Glutamate-dependent astrocyte modulation of synaptic transmission between cultured hippocampal neurons

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Abstract

The idea that astrocytes merely provide structural and trophic support for neurons has been challenged by the demonstration that astrocytes can regulate neuronal calcium levels. However, the physiological consequences of astrocyte–neuron signalling are unknown. Using mixed cultures of rat hippocampal astrocytes and neurons we have determined functional consequences of elevating astrocyte calcium levels on co-cultured neurons. Electrical or mechanical stimulation of astrocytes to increase their calcium level caused a glutamate-dependent slow inward current (SIC) in associated neurons. Microinjection of 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) into astrocytes to prevent the stimulus-dependent increase in astrocyte calcium level, blocks the appearance of the neuronal SIC. Pharmacological manipulations indicate that this astrocyte-dependent SIC is mediated by extracellular glutamate acting on N-methyl-D-aspartate (NMDA) and non-NMDA glutamate receptors. Additionally, stimulation of astrocytes reduced the magnitude of action potential-evoked excitatory and inhibitory postsynaptic currents through the activation of metabotropic glutamate receptors. The demonstration that astrocytes modulate neuronal currents and synaptic transmission raises the possibility that astrocytes play a neuromodulatory role by controlling the extracellular level of glutamate.

Introduction

The role of astrocytes in the nervous system has been traditionally considered to be restricted to structural and trophic support for neurons. However, several observations suggest that astrocytes may have a more active role in the nervous system. Indeed, astrocytes exhibit a form of excitability and communication based on intracellular Ca\(^{2+}\) variations that can result in propagating intercellular Ca\(^{2+}\) waves (Cornell-Bell et al., 1990; Charles et al., 1991; Finkbeiner, 1992; Dani et al., 1992). Astrocytes express several ligand-gated ion channels that can trigger these Ca\(^{2+}\) waves (Cornell-Bell et al., 1990; Jensen & Chiu, 1990; Charles et al., 1991; Dani et al., 1992; Murphy et al., 1993; Krieglert & Chiu, 1993; Duffy & MacVicar, 1995). Neuronal activity may induce astrocyte Ca\(^{2+}\) waves in vitro (Dani et al., 1992) and in situ (Porter & MacCarthy, 1996), indicating that neurons can signal to astrocytes. Finally, astrocytes may in turn signal to neurons, as astrocyte stimulation can evoke neuronal calcium elevations (Nedergaard, 1994; Parpura et al., 1994; Charles, 1994; Hassinger et al., 1995).

Recent studies have demonstrated, using Ca\(^{2+}\) imaging techniques, that either mechanical or electrical stimulation of astrocytes can induce a wave of elevated calcium in astrocytes and cause a delayed calcium signal in adjacent neurons (Nedergaard, 1994; Parpura et al., 1994; Charles, 1994; Hassinger et al., 1995). We now extend these earlier studies by determining the consequences of astrocyte stimulation on electrophysiological properties of hippocampal neurons, and by asking whether this astrocyte–neuron signalling regulates synaptic transmission.

We demonstrate that astrocyte stimulation induces a long-lasting, slow inward current mediated by the activation of both N-methyl-D-aspartate (NMDA) and non-NMDA glutamate receptors. Because this current was insensitive to bath-applied tetanus toxin, which prevents neuronal exocytosis, the glutamate must be directly released from astrocytes. Finally, we show that astrocyte stimulation reduces both excitatory and inhibitory synaptic transmission through the activation of selective presynaptic metabotropic glutamate receptors (mGlRs).

Materials and methods

Culture preparation

Primary cultures of mixed hippocampal neurons and astrocytes from 1- to 3-day-old postnatal rats were prepared as previously described (Basarsky et al., 1994), and were used after 8–20 days in culture. Enriched type-1 astrocytes were prepared as previously described (Parpura et al., 1995a). Cells were initially plated into culture flasks for 8–10 days, and then, following purification, astrocytes were replated onto glass coverslips and used in experiments after 3–4 days. Purity of astrocyte cultures was confirmed by labelling against glial fibrillary acidic protein (GFAP) and found to be greater than 95%.

Electrophysiology

Whole-cell patch clamp recordings were obtained from neurons with an Axopatch-1C amplifier and pClamp software (Axon Instru-
ments; Foster City, CA, USA). External control solution contained (in mM): NaCl, 140; KCl, 5; MgCl₂, 2; CaCl₂, 2; HEPES, 10; glucose, 10; sucrose, 6 (pH 7.35). In Mg²⁺-free solution, Mg²⁺ was substituted by Ca²⁺ and 10 µM glycine was added. Patch pipette solution contained (in mM): K-glucuronate, 140; EGTA, 10; Mg-ATP, 4; Tris-GTP, 0.2; HEPES, 10 (pH 7.35). Unless stated otherwise, the membrane potential was held between −70 and −60 mV in some cases while studying postsynaptic currents (PSCs), the holding potential was −40 mV, while glutamatergic excitatory postsynaptic currents (EPSCs) and GABAergic inhibitory postsynaptic currents (IPSCs) appeared as inward and outward currents, respectively (Basarsky et al., 1994; Tradewell et al., 1996).

The morphological identification of neurons was confirmed electrophysiologically by their ability to generate tetrodotoxin (TTX)-sensitive Na⁺-mediated action potentials and by the presence of fast synaptic currents. Confluent astrocytes, ≈ 25–150 µm from the soma of the neuron recorded from patch clamp recordings, were stimulated electrically or mechanically using glass micropipettes filled with external saline. For each neuron, up to four different astrocytes were stimulated at intervals of greater than 1 min using four voltage pulses (1 ms duration, 150 V) delivered at 10 Hz over the cellular surface. Similar results were obtained by mechanical stimulation elicited by gently tapping the astrocytes with a micropipette (Nedergaard, 1994). Unless stated otherwise, at least eight astrocytes were stimulated in each parallel control and test condition, and data were obtained from at least three different cultures (i.e. at least 24 astrocytes were stimulated in each condition). In some experiments, cells were incubated for 30–60 min with 1 µM taspigrargin.

Presynaptic neurons were extracellularly stimulated with glass micropipettes filled with external solution and placed over the soma. Suprathreshold voltage pulses (0.5–1 ms duration, 20–150 V) were continuously delivered at 0.5–1 Hz. Reductions in evoked synaptic currents were determined to be significant for each astrocyte–neuron pair by comparing the mean amplitude of synaptic currents evoked 30 s before and after astrocyte stimulation (Student’s t-test comparison). Astrocyte-induced changes in spontaneous postsynaptic current (sPSC) frequency were determined for each cell pair by a similar method by comparing the mean frequency 15–30 s before and after astrocyte stimulation. The incidence of astrocyte-induced responses was defined as the proportion of responses relative to the total number of astrocytes on each experimental day. Therefore, for this variable, n values correspond to the number of experiments, whereas for the other variables, e.g. the amplitude of the slow inward current (SIC), n represents the number of cells examined. Statistical differences were established using the Student’s t-test unless stated otherwise. All experiments were performed at room temperature (20–23 °C). Data are expressed as mean ± SEM.

Tetanus toxin treatment

In some experiments, we treated cultures with tetanus toxin to cleave the neuronal protein synaptobrevin and to block synaptic transmission. Cells were incubated overnight with 6.6–33 nM of tetanus toxin (C-FITC, List Biological Laboratories, Campbell, CA, USA) by a modification of a previously described procedure (Charles, 1994). Cells were incubated for 1 h in 10 µg/mL of C-FITC at 37 °C. Following washing, cells were either viewed live, or in other experiments were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 30 min for parallel immunocytochemistry. A monoclonal antibody (clone 69.1, 1:500; provided by Dr R. Jahn) was used to probe for synaptobrevin II. Visualization was accomplished using rhodamine-conjugated secondary antibody and conventional epi-fluorescence microscopy.

Calcium measurements

The ability of electrical stimuli to evoke a wave of elevated calcium in astrocytes was monitored by fluorescence microscopy using fluo-3 as a calcium indicator. Cultures were loaded with fluo-3 by incubation at 37 °C for 45 min in the presence of fluo-3-AM (10 µg/mL; Molecular Probes, Eugene, OR). After washing, indicator was allowed to de-esterify for 45 min. Coverslips containing fluo-3-loaded cells were visualized using a silicon-intensified target (SIT) camera (Hamamatsu, Bridgewater, NJ, USA) or IC-300 intensified CCD camera (Photon Technology International, Monmouth Junction, NJ, USA) attached to a Nikon 500 inverted microscope and a NeDLC optical workstation (Prairie Technologies, LLC, Waunakee, WI, USA).

Microinjection of BAPTA into astrocytes

In some experiments we injected the calcium chelator 1,2-bis(2-amino-phenoxo)ethane-N,N,N’,N’-tetraacetic acid (BAPTA) into individual astrocytes to determine the requirement for a calcium elevation in astrocytes for astrocyte–neuron signalling. Microinjection pipettes with a tip diameter of ≈ 400 nm were pulled from Kwik-Fil borosilicate glass capillaries (World Precision Instruments, Sarasota, FL, USA) using a Sutter P-2000 micropipette puller (Sutter Instrument, Novato, CA, USA). Pipettes were filled with a solution containing 0.375 mM BAPTA (pH 7.2) and 0.25 mM fluoro-ruby. This solution was pressure-injected into single astrocytes by a 300 ms pulse of 15 p.s.i. using an Eppendorf micromanipulator and a Narishige IM-200 microinjector (Narishige, Greenvale, NY, USA). Based on quantification of the fluoro-ruby fluorescence, we estimated that the injection led to about a 250-fold dilution of the pipette solution, resulting in a final intracellular BAPTA concentration of 1–2 mM.

Results

Neuronal responses to astrocyte stimulation

To study astrocyte–neuron signalling, we established mixed cultures of astrocytes and neurons isolated from postnatal hippocampus. A single astrocyte was electrically or mechanically stimulated (Fig. 1A) to cause an elevation of calcium in that cell. Subsequently, this calcium elevation spread to neighbouring astrocytes in a propagating wave. To detect neuronal responses, we recorded in the whole cell configuration from adjacent neurons. Stimulation of astrocytes evoked three types of neuronal responses: (i) a long-lasting SIC; (ii) a long-lasting increase in the frequency of the sPSCs (excitatory and/or inhibitory, e.g. Fig. 1B2 and Fig. 7B); or (iii) both (Fig. 1B). This increase in postsynaptic currents is likely to be the result of astrocytes activating synapses which in turn release transmitter onto the neuron that is recorded (see below).

The astrocyte-evoked neuronal responses were elicited by 75 ± 2% (mean ± SEM; n = 26 experiments) of stimulated astrocytes, with the SIC being detected in 46 ± 3%. The SIC developed slowly, reaching a maximum amplitude of 37.5 ± 2.4 pA (n = 235; only currents ≥ 10 pA were considered to be astrocyte-evoked SIC, i.e. at least three times higher than the standard deviation of the baseline noise) after 1–15 s and lasted for a duration of up to 75 s (Fig. 1B1).

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Fig. 1. Neuronal responses to electrical stimulation of astrocytes. (A) Schematic diagram representing a confluent layer of astrocytes (thin lines) and two neurons (thick lines). Astrocytes were mechanically or electrically stimulated with a glass micropipette, and whole-cell currents were recorded from adjacent neurons. To study the astrocyte-induced effects on synaptic transmission, the presynaptic neuron was electrically stimulated using a third electrode. (B) Three typical neuronal responses were evoked by astrocyte stimulation: a long-lasting, SIC (B1), a long-lasting increase of spontaneous PSCs (B2), or both (B3). Asterisks indicate stimulus artifacts. Note that these artifacts were only present when electrical stimuli were provided. (C) Relative number of stimulated astrocytes that evoked neuronal responses and percentage of each type of astrocyte-evoked neuronal response (n = 26). Bars represent mean ± SEM.

The sPSC frequency increase was elicited by 18 ± 2% of the stimulated astrocytes. The mechanism of the astrocyte-induced modulation of spontaneous synaptic transmission was not further investigated in this study. Astrocyte-induced neuronal responses were not prevented by 1 µM TTX (73 ± 8% and 75 ± 6% in control and TTX, respectively; n = 4 experiments). In agreement with the presence of the SIC, stimulation of astrocytes elicited a slow, long-lasting neuronal depolarization that could trigger trains of action potentials (not shown) when recorded in current clamp mode, as demonstrated previously by Hassinger et al. (1995).

Tetanus toxin C-FITC labelling of neuronal processes

In these experiments, we applied stimuli from a pipette that is immediately above an astrocyte. Because phase-contrast optics were used to position the stimulating pipette, it is possible that a neuronal process as well as an astrocyte was stimulated. We therefore developed a methodology that would reveal the presence of neuronal processes in living preparations so that we could selectively stimulate neurite-free regions of astrocytes. Previous studies have indicated that neurons, but not astrocytes, express receptors for tetanus toxin (Charles, 1994; Ahnert-Hilger & Bigalke, 1995). We confirmed the selective presence of tetanus toxin receptors in our cultures using the FITC-labelled C fragment of tetanus toxin. In mixed neuron and astrocyte cultures, we found that tetanus toxin C-FITC labelled the soma and neurites of neurons (Fig. 2A). By contrast, when purified astrocyte cultures were used, tetanus toxin labelling was absent (Fig. 2B). Therefore, tetanus toxin C-FITC can be used to selectively label neurons in mixed cultures containing both astrocytes and neurons.

We made use of the ability to selectively label neurons to identify the location of neurites in mixed astrocyte–neuron cultures using tetanus C-FITC and phase-contrast microscopy. We guided our stimulating electrode to a region of an astrocyte which was devoid of neuronal processes (Fig. 2C,D), provided a local stimulus, and determined the neuronal electrophysiological response. When the stimulation pipette was guided to a neurite-free region of the astrocyte, specific stimulation of the astrocyte evoked a SIC in 75% of the recorded neurons (n = 12). Since this labelling method might not reveal all neuronal processes, we also used fluo-3, which we have demonstrated discloses all neurites (Parpura et al., 1994), to guide the stimulating electrode specifically to an astrocyte. Again, stimulation of the astrocyte reliably evoked neuronal SIC.

Because direct stimulation of the astrocyte leads to a neuronal response, these data strengthen the notion that the neuronal responses shown in Fig. 1 represent responses to stimulation of the astrocyte rather than an effect of direct stimulation of the neuron. However,

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because of the concern that our labelling methods may not have disclosed all neuronal processes, we determined the consequences of neuronal stimulation on postsynaptic recordings. We visually located neuronal processes and stimulated them extracellularly while recording the response in the soma, or alternatively, we stimulated presynaptic neurons. Direct stimulation of a process led to an action potential that was recorded in the soma as a fast inward current (\( n = 14 \)), while presynaptic stimulation evoked synaptic currents (see Figs 7–10). These short latencies events were not detected when we applied the stimulus to the astrocyte, further supporting the argument that we can selectively stimulate an astrocyte which gives rise to the neuronal responses shown in Fig. 1.

**Astrocyte calcium elevation is necessary for astrocyte-induced neuronal SIC**

To further control for non-specific effects of astrocyte stimulation, we determined whether neuronal responses required the presence of a calcium wave in the astrocyte network. Charles et al. (1993) have previously demonstrated that calcium wave propagation between astrocytes is IP3-mediated and requires functional internal calcium stores. By imaging fluo-3-loaded astrocytes, we confirmed their earlier observation that depletion of internal stores by treatment with the Ca\(^{2+}\)-ATPase inhibitor, thapsigargin (1 \( \mu \)M), blocks the spread of the calcium wave between astrocytes (Fig. 3A–D). We then incubated cultures in 1 \( \mu \)M thapsigargin while making electrophysiological recordings of neuronal currents. After thapsigargin treatment, the ability of astrocyte stimulation to evoke the SIC was significantly reduced (Fig. 3E; \( n = 6 \), \( P < 0.001 \)). When the SIC was detected in thapsigargin, its mean amplitude (36.9 ± 4.8 pA) was not significantly different from that recorded in the control (28.9 ± 3.4 pA). Because the incidence but not the amplitude of the SIC was affected by thapsigargin, and because similar reductions were observed in the incidence of Ca\(^{2+}\) waves, it is likely that the remaining responses in thapsigargin were due to incomplete effects of the treatment. Taken together, these results are consistent with the hypothesis that the SIC is a result of the calcium wave in the astrocyte network.

Because thapsigargin could affect neuronal calcium homeostasis as well as astrocyte calcium waves, we developed a method that...
would selectively prevent calcium elevations in astrocytes. We micro-injected the calcium chelator BAPTA into single astrocytes, together with a fluorescent indicator, fluoro-ruby, to label the injected cell. As fluoro-ruby contains a dextran moiety (10 000 MW) conjugated to tetramethylrhodamine, it does not pass through gap junctions and is maintained within the injected cell. First, we determined whether microinjection per se and whether injection of BAPTA affected calcium signalling in response to mechanical stimuli. Using cultures of purified astrocytes loaded with the calcium indicator fluo-3, we found that mechanical stimulation of uninjected cells increased calcium in the majority of cells that were directly stimulated (82 ± 8%, \( n = 9 \) coverslips; Fig. 4C). To evaluate the ability of this stimulus to induce a calcium wave amongst adjacent astrocytes, we determined the proportion of non-stimulated cells within the field of view which responded with a calcium elevation (zero represents no wave, 100% represents a wave amongst all cells in the field of view). On average, 67% of cells within one field of view participated in a calcium elevation (\( n = 19 \) trials; Fig. 4D). In parallel to these experiments on

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Fig. 4. Microinjection of the calcium chelator BAPTA into an astrocyte prevents astrocyte calcium waves and blocks astrocyte-induced neuronal signalling. (A), (B) Purified astrocyte cultures were loaded with the calcium indicator fluo-3 to monitor the ability of mechanical stimulation to induce calcium elevations in astrocytes. (A) A control experiment is shown in which a single astrocyte was microinjected with a carrier solution containing fluoro-ruby (left panel). The centre and right panels show two images in pseudocolour mode representing intensity of fluo-3 emission. The central panel is taken prior to mechanical stimulation of the fluoro-ruby-injected cell. Mechanical stimulation increases intracellular calcium in the injected cell as well as in neighbouring unstimulated astrocytes (right panel). In contrast, (B) shows the result of injecting fluoro-ruby and BAPTA into one astrocyte (left panel). Mechanical stimulation of the injected cell did not change the fluorescent emission of fluo-3 either in the stimulated cell or the neighbouring astrocytes (compare the central and right panels taken before and 8 s after mechanical stimulation). (C), (D) Quantitative data taken from these experiments. BAPTA significantly reduces ($P < 0.01$) the ability of mechanical stimulation to elevate calcium levels in the directly stimulated cell (C) and the proportion of astrocytes involved in a stimulus-induced calcium wave ($P < 0.001$). In parallel studies, neuronal currents were recorded in response to mechanical stimulation of astrocytes. While injection of fluoro-ruby did not prevent an astrocyte-induced SIC (E, G), injection of BAPTA prevented the appearance of a SIC following astrocyte stimulation (F, G; chi-square test, $P < 0.01$), (**$P < 0.01$, *** $P < 0.001$). In all categories $n > 6$.
uninjected cells, single astrocytes were microinjected with fluoro-
ruby. Microinjection of fluoro-ruby did not significantly affect the
ability of a mechanical stimulus to induce a calcium elevation in the
directly stimulated cell (86%), nor the propagation of a calcium wave
(Fig. 4A,C,D).

We next microinjected BAPTA and fluoro-ruby into astrocytes,
and determined the ability of BAPTA (final concentration 1 mM)
to impair calcium waves. Stimulation of BAPTA-injected cells only
led to a calcium elevation in 28% of the directly stimulated cells
(Fig. 4B,C). Additionally, injection of BAPTA/fluoro-ruby into the
stimulated cell significantly attenuated the calcium wave amongst
astrocytes, since on average only 12% of adjacent cells responded
with a calcium elevation as compared to 84% in fluoro-ruby-injected
astrocytes (Fig. 4B,D; \( P < 0.001 \)). Thus, microinjection of BAPTA
specifically into one astrocyte significantly reduces the ability to
generate calcium responses.

We made use of BAPTA injection into astrocytes to ask whether
a calcium elevation within this non-neuronal cell is required for the
generation of the neuronal SIC. Individual astrocytes were injected
with BAPTA/fluoro-ruby or with fluoro-ruby alone. A fluoro-ruby-
positive cell was brought into the field of view and an adjacent
neuron was recorded in the whole-cell configuration. Stimulation of
BAPTA/fluoro-ruby-injected astrocytes only produced a neuronal
response in one out of seven experiments (14%, \( n = 7 \); Fig. 4F,G).
By contrast, stimulation of uninjected astrocytes, or stimulation of
fluoro-ruby-injected astrocytes, evoked neuronal SIC in 61 and 63%
of recorded cells, respectively (Fig. 4E,G). Taken together, these
experiments demonstrate that selective stimulation of astrocytes leads
to a calcium-dependent SIC in adjacent neurons.

The SIC is mediated by both NMDA and non-NMDA
glutamate receptors

Using Ca\(^{2+}\) imaging techniques, two different mechanisms have been
proposed to mediate astrocyte–neuron signalling. In mixed forebrain
cultures from embryonic rats, Nedergaard (1994) found that this
signal was attenuated by gap junction blockers, suggesting that it was
mediated through intercellular connections rather than through a
chemical transmitter. However, Parpura et al. (1994) reported that
astrocyte–neuron signalling in postnatal cultures of visual cortices
was mediated by glutamate released from astrocytes acting on NMDA
receptors, while Hassinger et al. (1995) found that both NMDA and
non-NMDA glutamate receptors were responsible for this signal.

To distinguish between a gap junction and a glutamate-mediated
generation of the neuronal SIC, we studied its pharmacology. The non-NMDA glutamate receptor antagonist 6-cyano-7-nitroquinox-
aline-2,3-dione (CNQX; 20 \( \mu \)M) reduced the incidence of the SIC
\( (P < 0.01) \), and the combination of CNQX and \( N \)-2-amino-5-phos-
phonopentanoic acid (D-AP5; 50 \( \mu \)M; an NMDA glutamate receptor
antagonist) abolished the SIC \( (P < 0.001) \) [Fig. 5A]. Removal of
external Mg\(^{2+}\) and the addition of 10 \( \mu \)M glycine (Mg\(^{2+}\)-free solution)
enhanced the magnitude of the SIC (Fig. 5B,C) without changing the
proportion of astrocytes that evoked the SIC. D-AP5 significantly
\( (P < 0.001) \) attenuated the magnitude of the SIC in Mg\(^{2+}\)-free
solution (to 49 ± 11 pA, \( n = 20 \), from 318 ± 60 pA, \( n = 19 \); Fig. 5C).

**Fig. 5.** Astrocyte-evoked SIC is mediated by both NMDA and non-NMDA
ionotropic glutamate receptors. (A) Percentage of astrocytes that evoked a SIC in
controls (Control), 0.5 mM MAP4, 0.5 mM MCPG, 20 \( \mu \)M CNQX, 50 \( \mu \)M D-AP5
and Mg\(^{2+}\)-free solution, and their respective control solutions in parallel cultures.
\( n \geq 3 \) for each group. (B) SIC in control and Mg\(^{2+}\)-free solution, where the membrane current noise,
probably NMDA receptor-mediated, conspicuously increased after astrocyte
stimulation. Asterisks indicate stimulus artefacts. (C) Mean amplitude of SIC
detected under the different recording conditions. \( n > 15 \) for each group
except for CNQX, where only four neurons had a detectable SIC due to the
reduction in the number of responses. In CNQX + D-AP5, SIC amplitude
was considered zero since no responses were observed. Significant differences
with respect to the control were established by the Student’s t-test at \( P < 0.05 \)
(∗), \( P < 0.01 \) (**) and \( P < 0.001 \) (***)
Neither the mGluR antagonist (S)-2-amino-2-methyl-4-phosphono-butyric acid (MAP4; 0.5 mM) nor (S)-α-methyl-4-carboxyphenyl-glycine (MCPG; 0.5 mM) altered the incidence or amplitude of the astrocyte-evoked SIC (Fig. 5A). Taken together, these results indicate that SIC was mediated by both NMDA and non-NMDA ionotropic, but not metabotropic, glutamate receptors.
The astrocyte-induced glutamate-dependent SIC is insensitive to tetanus toxin

While astrocytes were directly stimulated in our experiments, it is feasible that an astrocyte signal modulated glutamate release from neurons to produce the SIC. To test this possibility, we incubated cultures in tetanus toxin, a highly selective protease that cleaves the synaptic protein, synaptobrevin, and renders neuronal synapses non-functional (Link et al., 1992; Schiavo et al., 1992). After treatment with tetanus toxin, we then asked whether it blocked the astrocyte-induced neuronal SIC.

Because high concentrations of toxin receptors are present on nerve cells, but are practically absent on glial cells (Ahnert-Hilger & Bigalke, 1995; Fig. 2A,B), this toxin should act selectively on neurons. Although it has been reported that tetanus toxin can reduce glutamate release from astrocytes (Jeflinia et al., 1997), it is clear that short to intermediate periods of toxin incubation do not (Bezzi et al., 1998). We confirmed that these receptors are preferentially expressed on neurons by incubating cultures in fluorescein-conjugated C-fragments of tetanus toxin (Figs 2 and 6A,B). To experimentally determine whether tetanus toxin acts selectively on neurons, we performed immunocytochemistry for the vesicle protein synaptobrevin, which is expressed in astrocytes as well as neurons, after incubation of cultures in the tetanus toxin holoprotein (Fig. 6C–F) [Parpura et al., 1995b]. Subsequent immunocytochemistry supported the selectivity of toxin action since it revealed an absence of synaptobrevin in neurons (Fig. 6D), but a retention of positive immunoreactivity in astrocytes (Fig. 6F).

As reported previously (e.g. Trudeau et al., 1996), incubation in tetanus toxin abolished both spontaneous and evoked synaptic transmission (Fig. 7A). Despite this abolition of synaptic transmission, stimulation of astrocytes still reliably evoked a SIC in neurons (Fig. 7B). Neither the proportion of astrocytes that evoked neuronal responses nor the mean SIC amplitude were significantly changed by bath application of tetanus toxin (Fig. 7C), indicating that the neuronal responses to astrocyte stimulation are due to glutamate released from the astrocytes. Because tetanus toxin abolished both inhibitory and excitatory synaptic currents, the astrocyte-induced increase in frequency of excitatory and inhibitory miniature synaptic currents (Figs 2 and 7) is likely to be due to the activation of interposed synaptic terminals.

Astrocytes modulate both excitatory and inhibitory synaptic transmission

As glutamate can modulate synaptic transmission (Forsythe & Clements, 1990; Baskys & Malenka, 1991; Zorumski et al., 1996), we explored the effects of stimulating astrocytes on synaptic transmission between cultured neurons. To monitor synaptic transmission, we recorded whole-cell from one neuron while stimulating another with an extracellular patch pipette. Control experiments in which the extracellular pipette was placed adjacent to the recorded neuron confirmed that each extracellular stimulus evoked a single action potential in the neuron. Additionally, when the pipette was placed over the neuron, stimuli that evoked action potentials did not simultaneously activate a calcium wave in the underlying astrocyte. After obtaining a baseline measurement of synaptic transmission, an astrocyte was stimulated to induce a wave of elevated calcium. Astrocyte stimulation reduced the amplitude of both action potential-evoked EPSCs and IPSCs (Figs 8 and 9, respectively). The transient decrease in evoked synaptic transmission lasted for tens of seconds and was usually accompanied by an increase in the number of failures in synaptic transmission (Figs 8B and 9B). The amplitude of evoked EPSCs and IPSCs was decreased by 33 ± 4% (n = 18) and 30 ± 4% (n = 10), respectively, and this reduction was induced by 62 ± 9% and 88 ± 13% of the stimulated astrocytes (n = 4 experiments). In control experiments, in which astrocytes were not stimulated, we confirmed that the magnitude of the synaptic current was essentially unchanged during the period of recording (99% of initial amplitude; n = 11). While evoked synaptic currents were reduced in magnitude by astrocyte stimulation, the mean amplitudes of miniature EPSCs (nEPSCs; n = 17) and IPSCs (nIPSCs; n = 12) [recorded in the presence of 1 µM TTX] were not significantly changed by astrocyte stimulation (Figs 8C and 9C, respectively; Kolmogorov–Smirnov test).

To begin to address the mechanism of synaptic modulation by astrocytes, we asked whether there was an obligatory relationship between the presence of the astrocyte-induced SIC and the reduction in evoked synaptic transmission. In 14 out of 41 examples, astrocyte stimulation elicited both a SIC and the modulation of evoked synaptic transmission. In 14 other examples, evoked synaptic transmission was modulated without an accompanying postsynaptic SIC, while in six others a SIC was detected without an associated modulation of the synapse. Therefore, the astrocyte-induced SIC is not necessary for the modulation of synaptic transmission.

Presynaptic mGluRs have been shown to reduce both excitatory and inhibitory synaptic transmission in hippocampal neurons. A depression of inhibitory synaptic transmission is mediated by L-AP4-insensitive, MCPG-sensitive mGluRs (groups I and II in CA1 and CA3 pyramidal neurons, respectively; Gereau & Conn, 1995; Ponce et al., 1995; Fitzsimonds & Dichter, 1996). Excitatory synaptic transmission reduction is ubiquitously due to the activation of MAP4-sensitive group III mGluRs (Baskys & Malenka, 1991), although group I (Gereau & Conn, 1995; Manzoni & Bockaert, 1995) or group II mGluRs (Manzoni et al., 1995; Ugolini & Bordi, 1995; Vignes et al., 1995; Bushell et al., 1996; Yoshino et al., 1996) can also be involved depending on the hippocampal area, age and species considered. To determine whether the astrocyte-induced modulation of the synaptic transmission required the activation of mGluRs by glutamate released after astrocyte stimulation, we determined the sensitivity of this modulation to mGluR antagonists. The proportion of astrocytes that modulated EPSCs was significantly decreased by 0.5 mM MAP4 (P < 0.05), but unchanged by 0.5 mM MCPG (Fig. 10A), whereas the astrocyte-induced modulation of IPSCs was insensitive to MAP4 but antagonized by MCPG (P < 0.01) (Fig. 10B).

Fig. 6. Tetanus toxin cleaves synaptobrevin II in neurons, but not in astrocytes. (A) Labelling of mixed astrocyte–neuron cultures with fluorescein-conjugated C-fragment of tetanus toxin specifically labels neurons in a punctate fashion. This punctate staining was apparent even after treatment with holoprotein tetanus toxin (TeTx) which cleaves synaptobrevin (B). The efficacy of tetanus toxin action on cells was determined by immunolabelling of cultures with antibody against synaptobrevin II. Punctate pattern of synaptobrevin II staining was present in untreated neurons (C; same culture field as in A), but not in tetanus toxin-treated neurons (D; same field as in B). To determine whether the active form of tetanus toxin selectively cleaves synaptobrevin in neurons, purified astrocyte cultures were treated with the toxin. Anti-synaptobrevin II staining of astrocytes was unaffected by the tetanus toxin treatment (E, F). (Note that the synaptobrevin immunostaining of astrocytes is less intense than neurons, making it necessary to both increase the excitation intensity and camera gain to detect positive immunoreactivity.) Scale bars indicate 20 µm.
FIG. 7. Tetanus toxin abolished spontaneous and evoked synaptic transmission, but did not prevent astrocyte-evoked neuronal responses. (A) EPSCs (five superimposed traces) evoked by extracellular stimulation of a neuron in control conditions were absent in tetanus toxin-treated cultures. The presence of EPSCs and IPSCs was assayed by extracellular stimulation of potential presynaptic neurons. Asterisks indicate stimulus artefacts. (B) Astrocyte-evoked neuronal responses in control and tetanus toxin-treated cells. Transient outward currents in control conditions correspond to astrocyte-evoked IPSCs. Note that after tetanus toxin treatment, astrocytes still evoked a neuronal SIC, but neither spontaneous IPSCs nor EPSCs. (C) Percentage of neurons with spontaneous and evoked postsynaptic currents (sPSCs and ePSCs, respectively; at least 16 postsynaptic and potential presynaptic neurons were tested in each group), and the percentage of astrocytes that evoked neuronal responses ($n \geq 34$ for each group), and amplitude of the SIC ($n \geq 21$ for each group) in control and tetanus toxin-treated cells.

Discussion

In this study we have used ion imaging and electrophysiological approaches to evaluate the functional consequences of astrocyte-neuron signalling on neuronal physiology. In order to study astrocyte-neuron signalling, it was essential that selective stimuli were applied to the astrocyte while responses of the neuron were recorded. One experimental approach would have been to use neuromodulators, which induce calcium elevations in astrocytes, and then to have determined the consequences for the neuron. However, the concern that neuronal responses may be mediated at least in part by these modulators mitigated this approach. Instead we used mechanical and electrical stimuli that can be directed to localized regions of a cell. Following delivery of either stimulus to astrocytes, we determined that neurons responded with a glutamate-receptor-dependent SIC and that this same stimulus could induce the modulation of action potential-evoked synaptic transmission.

While it is clear that mechanical and electrical stimuli raise astrocyte calcium levels, care was taken to establish the selectivity of the stimulus. By staining neuronal processes with tetanus toxin C-FITC, we were able to guide the simulating pipette to areas of astrocytes in which neuronal processes were absent. Additionally, two experimental manipulations that prevent the stimulus-induced astrocyte calcium wave, thapsigargin and BAPTA injection, prevented the stimulus-induced neuronal responses. Of particular importance was the microinjection of BAPTA. Because injection of this calcium chelator into one astrocyte prevented both the stimulus-induced astrocyte calcium elevation as well as the neuronal SIC, we can conclude that the glutamate-dependent neuronal modulation is a consequence of the calcium elevation in the astrocyte.

Our present data extend the previous observations of astrocyte-neuron signalling (Nedergaard, 1994; Parpura et al., 1994; Charles, 1994; Hassinger et al., 1995) by demonstrating that experimentally
induced calcium elevations in astrocytes cause a delayed glutamate-
dependent SIC in hippocampal neurons and a mGluR-dependent
inhibition of inhibitory and excitatory synaptic transmission, which
based on previous studies is likely to be presynaptic in origin (see
e.g. Forsythe & Clements, 1990; Baskys & Malenka, 1991; Gereau
& Conn, 1995). In support of a presynaptic modulation, we detected
an increase in the number of failures in evoked synaptic transmission
with no associated change in amplitude of miniature synaptic currents.
However, one might predict a simultaneous reduction in miniature
frequency. In our experiments though miniature synaptic current
frequency increased in some preparations. As mGluR antagonists did
not attenuate this increase in miniature frequency (not shown), we
anticipate that is due to the operation of a separate mechanism.
Despite the sustained presence of glutamate during the SIC, desensit-
ization of miniature synaptic currents was not detected. Because
even low concentrations of extracellular glutamate can lead to receptor
desensitization (Trussel & Fischbach, 1989; Zorumski et al., 1996),
our data suggest that glutamate released from astrocytes does not act
equally at all sites on the neuron. Perhaps there are discrete sites of
release which spare receptors in the postsynaptic aspect of the synapse
from desensitization but that permit activation of presynaptic mGluRs.
Alternatively, glutamate uptake mechanisms may spare the synaptic
cleft from a sustained elevation of glutamate during astrocyte–neuron
signalling. Interestingly, type 2 mGluRs have been described to be
localized at the preterminal zone rather than at the synaptic junction
in the mossy fibre-CA3 synapse (Yokoi et al., 1996), and such a
spatial distribution of ionotropic and metabotropic receptors could
account for the specific effects observed.

Astrocyte stimulation leads to three distinct neuronal consequences:
a non-NMDA and NMDA-dependent ionotropic SIC, a mGluR-
dependent presynaptic inhibition of transmitter release from synaptic
terminals, and an increase in the frequency of ‘spontaneous’ synaptic
currents. Because astrocyte stimulation can depolarize neurons beyond
their firing threshold, the astrocyte-induced increase in spontaneous
PSC frequency might easily be explained by assuming that these
PSCs are evoked by action potentials. However, as TTX did not
prevent that neuronal response, it is possible that the astrocyte directly
influences the presynaptic terminal. The particular nature of this action,
however, requires further investigation. Irrespective of mechanism, it
is likely to be independent of mGluRs because agonists for these
receptors either do not influence or reduce the frequency of TTX-
insensitive miniature synaptic currents (Gereau & Conn, 1995).

There are at least three potential sources for glutamate to mediate
the astrocyte–neuron signalling. First, astrocytes might stimulate the
release of glutamate from neuronal synaptic terminals to induce the
SIC. However, this is unlikely given that incubation of cultures in
tetanus toxin cleaves the neuronal synaptic protein synaptobrevin
and blocks neuronal exocytosis without impairing astrocyte–neuron
signalling (Fig. 7). Additionally, our previous demonstration (Parpura
et al., 1994) that elevated astrocyte calcium is both necessary and
sufficient to induce the release of glutamate into the superfusate in
purified astrocyte cultures indicates that glutamate can be provided
by the astrocyte. Second, stimulation of the astrocyte might induce a
depolarization-dependent reversal of glutamate transporters to elevate
the extracellular levels of glutamate in the immediate environment
of the neuron. Certainly, depolarization-induced transporter reversal
Fig. 9. Astrocyte stimulation decreased the amplitude of evoked IPSCs. (A) Averaged \((n = 10)\) IPSCs evoked by extracellular stimulation at 0.5 Hz at the times indicated by the numbers in (B). Holding potential was –60 mV. Currents are offset for illustration purposes. (B) IPSC amplitude versus time. Zero time corresponds to the time of astrocyte stimulation. (C) Cumulative probability distribution of the amplitude of mIPSCs (recorded in 1 µM TTX) 30 s before and after astrocyte stimulation (open and filled symbols, respectively) \([n = 12]\). Holding potential was between –30 and –10 mV.

Fig. 10. Astrocyte stimulation decreased evoked EPSCs and IPSCs through the activation of different mGluRs. (A, B) Proportion of astrocytes that when stimulated reduced the amplitude of evoked EPSCs and IPSCs, respectively, in control, 0.5 mM MAP4 and 0.5 mM MCPG. Significant differences with respect to the control were established by the Student’s \(t\)-test at \(P < 0.05\) (*) and \(P < 0.01\) (**).

can lead to glutamate actions on neurons when the astrocyte is recorded with a patch pipette containing a reservoir of glutamate (Mennerick & Zorumski, 1994). However, glutamate transport inhibitors do not prevent stimulus-induced astrocyte–neuronal signalling (Parpura et al., 1994). This is perhaps not surprising given that glutamate is rapidly converted to glutamine in astrocytes by glutamine synthetase, which would significantly reduce the concentration of this transmitter in the astrocyte cytosol. A third possibility is that a calcium-dependent exocytotic mechanism mediates glutamate release from astrocytes. While critical tests of this hypothesis have not been
performed, the calcium dependence of astrocyte–neuronal signalling supports such a hypothesis. Additionally, storage of glutamate in a vesicular compartment would overcome the problem of metabolism of cytosolic glutamate by glutamine synthetase activity.

Our studies point to a potential functional role for astrocytes in modulating neuronal activity and synaptic transmission in vivo. However, our study was performed in conditions far removed from the central nervous system (CNS) as we used artificial stimuli to elevate astrocyte calcium levels within a cell culture environment. The question that results is whether such a signalling pathway could be activated in vivo. A recent study by Pasti et al. (1997) suggests that there can be bi-directional glutamate-dependent communication between astrocytes and neurons in acutely isolated hippocampal slices. These data suggest that astrocyte modulation of neuronal activity and synaptic transmission could well play a role in integration within the CNS. What are the signals that initiate calcium elevations in astrocytes and evoke astrocyte-induced neuromodulation? It has already been determined that glutamate released from neurons can lead to calcium elevations in adjacent astrocytes (Dani et al., 1992; Porter & McCarthy, 1996). Thus, 'spillover' from glutamatergic synapses within the hippocampus might activate feedback modulation from astrocytes. Perhaps astrocytes contribute glutamate to the recently reported use-dependent activation of presynaptic metabotropic receptors (Scanziani et al., 1997). Another possibility is that the release of a neuronal transmitter, e.g. norepinephrine, a transmitter that has been demonstrated to elevate astrocyte calcium in acutely isolated slices (Duffy & MacVicar, 1995), would activate this astrocyte neuromodulatory pathway. While these possibilities need to be experimentally evaluated, we have previously demonstrated that a neuropeptide, bradykinin, which mobilizes specifically astrocyte calcium, causes glutamate-dependent astrocyte–neuron signalling in vitro (Parpura et al., 1994). Given that this physiological stimulus is able to induce astrocyte–neuron signalling, coupled with the recent demonstration of bidirectional communication between astrocytes and neurons in hippocampal slice (Pasti et al., 1997), one must consider the possibility that astrocytes play an additional role in the CNS in modulating electrical activity and synaptic transmission.

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