Hippocampal Astrocytes In Situ Exhibit Calcium Oscillations That Occur Independent of Neuronal Activity

WOLFGANG J. NETT, SCOTT H. OLOFF, AND KEN D. MCCARTHY
Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599-7365

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Nett, Wolfgang J., Scott H. Oloff, and Ken D. McCarthy. Hippocampal astrocytes in situ exhibit calcium oscillations that occur independent of neuronal activity. J Neurophysiol 87: 528–537, 2002; 10.1152/jn.00268.2001. Results presented in this study indicate that a large subpopulation (~65%) of hippocampal astrocytes in situ exhibit calcium oscillations in the absence of neuronal activity. Further, the spontaneous oscillations observed within individual hippocampal astrocytes generally developed asynchronously throughout the astrocyte's fine processes and occasionally spread through a portion of that astrocyte as a calcium wave but do not appear to spread among astrocytes as an intercellular calcium wave. Bath application of cyclopiazonic acid and injection of individual astrocytes with heparin blocked astrocytic calcium oscillations. Application of tetrodotoxin or incubation of slices with bafilomycin A1 had no effect on astrocytic calcium oscillations but did block evoked and spontaneous postsynaptic currents measured in CA1 pyramidal neurons. Application of a cocktail of antagonists for metabotropic glutamate receptors and purinergic receptors had no effect on the astrocytic calcium oscillations but blocked the ability of purinergic and metabotropic glutamatergic agonists to increase astrocytic calcium levels. These results indicate that the spontaneous calcium oscillations observed in hippocampal astrocytes in situ are mediated by IP3 receptor activation, are not dependent on neuronal activity, and do not depend on activation of metabotropic glutamate receptors or purinergic receptors. To our knowledge, this is the first demonstration that astrocytes, independent of neuronal input, may act as pacemakers to modulate neuronal activity in situ.

INTRODUCTION

In recent years, many studies have provided evidence for bi-directional communication between astrocytes and neurons. Cultured astroglia in vitro and astrocytes in situ express a variety of receptors for neurotransmitters (Deitmer et al. 1998; Porter and McCarthy 1997; Verkhratsky et al. 1998). It has been shown that synaptically released glutamate can trigger astrocytic calcium increases in situ (Pasti et al. 1997; Porter and McCarthy 1996), indicating that neurotransmitter released from neurons activates astrocytic receptors in situ. Furthermore, results from in vitro studies indicate that calcium elevations in astroglia lead to calcium elevations in adjacent neurons (Charles 1994; Hassinger et al. 1995; Nedergaard 1994; Parpura et al. 1994). More recently, it has been shown in culture that a calcium increase in astroglia is both necessary and sufficient to induce glutamate release from astroglia, which leads to changes in the synaptic transmission between adjacent neurons (Araque et al. 1998a,b). Other studies indicate that calcium-dependent glutamate release from astrocytes may also occur in situ (Bezzi et al. 1998). Overall, the results from a number of laboratories suggest that astrocytes may modulate neuronal activity in vivo through calcium-dependent glutamate release. The results of above-mentioned studies provide very good evidence that astrocytes respond to synaptically released neurotransmitter with either sustained or oscillatory increases in intracellular calcium in situ. In contrast, astrocytic signaling in situ, independent of neuronal activity, has not been demonstrated.

In this study we investigated spontaneous calcium oscillations in the cell bodies and processes of hippocampal astrocytes from 10- to 17-day-old mice. We found that astrocytic calcium oscillations occur independent of neuronal activity. Spontaneous astrocytic calcium oscillations frequently developed within discrete regions of astrocytic processes and occasionally spread through the astrocytic processes as a calcium wave. However, calcium waves did not appear to spread to adjacent astrocytes even though adjacent astrocytes were capable of exhibiting calcium responses. Pharmacological experiments indicated that the spontaneous astrocytic calcium oscillations were not due to the activation of either ATP or glutamatergic receptors but were antagonized by agents that block IP3 receptors or deplete calcium stores within the endoplasmic reticulum. Overall, our findings indicate that a subpopulation of astrocytes exhibit intrinsic signaling that may serve to modulate neuronal activity.

METHODS

Slice preparation

Parasagittal hippocampal slices (300 μm thick) were prepared from 10- to 17-day-old C57/BL6 mice (Jackson Laboratory, Bar Harbor, ME), using a Vibroslice (Campden Instruments, Sileby, UK). Slices were prepared in ice-cold, nominally calcium-free saline containing (in mM) 125 NaCl, 3.5 KCl, 1.25 CaCl2, 1 NaH2PO4, 26.6 NaHCO3, 25 glucose, and 0.1 kynurenic acid, bubbled with 5% CO2-95% O2. Subsequently, slices were stored at room temperature (21–23°C) in artificial cerebrospinal fluid (ACSF) containing (in mM) 125 NaCl, 3.5 KCl, 2.5 CaCl2, 1.3 MgCl2, 1 NaH2PO4, 26.6 NaHCO3, and 25 glucose, bubbled with 5% CO2-95% O2. In a few experiments...
the calcium concentration of ACSF was decreased from 2.5 to 2 mM; this change did not noticeably affect the calcium oscillations. Slices were continuously superfused with oxygenated ACSF once the slices were transferred to the analysis/perfusion chamber.

**Bulk loading of slices with AM dye and immunostaining**

Slices were incubated for 80–90 min at 35–37°C in oxygenated loading buffer consisting of 11 μM Calcium Green-1 AM and 0.07% pluronic acid in ACSF (final DMSO concentration: 0.4%). The Calcium Green was primarily sequestered by astrocytes as observed previously with slices from rats (Porter and McCarthy 1996). Loading of astrocytes was confirmed in five slices that were immunostained for the glial cell markers GFAP and S100 after the calcium measurements were performed. Slices were fixed for 2–6 h in ACSF containing 40 mg/ml 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), which preserves the Calcium Green-1 within the cells (Fern 1998; Tymianski et al. 1997). Slices were then washed in PBS containing 0.2 M glycine, and 0.1% BSA for 1 h, and immunostained for GFAP and S100 to identify astrocytic processes and cell bodies, respectively (Schmidt-Kastner and Szymas 1990).

**Dye loading of individual astrocytes via patch pipettes**

Whole cell patch-clamp recordings were obtained from astrocytes using a Dagan 3900 (Dagan, Minneapolis, MN) or an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) and pClamp software (Axon Instruments). Experiments were performed on an Olympus BX50WI fixed-stage upright microscope (Olympus, Melville, NY) equipped with Nomarski optics and an infrared video camera (CCD100, Dage-MTI, Michigan City, IN). Patch pipettes were pulled from borosilicate glass (WPI, Sarasota, FL) on a Narishige PP-83 pipette puller (East Meadow, NY). The pipette resistance was 6–10 MΩ when filled with the intracellular solution containing 130 mM K-gluconate, 4 mM Na-creatine phosphate, 130 mg/ml creatine phosphokinase, and 200 mM 0.2 M glycin, and 0.1% BSA for 1 h, and immunostained for GFAP and S100 to identify astrocytic processes and cell bodies, respectively (Axon Instruments). Experiments were performed on an Olympus BX50WI fixed-stage upright microscope. The pipette was carefully withdrawn within 2–4 min after the whole cell configuration was established. Calcium imaging experiments were started after at least 15 min to allow for diffusion of the dye into the astrocytic processes. Since cells did not always survive withdrawal of the patch pipette, a calcium response to tACPD application was used as a positive control for cell viability.

**Calcium measurements**

Calcium measurements were performed with an Olympus GB200 confocal scanner attached to the upright BX50/WI microscope. The dyes were excited by the 488-nm line of a krypton/argon laser, and emission was collected from predefined regions of interest at >515 nm. Average fluorescence intensities from rectangular regions of interest (ROI) that were placed over cell bodies or processes were digitized (8 bit), normalized to baseline level (F/F₀), and plotted over time. Changes in the baseline level indicate increases in calcium concentration. Studies from our laboratory have shown previously that the vast majority of astrocytes respond to tACPD with an increase in calcium (Porter and McCarthy 1995). To make sure that the lack of spontaneous calcium increases in some astrocytes was not due to an inability of the cells to show a calcium increase, the mGlur agonist tACPD was applied at the end of each experiment as a positive control, and cells that did not respond to tACPD were excluded from the analysis.

**Materials**

Calcium Green-1 AM, Oregon Green 488 BAPTA-1, Pluronic acid, and Alexa-488–labeled goat anti-rabbit antibodies were obtained from Molecular Probes (Eugene, OR). Rabbit polyclonal primary antibodies were purchased from Dako (GFAP and S-100; Carpinteria, CA). tACPD [(±)-1-aminocyclopentane-trans-1,3-dicarboxylic acid] and bafilomycin A1 were obtained from Alexis (San Diego, CA). Kynurenic acid, cyclopiazonic acid, 8(p-sulfophenyl)theophylline, and suramin were purchased from Sigma-RBI (St. Louis, MO). RS-1-aminoindan-1,5-dicarboxylic acid (AI1). RS-α-cyclopropyl-4-phosphonophenylglycine (CPPG), 2-methyl-6-(phenylethyl) pyridine (MPEP), and pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid (PAPDS) were purchased from Tocris Cookson (Ballwin, MO). Heparin, 9,21-dehydro-ryanodine, dantrolene, and EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide] were purchased from Calbiochem (San Diego, CA). TTX was purchased from Alomone Labs (Jerusalem, Israel).

**RESULTS**

To confirm that the calcium-sensitive dye was primarily taken up by astrocytes, Calcium Green AM–loaded slices were immunostained for the glial cell markers GFAP and S100. Slices were fixed with EDC, which preserves the Calcium Green-1 within the cells (Fern 1998; Tymianski et al. 1997). Figure 1A shows Calcium Green-1 staining in CA1 stratum radiatum after fixation of a hippocampal slice. The corresponding GFAP/S100 immunostaining is shown in Fig. 1B. The overlay image (Fig. 1C) demonstrates that the vast majority of the cells in the stratum radiatum (s.r.) that were loaded with Calcium Green-1 were also immunopositive for GFAP/S100 (double-staining is indicated by the yellow color). In this field, only one cell in the s.r. was filled with Calcium Green-1 but GFAP/S100 negative (yellow arrow). In 5 slices, 42 of 45 cells that were filled with Calcium Green-1 were also immunopositive for GFAP/S100.

**Astrocytes in situ show spontaneous calcium oscillations**

Changes in intracellular calcium concentration were measured in five astrocytes within the slice shown in Fig. 1, A–C (yellow boxes), and plotted over time (Fig. 1D). Four astrocytes exhibited spontaneous oscillations in intracellular calcium over a recording time of 10 min. All five cells responded to bath application of the metabotropic glutamate receptor agonist tACPD (50 μM, 1 min) with an increase in calcium. Overall, 64% (150/234 cells, 49 slices) of the astrocytes that were examined in the CA1 s.r. exhibited intracellular calcium oscillations without prior application of neurotransmitter receptor agonists.

The frequency of the spontaneous astrocytic calcium oscillations was very variable, even between astrocytes in the same slice. Of the 150 astrocytes that had spontaneous calcium oscillations, 56 (37%) had irregular increases with intervals of typically more than 2 min (e.g., 2 and 5 in Fig. 1D). In 94
astrocytes (63%) calcium oscillations occurred more frequently, either relatively evenly distributed over time with intervals between 0.5 and 2 min (e.g., 1 in Fig. 1D) or as bursts of calcium oscillations that were followed by periods of lower activity (e.g., 3 in Fig. 1D). Calcium increases were typically not synchronized between adjacent cells. Only in 4 of the 49 slices examined (11 of the 150 cells that exhibited oscillations) were calcium increases observed that occurred within a short time interval in adjacent cells, reminiscent of a calcium wave. Slices were used for recordings 2–6 h after the animals were killed, and up to 4 h after loading with the calcium dye. There was no noticeable difference in the frequency of calcium oscillations or the number of astrocytes that exhibited spontaneous oscillation between slices that were recorded within that period of time. One concern with confocal microscopy is phototoxicity due to excitation of the fluorescent dye with laser light. Therefore control experiments were carried out to determine whether the spontaneous calcium oscillations were dependent on exposure period or laser intensity. When the normal laser power was used, the frequency of astrocytic calcium oscillations was typically stable over 30–60 min. Increasing the laser power by 200% did not affect the calcium oscillations within that period of time. Furthermore, spontaneous calcium oscillations could also be observed in primary astroglial cultures from C57/BL6 mice on a fura-2 imaging system (not shown). These findings indicate that astrocytic calcium oscillations were not caused by phototoxicity.

\[ \text{Ca}^{2+} \text{ oscillations can be confined to single processes} \]

To study calcium changes in astrocytic processes, astrocytes were individually filled with fluorescent dye via patch pipettes. With this method, background fluorescence was much lower than in AM-loaded slices. This led to an improved spatial
resolution and enabled us to examine calcium changes in processes as small as 2 μm diam.

Figure 2A shows a fluorescence image of an astrocyte that was filled with Oregon Green BAPTA-1. A total of 26 regions of interest were defined along four major processes (a–d), and the average fluorescence was plotted over time (Fig. 2B). The four major processes exhibited very different patterns of spontaneous calcium oscillations. Processes b and d showed spontaneous calcium oscillations at the beginning of the recording, while the first calcium oscillations in processes a and c occurred after 1.5 and 3 min, respectively. Note that the first calcium increase in process b could also be observed in box 13 of process a (I), but was not propagated into the rest of process a. Within a single process, the differences in the pattern of calcium oscillations were more moderate (IV). During the indicated time interval, the proximal parts of the process exhibit three individual increases in calcium, while the distal parts show four calcium increases. A transitional area can be identified between region of interest (ROI) 5 and 6. A movie illustrating spontaneous calcium oscillations within an astrocyte can be viewed on the Journal of Neurophysiology web site.

To show the time course of a single calcium increase, the box marked by II has been expanded in Fig. 2C. The expanded traces show that the calcium increase started in ROI 9 or 10 and then spread into the rest of the process as an intracellular calcium wave. Due to limitations in the temporal resolution of the data acquisition system, it was not possible to determine the origin of the calcium oscillation with higher accuracy. A calcium increase could also be seen in process b. Time course and the decay in amplitude between ROI 14 and 16 indicate that this calcium increase was part of the wave that started in ROI 9 or 10 and then spread into process b. Box III in Fig. 2B shows an example of an intracellular calcium wave that started in the same area of process a, but was not propagated into the distal parts of the process. Although these two consecutive calcium waves started in the same area of the process, it was generally not possible to define one single origin of activity where all calcium oscillations within a particular process started.

**FIG. 2.** Calcium oscillations can be confined to single processes. Fluorescence image (A) and calcium recordings (B and C) from a hippocampal astrocyte that was filled with Oregon Green BAPTA-1 via a patch-clamp electrode. **A**: regions of interest (ROI) for calcium measurement were defined along the 4 major processes (a–d). **B**: calcium recordings from ROIs 1–26. The 4 major processes exhibited very different patterns of spontaneous calcium oscillations. Within a single process, the differences in the pattern of calcium oscillations were more moderate. The box marked by II has been expanded in C. The expanded traces show that the calcium increase started in ROIs 9 or 10 and then spread into the rest of the process as an intracellular calcium wave. Supplemental video related to this figure may be found at http://jn.physiology.org/cgi/content/full/87/1/528.
Due to limitations in the dynamic range of the data acquisition system, it was usually not possible to measure calcium changes in the soma (high fluorescence intensity) and in the processes (low fluorescence intensity) simultaneously. Where it was possible, the frequency of spontaneous calcium increases was higher in the processes than in the soma (data not shown).

The results indicate that astrocytic calcium increases can be confined to parts of a single process, suggesting that these regions act as microdomains, where calcium signaling can be largely independent of other processes and of the cell body. The results further suggest that calcium increases can be propagated over varying distances along a single process. A total of three individual astrocytes was analyzed in this manner; all three showed similar compartmentalized responses.

**Astrocytic Ca**^{2+} oscillations are mediated by IP3 receptor activation

It is widely accepted that astrocytic responses to neurotransmitter release often involve activation of metabotropic receptors and subsequent calcium release from intracellular stores (Porter and McCarthy 1995, 1996). Evidence also exists that astrocytes express ionotropic neurotransmitter receptors (Porter and McCarthy 1996; Shelton and McCarthy 1999; Steinhauser et al. 1994), which can mediate calcium increases. The presence of voltage-operated calcium channels in acutely isolated astrocytes has been reported (Duffy and MacVicar 1994), although the presence of these channels in situ has been controversial (Carmignoto et al. 1998). We carried out experiments to assess whether spontaneous calcium oscillations were due to calcium release from intracellular stores.

Inhibitors of the endoplasmic reticulum calcium ATPase such as cyclopiazonic acid (CPA) can deplete intracellular calcium stores and thereby inhibit cytoplasmic calcium increases that are due to release of calcium from these stores. Bath application of CPA (10–20 μM for 1–5 min) blocked spontaneous calcium increases in 10 of 10 astrocytes in 5 slices that were loaded with Calcium Green AM (Fig. 3). Initially, CPA caused an increase in calcium, after which the calcium in most cells returned to baseline level within a few minutes. CPA also blocked tACPD-induced calcium increases, and the effect of CPA was partially reversible after a wash out period of at least 10 min (data not shown). These results indicate that spontaneous calcium increases in astrocytes rely on calcium release from intracellular stores. Since bath application of cyclopiazonic acid affects calcium stores in astrocytes and neurons, these experiments could not rule out the possibility that the inhibition of astrocytic calcium oscillations was secondary to depletion of neuronal calcium stores.

To selectively interfere with calcium release from astrocytic calcium stores, individual astrocytes were injected with heparin, a competitive inhibitor of IP3 receptors (Ghosh et al. 1988). Heparin was used in the intracellular solution at a relatively high concentration (5 mM) because the short time in whole cell mode (2–4 min) does not allow for equilibration of the pipette content with the intracellular milieu. Fluorescence recordings from a control cell (top trace) and a cell that was injected with heparin (bottom trace) are shown in Fig. 4. While 12 of 13 cells that were voltage clamped with regular intracellular solution exhibited spontaneous calcium oscillations, 8 of 10 cells that were voltage clamped with heparin-containing solution did not show any spontaneous calcium increase. In the two remaining cells a single calcium increase was observed over a period of 20 min. As expected (given that heparin is a competitive IP3R antagonist), most cells that were patched with pipettes containing heparin responded to bath application of tACPD with an increase in calcium. However, the calcium increase observed in the presence of heparin was delayed and of shorter duration compared with tACPD-induced calcium increases in control cells. The response to tACPD application could therefore be used as a positive control for cell viability. Cells that did not respond to tACPD application with a calcium increase were excluded from the analysis. Application of dantrolene (50 μM) or dehydroxyanodine (20 μM), which both interfere with calcium release from ryanodine-sensitive stores, had no effect on astrocytic calcium oscillations (data not shown). These findings indicate that spontaneous calcium increases in astrocytes rely on IP3-mediated calcium release from intracellular stores.

**FIG. 3.** Calcium oscillations are blocked by depletion of intracellular stores. Calcium recordings from 3 cells filled with Calcium Green AM. Bath application of cyclopiazonic acid (CPA) led to a calcium increase that was transient in most cells. CPA application blocked calcium oscillations in 10 of 10 astrocytes in 5 slices.

**FIG. 4.** Calcium oscillations are blocked by inhibition of IP3 receptors. Calcium recordings from 2 astrocytes in the same brain slice that were filled with Oregon-Green BAPTA-1 via whole cell patch clamp. The bottom trace was recorded from a cell where 5 mM heparin was included in the pipette buffer solution. Twelve of 13 control cells (patched without heparin) exhibited multiple spontaneous calcium oscillations. In contrast, only 2 of 10 cells that were patched with heparin-containing electrodes exhibited spontaneous calcium increase during a 20-min recording. The other 8 heparin-filled cells did not show any spontaneous increases in calcium.


**Ca**$^{2+}$ oscillations can occur independently of neuronal activity

It is well established that astrocytes in situ can respond with calcium increases to synaptically released neurotransmitters (Porter and McCarthy 1996). We therefore tested the hypothesis that spontaneous calcium oscillations in astrocytes were caused by transmitter release from neurons. Neurotransmitter release due to action potential–dependent neuronal activity can be effectively blocked by bath application of tetrodotoxin (TTX), which blocks neuronal sodium channels. Bath application of 1 μM TTX, however, had no noticeable effect on the spontaneously occurring calcium oscillations in astrocytes that were loaded with Calcium Green AM (Fig. 5, 26 cells, 7 slices). To confirm that TTX blocked neuronal activity in our experiments, field potentials were recorded in the CA1 stratum radiatum while the Schaffer collaterals (SC) were electrically stimulated. TTX completely and reversibly blocked stimulus-evoked field potentials within 2–3 min of application in three of three slices (data not shown). Current-clamp recordings from CA1 pyramidal neurons were performed to further assess changes in neuronal activity during TTX application. Cells were kept at a baseline membrane potential between −65 and −75 mV by current injection, and a chloride-based intracellular solution was used ($E_{Cl} = 0$ mV) such that glutamatergic and GABAergic postsynaptic potentials were both depolarizing. In the absence of TTX, the pyramidal neurons responded with large depolarizations and action potentials to SC stimulation. TTX completely blocked SC stimulation–induced postsynaptic potentials and action potentials (Fig. 7B, middle trace), suggesting that action potential–dependent neurotransmitter release was indeed blocked in these experiments. TTX applications, however, did not affect the frequency of spontaneous postsynaptic potentials (Fig. 7A, middle trace). The frequency of spontaneous postsynaptic potentials was 42/min in the absence and 40/min in the presence of TTX. Similar results were found in two other slices. These results suggest that action potential–dependent neuronal activity is not responsible for spontaneous astrocytic calcium oscillations. However, the results leave open the possibility that the spontaneous calcium oscillations are due to spontaneous, action potential–dependent neurotransmitter release from neurons.

Spontaneous and evoked postsynaptic potentials in hippocampal slice preparations can be inhibited by incubation of slices with bafilomycin A1 (Zhou et al. 2000). Bafilomycin A1 inhibits the vacuolar-type proton pump (V-ATPase), which is responsible for establishing pH and electrical gradients across the membrane of synaptic vesicles (Dröse and Altendorf 1997). Bafilomycin A1 thereby blocks uptake of neurotransmitter into synaptic vesicles (Maycox et al. 1990). Hippocampal slices were incubated with bafilomycin A1 (4 μM) during loading with Calcium Green-1 and maintenance in ACSF (~110 min), while control slices were incubated in the absence of bafilomycin A1. Incubation with bafilomycin A1 did not prevent astrocytic calcium oscillations (Fig. 6). In 4 slices that were incubated with bafilomycin A1, 11 of 19 cells (58%) showed calcium oscillations, while 21 of 35 cells (60%) in 4 control slices showed oscillations. To confirm the effect of bafilomycin A1 on neuronal activity, current-clamp recordings were performed from CA1 pyramidal neurons in control and bafilomycin A1–incubated slices that were used for calcium recordings. Compared to the control slice, the frequency of spontaneous postsynaptic potentials was greatly reduced in the bafilomycin A1–incubated slice (Fig. 7A, bottom trace, 3/min). Bafilomycin A1 also markedly reduced the depolarization that was induced by SC stimulation (Fig. 7B, bottom trace). Similar results were found in two other pairs of control and bafilomycin A1–incubated slices. These results indicate that astrocytic calcium oscillations are independent of neuronal action potentials and spontaneous vesicular neurotransmitter release from neurons.

**Astrocytic Ca**$^{2+}$ oscillations are not mediated by metabotropic glutamate receptors or purinergic receptors

To assess the possibility that astrocytic calcium oscillations were due to responses of astrocytes to glutamate or ATP release from these cells in situ, a cocktail of antagonists for purinergic receptors and metabotropic glutamate receptors was bath applied to hippocampal slices. Bath application of an antagonist cocktail consisting of AIDA (200 μM, mGlu1a), MPEP (10 μM, mGlu5), CPPG (5 μM, group II/III mGlu), PPADS (100 μM, P2Y1,4,6), suramin (100 μM, P2Y2,6,11), 8(p-sulfophenyl)theophylline (100 μM, A1, A2), and TTX (1 μM) did not affect astrocytic calcium oscillations while completely blocking astrocytic responses to the simultaneous addition of tACPD (50 μM) and ATP (20 μM; Fig. 8, 3 slices, 10 cells). These results indicate that neither metabotropic glutamate receptors nor purinergic receptors mediate spontaneous astrocytic calcium oscillations.


**DISCUSSION**

The major finding of this study is that hippocampal astrocytes in situ exhibit spontaneous oscillations in intracellular calcium that are independent of neuronal activity. To our knowledge, this is the first demonstration that astrocytes in situ initiate signaling cascades in the absence of extrinsic stimulation. This finding coupled with the observation of others that astroglia in vitro and astrocytes in situ release glutamate in a calcium-dependent manner (Araque et al. 1998a; Bezzi et al. 1998) suggest that in vivo astrocytes may serve as an independent modulator of neuronal excitability. Our observation that astrocytic calcium spikes often occurred localized within microdomains of astrocytic processes and were asynchronous within a given astrocyte suggests that these microdomains may be influencing neuronal activity in a synapse-specific manner. Further, the observation that calcium spikes occasionally propagated through a distinct subset of astrocytic processes suggests that, under certain conditions, astrocytes have the capability of synchronously influencing a larger synaptic field. It is also significant that intercellular calcium waves were not observed between adjacent astrocytes. This is in striking contrast to findings in vitro where astroglia routinely propagate intercellular calcium waves (Charles et al. 1991; Cornell-Bell et al. 1990). Sixty-four percent of the astrocytes in bulk-loaded hippocampal slices exhibited spontaneous calcium oscillations. The difference in behavior between oscillating and nonoscillating astrocytes did not manifest in morphology, immunostaining, or electrophysiological properties. All astrocytes that were included in this study were able to respond with a calcium increase to application of the mGlu-R agonist tACPD at the end of each experiment. This suggests that there are subtle differences between subpopulations of oscillating and non-oscillating astrocytes. There was no noticeable difference in calcium oscillations within 6 h after slice preparation. It is therefore unlikely that spontaneous calcium oscillations were due to deterioration of the slice over time. When calcium oscillations occurred in astrocytes, the oscillation pattern was usually stable over at least 1 h during prolonged recordings. Furthermore, increasing the intensity of the laser light that was used to excite the calcium dye had no effect on the oscillations. This argues against the possibility that spontaneous astrocytic calcium oscillations are due to photodamage. This conclusion is supported by the finding that astrocytic calcium oscillations were observed at the same time evoked and spontaneous neuronal synaptic activity within the same CA1 region appeared normal.

Neither bath application of TTX nor incubation of hippocampal slices with bafilomycin A1 inhibited spontaneous astrocytic calcium oscillations. TTX is an inhibitor of neuronal sodium channels and completely blocked action potentials and evoked postsynaptic potentials in CA1 pyramidal neurons. Incubation with bafilomycin A1, an inhibitor of vesicular transport, greatly reduced spontaneous and evoked postsynaptic potentials in CA1 pyramidal neurons while having no apparent effect on astrocytic calcium oscillations. The lack of effect of these drugs on the spontaneous calcium oscillations in astrocytes indicates that spontaneous astrocytic calcium oscillations are independent of spontaneous or action potential–dependent release of neurotransmitters from neurons. In addition, this

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**FIG. 7.** Spontaneous and stimulation-induced neuronal activity. Current-clamp recordings from CA1 pyramidal neurons. Cells were kept at a baseline membrane potential between −65 and −75 mV by current injection. The thin horizontal line at the beginning of each trace indicates −70 mV. A: recordings of spontaneous postsynaptic potentials (PSPs). PSP frequency was not affected by TTX, but greatly reduced by incubation with bafilomycin A1. Action potentials are clipped and marked by asterisk. B: responses to electrical stimulation of the Schaffer collaterals (SC). Responses to SC stimulation are blocked by TTX and greatly reduced by bafilomycin A1.

**FIG. 8.** Astrocytic calcium oscillations are not mediated by metabotropic glutamate receptors or purinergic receptors. Calcium recordings from 4 calcium green AM–loaded astrocytes. Bath application of an antagonist cocktail for metabotropic glutamate receptors and purinergic receptors did not affect astrocytic calcium oscillations while completely blocking astrocytic responses to tACPD and ATP (3 slices, 10 cells). The double diagonal bars indicate an 8-min break in the recording. The antagonist solution caused a decrease in baseline fluorescence intensity because of absorption. Therefore the traces in the presence of the antagonist cocktail were normalized to this new baseline.
finding indicates that the amount of neurotransmitter necessary for eliciting a spontaneous postsynaptic potential in CA1 pyramidal neurons is not sufficient to elicit calcium increases in astrocytes. While not generally observed, one report suggests that cultured astroglia may also exhibit spontaneous calcium oscillations (Fatatis and Russell 1992), further supporting the view that the astrocytic calcium oscillations we observe in situ are not dependent on neuronal activity. The work presented in our study utilized hippocampal slices prepared from 10- to 17-day-old mice. We did not find any difference in the frequency of oscillating astrocytes as a function of age within this group, making it unlikely that the spontaneous calcium oscillations represent a developmental process that does not occur in mature hippocampus. This view is further supported by reports indicating that mature hippocampal astrocytes exhibit calcium responses to neurotransmitters similar to immature astrocytes (Shelton and McCarthy 1999) and that astrocytes from 21-day-old hippocampus exhibit their mature complement of ion channels (Bordey and Sontheimer 1997). Spontaneous astrocytic calcium oscillations were blocked by bath application of cyclopiazonic acid or injection of heparin directly into astrocytes. Cyclopiazonic acid has been shown to cause depletion of predominantly IP$_3$-sensitive intracellular calcium stores in cultured astroglia (Golovina and Blaustein 2000). Heparin, a competitive inhibitor of IP$_3$ receptors, blocked spontaneous calcium oscillations. These findings suggest that calcium release from IP$_3$-sensitive intracellular stores in the astrocytes is essential for spontaneous calcium oscillations.

Recent studies with neuron-astroglia co-cultures suggest that astroglia in culture can release glutamate by a mechanism that is blocked by bafilomycin A1 (Araque et al. 2000). Another study has found evidence for a vesicular release mechanism in astrocytes in hippocampal slices (Bezzi et al. 1998). These mechanisms were most likely blocked in our experiments after incubations with bafilomycin A1. Therefore our experiments also suggest that spontaneous astrocytic calcium oscillations are independent of vesicular release of glutamate from astrocytes.

It is worth noting that intercellular calcium waves between astrocytes were rarely, if ever, observed in our experiments. A temporal correlation in the rise of indicator dye fluorescence between adjacent astrocytes was observed in only 4 of 49 slices (11/150 cells); these slices generally contained multiple astrocytes exhibiting spontaneous calcium oscillations. More typically, there was no temporal correlation between calcium spikes in adjacent cells (see Fig. 1). Our current view is that those few times we observed a temporal correlation in the rise of calcium between adjacent astrocytes were serendipitous. The lack of observable intercellular calcium waves in situ is surprising, given that astroglia in vitro routinely exhibit intercellular calcium waves. However, the morphology of astrocytes in situ and astroglia in vitro is strikingly different. In vitro, astroglia generally contact one another at all borders markedly increasing their direct contact relative to the situation in situ. Our current view is that while we cannot exclude the possibility that intercellular waves may occur between the distal regions of processes of adjacent astrocytes, intercellular calcium waves do not propagate through a syncytium of astrocytes in situ following a spontaneous rise in calcium within a single astrocyte. It remains possible that astrocytes responding to neurotransligands or other forms of stimulation may respond with increases in intracellular calcium that propagate among astrocytes as an intercellular calcium wave. Injecting individual astrocytes with a calcium dye via patch pipettes allowed us to monitor calcium oscillations in astrocytic processes as small as 2μm diam. These recordings revealed that the most common pattern of calcium oscillations was asynchronous calcium spikes occurring throughout astrocytic processes. Occasionally, calcium oscillations started in a small area of a process and then propagated as an intracellular wave through that process and into a subset of alternate astrocytic processes. These results indicate that a large number of regions within astrocytic processes can act as independent microdomains and that under certain conditions intracellular calcium waves propagate directionally through a subset of the astrocyte’s processes. A study that was done in Bergmann glial cells of cerebellar slices found that stimulation of the parallel fibers led to increases in calcium within astrocytic microdomains (Grosche et al. 1999). In the absence of stimulation of parallel fibers, increases in calcium within the Bergmann glia were observed, which coincided with regions that responded to stimulation of parallel fibers with calcium increases. While not examined, it seems likely that the “spontaneous” responses observed in Bergmann glia were due to the release of neurotransmitter from neurons given that these were the same regions that responded to neuronal stimulation with increases in calcium. No experiments were presented in that study, indicating that calcium oscillations occurred in astrocytes that were independent of neuronal activity.

Studies carried out using astroglial cultures suggest that propagation of intercellular calcium waves between astroglia involve the release of ATP, which then activates the purinergic receptors of neighboring astrocytes (Cotrina et al. 1998, 2000; Wang et al. 2000). Furthermore, astroglial cells have been reported to release glutamate by vesicular and nonvesicular mechanisms (Araque et al. 2000; Szatkowski et al. 1990). These findings led us to test the hypothesis that the spontaneous oscillations in astrocytic calcium observed in this study were the result of astrocytic release of ATP or glutamate, which then activated their purinergic or glutamatergic receptors in an autocrine manner. However, bath application of an antagonist cocktail that blocked astrocytic calcium responses to ATP and tACPD had no effect on the spontaneous calcium oscillations. This finding indicates that activation of metabotropic glutamate receptors or purinergic receptors is not involved in generating spontaneous calcium oscillations in astrocytes.

The mechanism underlying spontaneous calcium oscillations in astrocytes remains unknown. Neither neuronal activity nor activation of glutamatergic or purinergic receptors are necessary for astrocytic calcium oscillations. This is in marked contrast to the calcium oscillations observed in neocortical neurons during development where the majority (~95%) of neuronal oscillations were blocked by a drug combination that blocked action potentials, ionotropic glutamatergic synaptic transmission, and group 1 metabotropic glutamate receptors (Flint et al. 1999). A number of studies indicate that growth cones exhibit spontaneous calcium oscillations and that these are important in the guidance of neurons (Gomez and Spitzer 2000). The mechanism underlying the calcium oscillations occurring in growth cones has not yet been identified but appears to involve both an unidentified calcium (Gomez et al.
1995) channel and calcium release from internal stores (Takei et al. 1998). It is possible that a similarly unidentified calcium channel is present in astrocytes and important in the calcium oscillations observed in this study. Such a calcium channel could lead to the “over-filling” of internal calcium stores and subsequent IP$_3$-dependent calcium release due to the enhanced IP$_3$ sensitivity of these stores as a result of their calcium load (Berridge 1998). Alternatively, it is possible that specific phospholipase C (PLC)–linked receptors present on astrocytes are constitutively active resulting in increases in IP$_3$ and diacylglycerol. Hippocampal astrocytes express mGluR5 (Lujan et al. 1996) and the calcium oscillations observed following activation of mGluR5 in vitro appear to be due to the phosphorylation of mGluR5 by protein kinase C. This phosphorylation inhibits mGluR5 activation of PLC leading to a decrease in IP$_3$ production and calcium release from internal stores (Kawabata et al. 1996). The spontaneous calcium oscillations in our study may be due to constitutively active mGluR5 receptors in a subpopulation of these cells. Further studies will be required to sort out the processes underlying the spontaneous calcium oscillations observed in our experiments.

Potential significance of spontaneous astrocytic calcium oscillations

Essentially all hypotheses concerning astrocyte function assume that astrocytes are responding to a neuronal signal. The observation that astrocytic calcium oscillations occur in the absence of neuronal activity suggests that astrocytes may initiate signaling events rather than only respond to signals arriving from neurons or other cells. Findings from a number of in vitro (Araque et al. 1998a,b) as well as in situ (Bezzi et al. 1998) studies indicate that astrocytes release glutamate in a calcium-dependent manner. Further, it is evident that the release of glutamate from astroglia in vitro is sufficient to elicit activation of neuronal glutamatergic receptors with concomitant changes in neuronal excitability (Araque et al. 1998a,b). Given that similar mechanisms appear to underlie calcium-dependent glutamate release from astrocytes and neurons, it is becoming increasingly difficult to sort out the cellular origin of modular regulation of synaptic transmission in situ.

The spontaneous calcium oscillations seen in hippocampal astrocytes generally occur asynchronously in small compartments within astrocytic processes. This observation suggests that astrocytes may be comprised of microdomains that function autonomously from one another to affect local events, possibly nearby synaptic transmission. While current focus in the area of astrocyte signaling is on glutamate release, the calcium oscillations observed in astrocytes could affect a variety of astrocytic functions to modulate neuronal activity. For example, oscillations in astrocytic calcium could modulate glutamate transporters, affect the permeability of K$_r$ channels, alter local cell volume, regulate the release of trophic factors, change the local morphology of astrocytic microdomains, and/or alter synaptic connections. Affecting any of these parameters could lead to changes in neuronal excitability. While a conceptual shift, our findings suggest that astrocytes may act as an independent modulator that influences neuronal activity independent of incoming neuronal signals. Results emerging in this field strongly suggest that astrocytes may play a much larger and dynamic role in neural signaling than imagined just a few years ago.

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