized to baseline STCs occurring prior to HFS. These findings are compelling, because they introduce the possibility that glutamate uptake in astrocytes is regulated bidirectionally depending on the balance of signaling molecules activated in astrocytes. By inhibiting PKC, upregulation of astrocyte glutamate uptake is prevented, which may unmask the effects of signaling molecules activated in astrocytes by afferent stimulation that acutely depresses astrocyte glutamate uptake. Cultured studies suggest that Gs GPCRs and activation of PKA downregulate glutamate uptake (Adolph et al. 2007; Rath et al. 2008). In future studies, it will be interesting to determine the potential role of PKA in modulation of astrocyte glutamate uptake in intact tissue preparations.

We used MrgA1R transgenic mice to selectively stimulate an astrocytic, Gq-coupled GPCR, analogous to group I mGluRs in the slice preparation. Activation of these receptors has been previously demonstrated to evoke Gq GPCR responses similar to those evoked by group I mGluRs (Fiacco et al. 2007). Stimulation of astrocytic MrgA1Rs was found not to be coupled to release of glutamatergic transmitters from hippocampal astrocytes (Agulhon et al. 2010; Fiacco et al. 2007; Petravicz et al. 2008), which might otherwise cloud interpretation of changes in astrocyte glutamate uptake. Application of the MrgA1R agonist FMRF resulted in significant increases in isolated astrocytic STCs in MrgA1R mice but not in littermate controls, suggesting that astrocyte-specific activation of Gq GPCRs is sufficient to potentiate glutamate uptake. However, compared with the HFS-induced potentiation of astrocytic glutamate uptake, the increase in the evoked STC amplitude and charge transfer following FMRF application occurred much more rapidly (significant increase was observed 5 min after FMRF wash-in) and was shorter lasting. The more rapid effect on the STCs produced by isolated stimulation of astrocytic Gq GPCRs matches the time points in which the transporter currents were reduced in the presence of the PKC inhibitor PKC 19–36 following HFS. This supports the hypothesis that PKC-
mediated potentiation of astrocyte glutamate transporter currents is important for maintenance of glutamate uptake shortly after HFS.

K⁺ uptake, unlike glutamate uptake, may be affected greatly by LTP induction, as the source of glutamate is presynaptic, whereas K⁺ is released by the presynaptic and postsynaptic neurons in the milieu of the same astrocyte. Previous work suggests that inducing LTP contributes to increased K⁺ influx in astrocytes. Enhanced astrocytic K⁺ influx following LTP induction using a 100-Hz, 1-s stimulus has been reported to be a passive reflection of increased extracellular K⁺, released by newly inserted, postsynaptic AMPARs (Ge and Duan 2007). However, in addition to upregulation of K⁺ uptake following HFS, we found an increase in K⁺ current amplitude of almost identical magnitude at each time point following stimulation of the astrocyte-specific MrgA1R, suggesting that K⁺ uptake can be regulated intrinsically by astrocytes, independent of the passive increase observed after LTP-inducing protocols. During our work on this project, Wang et al. (2012) also reported that astrocytic MrgA1R stimulation potentiates K⁺ uptake by astrocytes. Our findings further suggest that this potentiation is PKC dependent, as astrocyte-specific inhibition of PKC prevented the potentiation of K⁺ uptake after HFS. The most straightforward explanation for the PKC inhibitor preventing HFS-induced enhancement of K⁺ uptake by astrocytes, despite an increase in extracellular K⁺ after HFS, is a reduction in astrocytic uptake capacity for K⁺.

The major inwardly rectifying (ir) K⁺ channel in astrocytes responsible for K⁺ uptake is Kᵢ₃.₄.₁ (Butt and Kalsi 2006). Whereas GPCR modulation of K⁺ channels of the Kᵢ₃.₃ family has been well documented (Stanfield et al. 2002), it is unknown whether Kᵢ₃.₄.₁ undergoes similar modulation. Our results, showing an increase in K⁺ current following MrgA1R activation, suggest that Kᵢ₃.₄.₁ can also be modulated by astrocytic GPCR activation. Given the synergism between glutamate and K⁺ uptake by astrocytes, it is not surprising that both are modulated by astrocytic Gq GPCRs.

In summary, we report here that astrocytic group I mGlurS rapidly potentiate astrocyte glutamate and K⁺ uptake, and the potentiation is PKC dependent, based on direct recording of the isolated transporter and K⁺ currents in astrocytes. It will be important to determine in future experiments if astrocyte glutamate uptake is modulated bidirectionally, depending on the balance of GPCR signaling molecules activated in astrocytes. Whereas it is unlikely that rapid modulation of astrocyte glutamate uptake in the hippocampus affects kinetics of synaptic AMPAR or NMDAR currents (Bergles et al. 1999; Tzingounis and Wadiche 2007), it might shape the spatial extent of glutamatergic signaling to extrasynaptic receptors or spillover to adjacent synapses (Kullmann and Aszety 1996). Within the domain of a single astrocyte, which encompasses a volume that includes thousands of synapses, dynamic up- or downregulation of glutamate uptake in astrocyte subcompartments may locally modulate synaptic function. Rapid potentiation of astrocyte K⁺ uptake by astrocytic group I mGlurS suggests synergistic modulation of glutamate and K⁺ uptake by these astrocytic Gq GPCRs. Further experiments will be necessary to elucidate the molecular mechanisms involved in the regulation of glutamate and K⁺ uptake in astrocytes.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: T.A.F. conception and design of research; P.D., M-Y.S., and T.L.M. performed experiments; P.D., M-Y.S., T.L.M., and K.L. analyzed data; P.D., M-Y.S., and T.A.F. interpreted results of experiments; P.D., M-Y.S., and T.A.F. prepared figures; P.D. drafted manuscript; P.D. and T.A.F. edited and revised manuscript; T.A.F. approved final version of manuscript.

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