Synaptically Released Acetylcholine Evokes Ca^{2+} Elevations in Astrocytes in Hippocampal Slices

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Recent results have demonstrated the existence of bidirectional communication between glial cells and neurons. We investigated in brain slices whether rat hippocampal astrocytes respond to acetylcholine synaptically released by an extrinsic pathway. We stimulated the stratum oriens/alveus, which contains cholinergic afferents from the septum and diagonal band of Broca, and recorded whole-cell membrane currents and intracellular Ca^{2+} levels of astrocytes located in the hippocampal stratum oriens. Nerve-fiber stimulation evoked a long-lasting inward current and increased the Ca^{2+} levels in astrocytes. Both astrocytic responses were abolished by tetrodotoxin or Cd^{2+} and were increased by 4-aminopyridine, indicating that the responses were attributable to synaptically released neurotransmitter. The inward current was inhibited by glutamate transporter antagonists, indicating that it was attributable to the electrogenic glutamate transporter activity. The synaptically evoked intracellular Ca^{2+} elevations were not affected by glutamate receptor antagonists but were abolished by atropine, indicating that they were mediated by muscarinic cholinergic receptors. Thapsigargin prevented the Ca^{2+} elevation but did not modify the inward current, indicating that the Ca^{2+} signal was attributable to intracellular Ca^{2+} mobilization. These results indicate that hippocampal astrocytes respond to acetylcholine released by synaptic terminals. The synaptically released acetylcholine acts on muscarinic receptors, mobilizing Ca^{2+} from the intracellular stores. Different regions in the recorded astrocytes showed independent stimulus-induced Ca^{2+} variations, suggesting the existence of subcellular domains in the astrocytic responses evoked by the synaptic cholinergic activity. Therefore, our results show the existence of cholinergic neuron–astrocyte signaling and suggest that astrocytes are a target of axonal inputs from different brain areas.

Key words: intracellular calcium; astrocytes; muscarinic cholinergic receptors; glutamate transporters; hippocampal slices; synaptic transmitter release

Astrocytes possess a form of excitability based on intracellular Ca^{2+} variations (Cornell-Bell et al., 1990; Charles et al., 1991; Newman and Zahs, 1997) that can be triggered by synaptically released glutamate (Dani et al., 1992; Porter and McCarthy, 1996; Pasti et al., 1997). Furthermore, physiological astrocytic Ca^{2+} elevations evoke Ca^{2+}-dependent glutamate release from astrocytes that signal to adjacent neurons (Parpura et al., 1994; Pasti et al., 1997; Araque et al., 1998a,b, 2000; Bezzi et al., 1998; Parpura and Haydon, 2000), modulating the neuronal excitability (Araque et al., 1998a; Newman and Zahs, 1998) and synaptic transmission (Araque et al., 1998a,b; Kang et al., 1998). Neuron–glia interaction has also been reported in the peripheral nervous system, in which perisynaptic Schwann cells respond to neurotransmitter release and modulate synaptic transmission (Robitaille, 1998; Rochon et al., 2001). These results suggest the existence of bidirectional communication between astrocytes and neurons in which glutamate plays a pivotal role as the signal that mediates this new form of communication in the nervous system (Carmignoto, 2000; Araque et al., 2001; Haydon, 2001).

Astrocytes express many neurotransmitter receptors coupled to intracellular Ca^{2+} mobilization (Verkhratsky and Kettenmann, 1996; Porter and McCarthy, 1997). Studies using brain slices have demonstrated that several neurotransmitters may regulate Ca^{2+} levels of astrocytes (Porter and McCarthy, 1996; Pasti et al., 1997; Kang et al., 1998; Kulik et al., 1999; Shelton and McCarthy, 1999, 2000). Nevertheless, the activation of these receptors by synaptically released neurotransmitters has not been fully determined. Indeed, most of the *in situ* studies have reported that glutamate is the neurotransmitter that controls astrocytic Ca^{2+} (Porter and McCarthy, 1996; Pasti et al., 1997). Kang et al. (1998) showed that stimulation of hippocampal interneurons can elevate astrocytic Ca^{2+} levels through activation of $GABA_B$ receptors. Bergmann glial Ca^{2+} can be regulated by stimulation of either the molecular- or granular-cell layer of the cerebellum through α_1-adrenoreceptor activation (Kulik et al., 1999). However, whether other receptors can be activated by synaptically released neurotransmitters and, therefore, whether they participate in the bidirectional communication between astrocytes and neurons is unknown.

Our present knowledge of bidirectional communication between astrocytes and neurons indicates that astrocytes are activated by neurotransmitters released from immediately adjacent synapses and from axon terminals of neurons belonging to the local circuit in which the astrocytes are immersed (for review, see Araque et al., 1999). However, whether extrinsic axons are able to act on target astrocytes in a different brain area remains unknown.

We have investigated whether an extrinsic cholinergic pathway to the hippocampus can signal to hippocampal astrocytes regulating their intracellular Ca^{2+}. Using electrophysiological and

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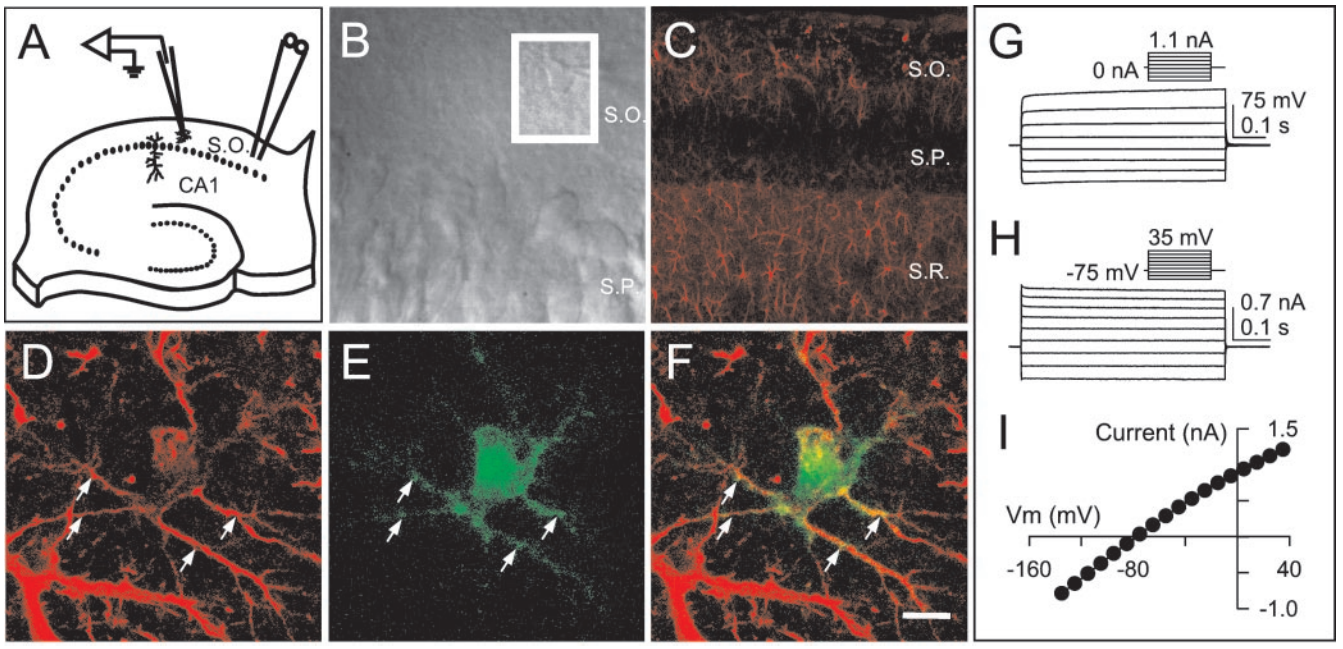

Figure 1. Morphological, immunocytochemical, and electrophysiological identification of astrocytes in the hippocampal stratum oriens is shown. *A*, Schematic drawing of the experimental arrangement showing the position of the stimulating (*right*) and recording (*left*) electrodes in the hippocampal slice preparation. *B*, Infrared differential interference contrast image showing the hippocampal pyramidal layer (*bottom*) and the recorded astrocyte (*top*) (note the recording pipette on the right side of the astrocyte). The fluorescence intensity was collected by the photomultiplier tube from the window depicted by the *box* around the astrocyte. *C*, Fluorescence image of the GFAP-stained CA1 hippocampus. *D, E*, Fluorescence images of GFAP-stained cells and a fluo-3-filled cell, respectively, obtained by laser-scanning confocal microscopy constructed from a stack of 15 successive images (1.5 μm deep). *F*, Combination of the images shown in *D* and *E*, showing that the fluo-3-filled cell was GFAP-positive. *Arrows* in *D–F* indicate some dual-labeled processes. *G*, Current-clamp recordings of the astrocytic membrane potential variations evoked by hyperpolarizing and depolarizing current pulses. *H*, Whole-cell currents evoked by hyperpolarizing and depolarizing voltage pulses. *I*, Current–voltage relationship of the steady-state membrane currents. *S.O.*, Stratum oriens; *S.P.*, stratum pyramidale; *S.R.*, stratum radiatum. Scale bars: *B*, 15 μm, *C*, 75 μm, *D–F*, 8 μm.

single-cell fluorescence photometric Ca^{2+} techniques in hippocampal slices, we have found that stimulation of the stratum oriens/alveus of the hippocampus, which contains cholinergic afferents from the septum and diagonal band of Broca, increased the intracellular Ca^{2+} of astrocytes in the stratum oriens through activation of muscarinic cholinergic receptors (mAChRs). These results demonstrate that astrocytes are a target of extrinsic axons arising from a different brain area, adding a new element of complexity to the signaling pathways in the nervous system.

MATERIALS AND METHODS

Hippocampal slice preparation. Acute hippocampal slices were obtained as described previously (Borde et al., 1995). Briefly, Wistar rats (12–17 d of age) were decapitated; brains were removed rapidly and placed in ice-cold artificial CSF (ACSF) gassed with 95% O_2 and 5% CO_2, pH 7.3. Brain slices (350–450 μm thick) were cut with a Vibratome (Pelco 101, Series 1000; Vibratome, St. Louis, MO) and incubated for >1 hr at room temperature (21–24°C) in ACSF. The ACSF contained (in mM): 124 NaCl, 2.69 KCl, 1.25 KH_2PO_4, 2 $MgSO_4$, 26 $NaHCO_3$, 2 $CaCl_2$, and 10 glucose; it was gassed with 95% O_2 and 5% CO_2. Because astrocytic responses were enhanced by 4-aminopyridine (4-AP), in some cases the control ACSF contained 100 μM 4-AP. Slices were then transferred to an immersion recording chamber and superfused with gassed ACSF. Cells were visualized under an Olympus (Tokyo, Japan) BX50WI microscope equipped with infrared and differential interference contrast imaging devices, and with a 40× water immersion objective.

Electrophysiology. Simultaneous fluorescence photometric Ca^{2+} measurements (see below) and electrophysiological recordings from astrocytes located in the stratum oriens of the CA1 hippocampal region were made using the whole-cell configuration of the patch-clamp technique. Patch electrodes were fabricated from borosilicate glass capillaries and had resistances of 6–10 MΩ when filled with the internal solution that contained (in mM): 100 $KMeSO_4$, 50 KCl, 10 HEPES, and 4 ATP–Na_2, pH 7.3. Recordings were obtained with an Axoclamp-2A amplifier (Axon Instruments, Foster City, CA) either in the current-clamp bridge mode or the continuous single-electrode voltage-clamp mode. Fast and slow whole-cell capacitances were neutralized and series resistance was compensated (~70%). In voltage-clamp experiments, the membrane potential was held at V_r. Signals were fed to a Pentium-based personal computer through a DigiData 1320 interface board (Axon Instruments). pClamp 8 software (Axon Instruments) was used for stimulus generations and for data display, acquisition, and storage.

Astrocytes were identified according to the following morphological and electrophysiological criteria (Pasti et al., 1997; Bergles and Jahr, 1997, 1998; Bezzi et al., 1998; Bergles et al., 2000): small round soma (<15 μm) without thick processes (Fig. 1*B*), numerous thin radiating processes (detected after loading the cell with the fluorescent indicator) (Fig. 1*E*), high resting membrane potential ($V_r = -73 \pm 1$ mV; $n = 144$), high membrane conductance (22 ± 2 nS; $n = 144$), and absence of action potentials (Fig. 1*G–I*). In some cases, the recorded fluo-3-filled astrocytes were later studied immunocytochemically and examined using laser-scanning confocal microscopy (see below).

Experiments were performed at room temperature (21–24°C). All data are expressed as means ± SEM. Statistical differences were established using the Student *t* test.

Measurement of intracellular Ca^{2+} variations. Ca^{2+} levels in single astrocytes were monitored by fluorescence microscopy using the Ca^{2+} indicator fluo-3. Patch pipettes were filled with the internal solution containing 10–50 μM fluo-3 (Molecular Probes, Eugene, OR). Cells were illuminated with a xenon lamp at 490 nm using a monochromator Polychrome II (T.I.L.L. Photonics, Planegg, Germany). Fluorescence intensity was collected by a photomultiplier tube (model R928; Hamamatsu, Bridgewater, NJ) from a variable rectangular window (side: 25–50 μm) containing the recorded cell. Cells were illuminated during 20–200 msec every 500–1000 msec and the fluorescence signal collected was integrated using the T.I.L.L. Photonics photometry system. Ca^{2+} variations were estimated as changes of the fluorescence signal over baseline ($\Delta F/F_0$) after background subtraction. Astrocytes were considered to respond to the stimulation when the fluorescence signal increased two times above the SD of the basal signal.

Ca^{2+} imaging. In some cases, fluo-3-filled astrocytes were imaged using a CCD camera (SPOT RT Monochrome; Diagnostic Instruments, Sterling Heights, MI) attached to the Olympus microscope. Quantitative epifluorescence measurements were made using the ImageJ public domain software (developed at the National Institutes of Health, Bethesda, MD). Ca^{2+} variations were estimated as $\Delta F/F_0$ after background subtraction; regions of interest (ROI) were considered to respond to the stimulation when $\Delta F/F_0$ increased >5% for at least two consecutive images.

Immunocytochemistry. Recorded cells were initially identified according to their morphological and electrophysiological properties (Fig. 1). After recording, some cells were immunocytochemically studied using a mouse monoclonal antibody against glial fibrillary acidic protein (GFAP) (dilution 1:400; Sigma). Slices were fixed overnight with 4% paraformaldehyde in 0.1 M phosphate buffer at 4°C, incubated in GFAP antibody in 0.25% Triton X-100 with 3% normal goat serum in phosphate buffer. GFAP immunoreactivity was visualized using an Alexa 594-conjugated goat secondary antibody (1:1000; Molecular Probes). After washing, slices were mounted in glycerol (50% in phosphate buffer) and examined using laser-scanning confocal microscopy. Recorded cells were identified as astrocytes according to their dual label with fluo-3 and the GFAP antibody. In some cases, control experiments were performed by immunocytochemical processing in the absence of primary antibody; no GFAP-positive staining of fluo-3-filled astrocytes was observed.

Stimulation. Bipolar nichrome wire (80 μm) electrodes were connected to a stimulator and isolation unit (Grass S88; Grass Instruments, West Warwick, RI) and placed under visual guidance in the stratum oriens/alveus near the subiculum area, which contains cholinergic afferents from the diagonal band of Broca and the septum (Lewis and Shute, 1967; Amaral and Witter, 1995). Trains of stimuli at 30 Hz during 5 sec were delivered at 0.013 sec^{-1}, unless stated otherwise, and three to five responses were averaged.

Ionophoresis. ACh was ionophoretically delivered from a micropipette (5–15 MΩ) filled with 0.5 M ACh (in ACSF, pH 6) by 1- to 5-sec-duration current pulses (MicroIontophoresis Dual Current Pulse Generator 260; World Precision Instruments, Sarasota, FL). Likewise, glutamate (0.7 M in ACSF, pH 7.5–8) was ionophoretically applied.

Drugs. Thapsigargin, L-*trans*-pyrrolidine-2,4-dicarboxylate (t-PDC), (*S*)-α-methyl-4-carboxyphenylglycine (MCPG), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and D-(−)-2-amino-5-phosphonopentanoic acid (AP-5) were purchased from Tocris Cookson (Bristol, UK); dihydrokainate (DHK) was purchased from Ocean Produce International (Shelburne, Canada). All other drugs were purchased from Sigma.

RESULTS

Simultaneous intracellular Ca^{2+} levels and whole-cell currents of morphologically and electrophysiologically identified astrocytes in the stratum oriens were recorded (Fig. 1). It is well established that astrocytes in culture express receptors for numerous neurotransmitters, including ACh (Verkhratsky and Kettenmann, 1996; Porter and McCarthy, 1997). Furthermore, it has been shown that astrocytes located in the hippocampal stratum radiatum region respond to perfused ACh with intracellular Ca^{2+} variations (Shelton and McCarthy, 2000). However, it is not known whether astrocytes located in the stratum oriens, a CA1 hippocampal region that receives abundant cholinergic projections (Amaral and Witter, 1995), respond to ACh.

Therefore, we investigated whether stratum oriens astrocytes also responded to exogenously applied ACh. Ionophoretic application of ACh increased the intracellular Ca^{2+} levels in eight of eight recorded astrocytes. This response was inhibited by 10 μM atropine, an mAChR antagonist (to 14 ± 14% from control values; $n = 3$) (Fig. 2*A*), indicating that the ACh-induced Ca^{2+} increase was mediated by mAChR activation.

Electrical stimulation of the stratum oriens/alveus of the hippocampus evoked a long-lasting inward current in astrocytes (the mean amplitude from a representative sample of 68 astrocytes was −115.7 ± 13.3 pA) (Fig. 2*B,C*). Furthermore, ~70% of the astrocytes (54 from a representative sample of 79 astrocytes) responded to the stimulation with transient, long-lasting elevations in their intracellular Ca^{2+} levels. Figure 2*D,E* shows the dependence of both astrocytic responses on the frequency and duration of the stimulus. In addition, in 12 of 79 astrocytes, nerve-fiber stimulation evoked repetitive intracellular Ca^{2+} oscillations that persisted for several seconds after cessation of the stimulus (Fig. 2*F*). The present work was focused on the transient intracellular Ca^{2+} responses; the occasional subsequent oscillations were not considered further.

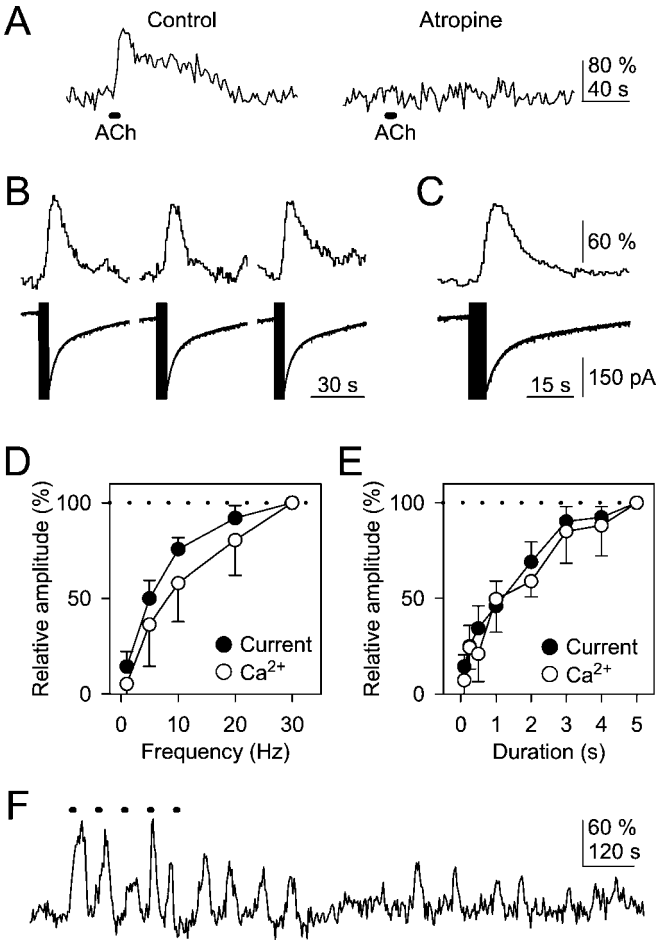

Figure 2. Astrocytic responses evoked by ionophoretically applied ACh and nerve-fiber stimulation are shown. *A*, Intracellular Ca^{2+} levels estimated from the fluorescence intensity recorded from a single astrocyte filled with the Ca^{2+} indicator fluo-3. ACh, ionophoretically delivered from a micropipette (0.5 M, 5 sec; *bottom line*), increased the astrocytic Ca^{2+} levels in control conditions (*left trace*). In the presence of atropine, ionophoretic application of ACh did not modify the Ca^{2+} levels (*right trace*). *B*, Representative astrocytic Ca^{2+} levels (*top traces*) and whole-cell membrane currents (*bottom traces*) elicited by nerve-fiber stimulation (30 Hz, 5 sec; as in all other figures). Three consecutive responses were evoked at 0.013 sec^{-1}. The *vertical black columns* on the current traces correspond to the stimulus artifact (as in all other figures). *C*, Averaged responses of the traces shown in *B*. *D, E*, Dependence of the maximum current amplitude (*solid circles*) and Ca^{2+} increase (*open circles*) on the stimulus frequency and duration, respectively. Values are relative to the responses evoked by a stimulus at 30 Hz for 5 sec (*dotted lines*). Each point represents mean values from at least four astrocytes. *F*, In 15% of the recorded astrocytes, repetitive nerve-fiber stimulation (*dotted line*) evoked intracellular Ca^{2+} elevations that were followed by intracellular Ca^{2+} oscillations that persisted for several seconds after cessation of the stimulus.

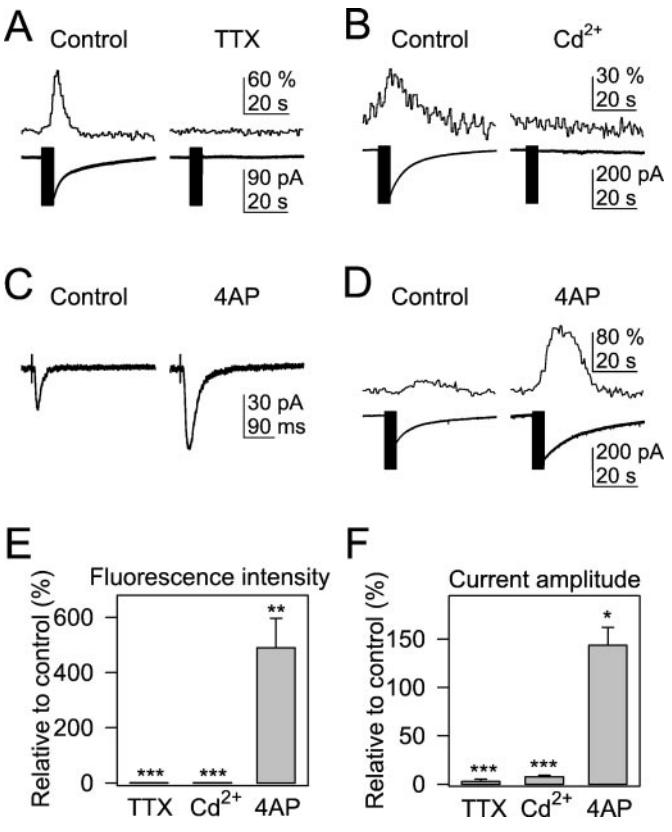

Figure 3. Astrocytic responses are evoked by synaptically released neurotransmitter. *A, B*, Astrocytic Ca^{2+} levels (*top traces*) and whole-cell membrane currents (*bottom traces*) evoked by nerve-fiber stimulation in control conditions and in the presence of 1 μM TTX and 100 μM Cd^{2+}, respectively. *C*, Averaged ($n = 15$) EPSCs evoked by Schaffer collateral–commissural stimulation and recorded from CA1 pyramidal neurons in controls and in the presence of 100 μM 4-AP. *D*, Astrocytic Ca^{2+} levels (*top traces*) and whole-cell membrane currents (*bottom traces*) evoked by nerve-fiber stimulation in control conditions and after superfusion with 100 μM 4-AP. *E, F*, Relative changes from control recordings of the fluorescence intensity and membrane current amplitudes, respectively, evoked by nerve-fiber stimulation in the presence of 1 μM TTX ($n = 4$), 100 μM Cd^{2+} ($n = 6$), and 100 μM 4-AP ($n = 14$). Significant differences were established by the Student *t* test at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Astrocytic responses are evoked by synaptically released neurotransmitter

The stimulus-induced Ca^{2+} elevations and inward currents were abolished by the sodium channel antagonist tetrodotoxin (TTX) (1 μM), which prevents action-potential generation, indicating that both responses depended on neuronal activity ($n = 4$) (Fig. 3*A*). We also tested whether these responses were attributable to synaptic transmitter release by using pharmacological tools that modulate synaptic transmission.

Evoked synaptic transmitter release requires Ca^{2+} influx through voltage-gated Ca^{2+} channels that can be blocked by extracellular Cd^{2+} (Hille, 1992). The astrocytic Ca^{2+} variations and the inward current evoked by nerve-fiber stimulation were abolished by 100 μM Cd^{2+} (Fig. 3*B*). 4-AP is a potassium-channel blocker that enhances the release of neurotransmitter from nerve terminals in hippocampal slices (Thesleff, 1980). To confirm that 4-AP increases synaptic transmitter release, we recorded the EPSCs evoked by Schaffer collateral–commissural stimulation in CA1 pyramidal neurons. The amplitude of the EPSCs was consistently increased by 100 μM 4-AP in all cells tested ($n = 12$) (Fig. 3*C*). The astrocytic Ca^{2+} elevations evoked by nerve-fiber stimulation were also increased in the presence of 100 μM 4-AP ($n = 14$) (Fig. 3*D,E*). Furthermore, the amplitude of the inward current was also increased by 4-AP ($n = 14$) (Fig. 3*D,F*). These results indicate that the astrocytic responses are evoked by synaptic activity and suggest that neurotransmitter release from synaptic terminals is required to elicit these astrocytic responses.

The astrocytic inward current is mediated by activation of electrogenic glutamate transporters

Astrocytes express high levels of two types of glutamate transporters (GLT-1 and GLAST) responsible for the clearance of glutamate from the extracellular space (Mennerick et al., 1996; Bergles and Jahr, 1997, 1998). It has been shown that glutamate released from Schaffer collateral–commissural synaptic terminals can activate nearby hippocampal astrocytes located in the stratum radiatum of the CA1 region (Bergles and Jahr, 1997, 1998). The activation of these glutamate transporters leads to the electrogenic uptake of glutamate into the astrocyte, which generates a net inward current across the membrane. In addition to cholinergic axons, the stratum oriens/alveus also contains glutamatergic axons (e.g., recurrent collaterals from CA1 pyramidal neurons) that make synaptic contacts in the stratum oriens with CA1 pyramidal neurons and interneurons (Ramón y Cajal, 1904; Klishin et al., 1995; Maccaferri and McBain, 1995). Therefore, it is feasible to think that stimulation of these axons could induce the activation of astrocytic glutamate transporters.

To test this hypothesis, we asked whether the astrocytic inward current evoked by nerve-fiber stimulation was affected by glutamate transporter inhibitors (Fig. 4*A*). After control recordings, slices were superfused with 1 mM DHK, a selective nontransportable antagonist of GLT-1 transporters, plus 0.3 mM t-PDC, a nonselective competitive uptake inhibitor. Although the glutamate transporter antagonists did not modify the amplitude of the stimulation-induced Ca^{2+} elevations, they reduced the amplitude of the inward current (to 19.3 ± 5.8% from control values; $n = 4$) (Fig. 4*A,C,D*). Furthermore, the replacement of the extracellular sodium by lithium, which inhibits the sodium-dependent glutamate uptake, significantly reduced the amplitude of the inward current (to 11.2 ± 5.3% from control values; $n = 7$; $p < 0.001$; data not shown). The remaining residual current observed after glutamate transporter blockade was not significantly changed by cholinergic or glutamatergic receptor antagonists (data not shown). Indeed, after inhibition of the glutamate transporters with DHK plus t-PDC, the relative amplitude of the residual current obtained from control (DHK plus t-PDC) values was 92.0 ± 7.4% ($n = 6$) in 50 nM methyllycaconitine (MLA) plus 50 μM atropine, and 89.3 ± 10.9% ($n = 5$) in 20 μM CNQX plus 50 μM AP-5 plus 0.8 mM MCPG. These results suggest that the residual current recorded after glutamate uptake blockade is not mediated by cholinergic or glutamatergic receptor activation; rather, it is probably attributable to the extracellular K^{+} accumulation during neuronal activity, as described previously in stratum radiatum astrocytes (Bergles and Jahr, 1997, 1998).

Together, these results indicate that the astrocytic inward current was primarily mediated by activation of electrogenic glutamate transporters, suggesting that astrocytes located in the stratum oriens are involved in the clearance of glutamate from the synaptic cleft. Furthermore, the differential sensitivity of the inward current and the Ca^{2+} elevation to glutamate transporter

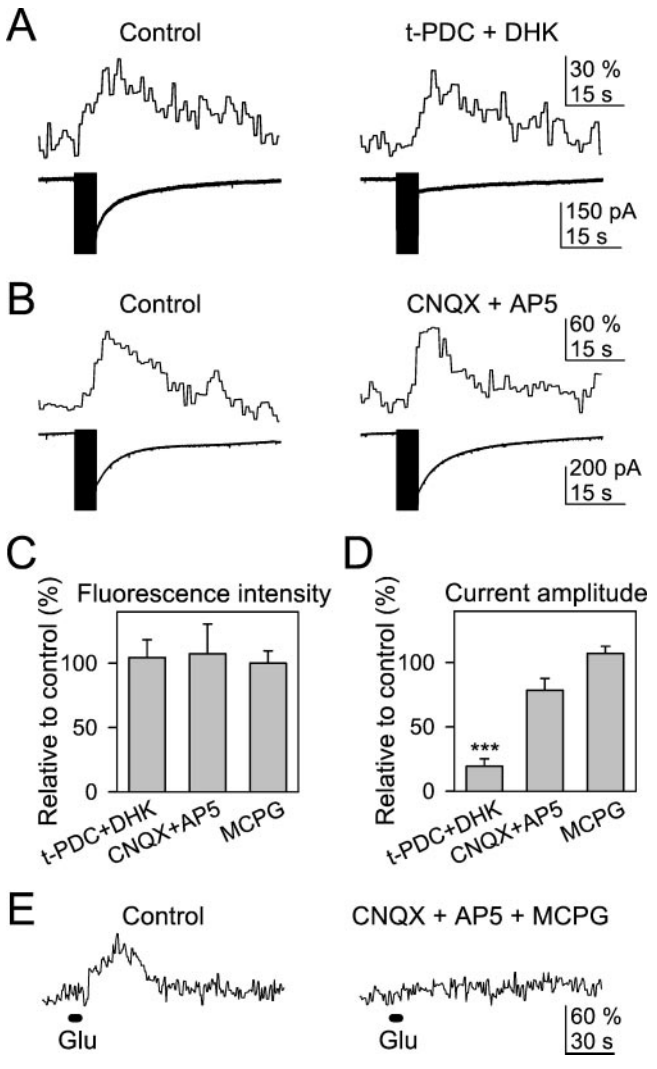

Figure 4. Participation of synaptically released glutamate in the astrocytic responses. *A, B*, Astrocytic Ca^{2+} levels (*top traces*) and whole-cell membrane currents (*bottom traces*) evoked by nerve-fiber stimulation in control conditions and in the presence of 0.3 mM t-PDC plus 1 mM DHK and 20 μM CNQX plus 50 μM AP-5, respectively. *C, D*, Relative changes from control recordings of the fluorescence intensity and membrane current amplitudes, respectively, evoked by nerve-fiber stimulation in the presence of t-PDC plus DHK ($n = 4$), CNQX plus AP-5 ($n = 6$), and 0.8 mM MCPG ($n = 6$). Significant differences were established by the Student's t test at $^{***}p < 0.001$. *E*, Intracellular Ca^{2+} variations evoked by glutamate ionophoresis estimated from the fluorescence intensity recorded from a single astrocyte filled with fluo-3. The astrocytic Ca^{2+} elevations evoked in control conditions (*left trace*) by glutamate ionophoresis (0.7 M; 5 sec; *bottom line*) were prevented in the presence of glutamate receptor antagonists (20 μM CNQX, 50 μM AP-5 plus 0.8 mM MCPG) (*right trace*).

inhibitors suggest that different mechanisms underlie both phenomena.

Astrocytic Ca^{2+} variations are not mediated by activation of glutamate receptors

It has been shown in hippocampal slices that stimulation of glutamatergic Schaffer collaterals evokes Ca^{2+} elevations in astrocytes located in the stratum radiatum of the CA1 area (Porter and McCarthy, 1996; Pasti et al., 1997; Bezzi et al., 1998). These Ca^{2+} elevations are mediated through activation of metabotropic glutamate receptors (mGluRs), because they are abolished by the mGluR antagonist MCPG (Porter and McCarthy, 1996; Pasti et al., 1997) and are mimicked by the mGluR agonist *trans*-(±)-1-amino-1,3-cyclopentanedicarboxylic acid (Pasti et al., 1997). Moreover, Bergles et al. (2000) have shown that Schaffer collateral–commissural stimulation evokes AMPA-receptor-mediated currents in stratum radiatum oligodendrocyte precursor cells.

Therefore, we investigated whether the Ca^{2+} elevations in stratum oriens astrocytes evoked by stratum oriens/alveus stimulation were similarly mediated by the activation of glutamate receptors. The inward current and the Ca^{2+} elevations were not significantly affected by either the mGluR antagonist MCPG (0.8 mM) or the ionotropic glutamate receptor antagonists CNQX (20 μM) and D-AP-5 (50 μM) (Fig. 4*B–D*), indicating that the astrocytic Ca^{2+} elevations induced by nerve-fiber stimulation were not mediated by activation of glutamate receptors.

We also tested the presence of functional glutamate receptors in stratum oriens astrocytes. Ionophoretic application of glutamate increased the astrocytic Ca^{2+} levels (eight of nine astrocytes; cf. Shelton and McCarthy, 1999) (Fig. 4*E*), indicating that the insensitivity of the astrocytic Ca^{2+} signal to glutamate antagonists was not attributable to the absence of functional glutamate receptors.

Astrocytic Ca^{2+} variations are mediated by activation of mAChRs

The stratum oriens/alveus of the hippocampus contains cholinergic afferents projecting from the septum and diagonal band of Broca to the CA1 area that make synaptic contacts in the stratum oriens (Lewis and Shute, 1967; Amaral and Witter, 1995). Therefore, we investigated the involvement of cholinergic receptors on the stimulus-induced astrocytic Ca^{2+} elevations.

Cultured hippocampal astrocytes express nicotinic cholinergic receptors (nAChRs), which contain the α_7 subunit and increase the intracellular Ca^{2+} through a Ca^{2+}-induced Ca^{2+}-release mechanism (Sharma and Vijayaraghavan, 2001), but their functional expression *in situ* remains unknown. Furthermore, synaptic activation of nAChRs has been shown in molluscan glial cells (Smit et al., 2001). To investigate the participation of nAChRs, we tested the sensitivity of the nerve-fiber-evoked astrocytic Ca^{2+} responses to 50 nM MLA, an antagonist of the α_7-containing nAChRs. Both the astrocytic Ca^{2+} elevations and the inward current were not significantly affected by 50 nM MLA (Fig. 5*A,D,E*).

We have shown that functional mAChRs are expressed by stratum oriens astrocytes, and it has been reported that mAChRs can increase intracellular Ca^{2+} levels (Fig. 2*A*) (Shelton and McCarthy, 2000). Therefore, we tested the effects of atropine on the nerve-fiber-evoked astrocytic Ca^{2+} responses (Fig. 5*B*). The Ca^{2+} elevations were reduced by 50 μM atropine (to 12.1 ± 8.2% from control values; $n = 8$) (Fig. 5*B,D*). However, the inward current was not significantly affected (Fig. 5*D*), suggesting that the Ca^{2+} signal reduction was not attributable to a reduction in synaptic activity. Therefore, these results indicate that the astrocytic Ca^{2+} elevations induced by stratum oriens/alveus stimulation were mediated through the activation of mAChRs.

mAChRs are coupled to G-proteins, and their activation leads to Ca^{2+} mobilization from the internal stores, thus elevating the intracellular Ca^{2+} levels (Porter and McCarthy, 1997; Shelton and McCarthy, 2000). We tested whether intact intracellular Ca^{2+} stores are required for the astrocytic responses by perfusing the slices with thapsigargin, which depletes the intracellular Ca^{2+} stores by inhibiting the Ca^{2+} ATPase (Charles et al., 1993;

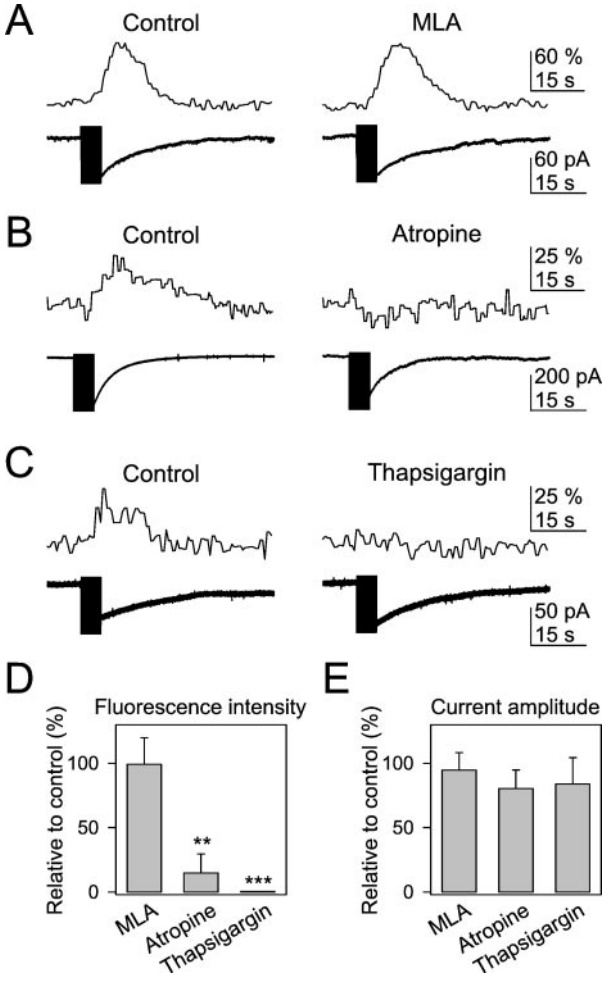

Figure 5. Astrocytic Ca^{2+} elevations are mediated by activation of mAChRs that mobilize Ca^{2+} from the intracellular stores. *A–C*, Astrocytic Ca^{2+} levels (*top traces*) and whole-cell membrane currents (*bottom traces*) evoked by nerve-fiber stimulation in control conditions and in the presence of 50 nM MLA, 50 μM atropine, and 1 μM thapsigargin, respectively. *D, E*, Relative changes from control recordings of the fluorescence intensity and membrane current amplitudes, respectively, evoked by nerve-fiber stimulation in the presence of 50 nM MLA ($n = 6$), 50 μM atropine ($n = 8$), and 1 μM thapsigargin ($n = 5$). Significant differences were established by the Student's t test at $^{**}p < 0.01$ and $^{***}p < 0.001$.

Araque et al., 1998a). After control recordings, the slices were perfused with 1 μM thapsigargin for 30–45 min. The stimulus-induced inward currents were not significantly affected by thapsigargin (Fig. 5*C,E*), suggesting that thapsigargin did not modify synaptic transmitter release (cf. Reyes and Stanton, 1996). In contrast, the astrocytic Ca^{2+} elevations were abolished by thapsigargin (Fig. 5*C,D*), indicating that the astrocytic Ca^{2+} elevations require the presence of intact intracellular Ca^{2+} stores.

Together, these results indicate that the stimulus-induced intracellular Ca^{2+} elevations in stratum oriens astrocytes are attributable to synaptically released ACh acting on mAChRs that mobilize intracellular Ca^{2+}.

Astrocytic Ca^{2+} variations are spatially defined

Discrete cellular microdomains that respond differentially to synaptically released neurotransmitters have been demonstrated in astrocytes and Bergmann glia (Pasti et al., 1997; Grosche et al., 1999). Therefore, we investigated the spatial cellular distribution

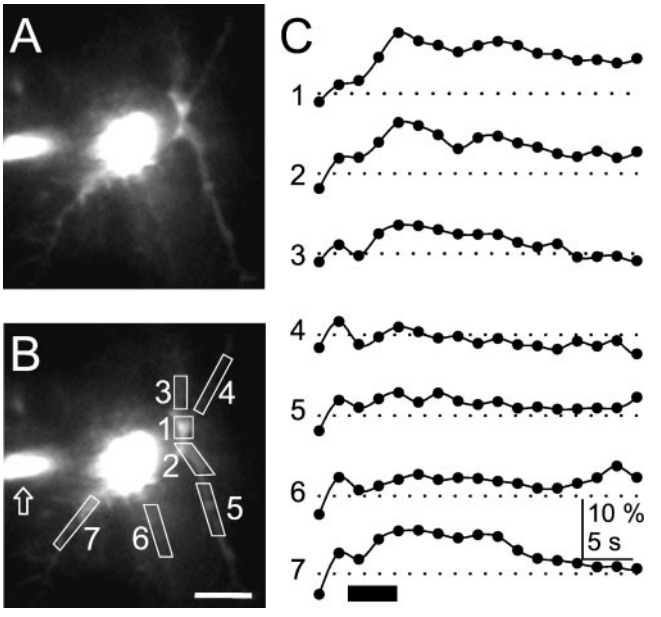

Figure 6. Nerve-fiber stimulation evokes local astrocytic Ca^{2+} elevations. *A, B*, Fluorescence images of an astrocyte filled with the Ca^{2+} indicator fluo-3 included in the patch pipette (*left side* of the astrocyte; *arrow* in *B*). *White boxes* in *B* indicate ROI (15–20 μm^2) in which fluorescence signals were measured. Scale bar, 9 μm. *C*, Normalized fluorescence intensity in regions shown in *B*. Nerve stimulation at 30 Hz for 5 sec is indicated by the *black box* in the *bottom trace*. *Dotted lines* indicate zero values estimated from the averaged resting values recorded before stimulation. While regions 1, 2, 3, and 7 increased their Ca^{2+} signal after nerve stimulation ($\Delta F/F_0 > 5\%$), regions 4–6 did not respond to stimulation.

of the cholinergic-mediated neuron–astrocyte signal. The intracellular Ca^{2+} levels of fluo-3-filled astrocytes were imaged (Fig. 6*A*). Several ROI of 15–20 μm^2, including different conspicuous processes, were defined (Fig. 6*B*). The stimulus-induced Ca^{2+} variations in those regions were analyzed and the ROI were considered to respond to the stimulation when $\Delta F/F_0$ increased by >5% for at least two consecutive images. Figure 6*C* shows the intracellular Ca^{2+} levels of the different ROI defined in Figure 6*A*. Although some regions increased their intracellular Ca^{2+} levels (ROI 1–3 and 7), other regions failed to respond to the nerve stimulation (ROI 4–6). Therefore, in close agreement with previous results that indicated subcellular microdomains for neuron–glia interaction (Grosche et al., 1999), our results indicate that subcellular regions of astrocytes may respond differentially to the synaptic cholinergic activity.

DISCUSSION

Recent data have demonstrated the existence of bidirectional communication between astrocytes and neurons, which is mediated by neurotransmitters released by both neurons and glia. Although astrocytes express numerous neurotransmitter receptors, the ability of synaptically released neurotransmitters to access these astrocytic receptors is largely unknown. We show that hippocampal astrocytes located in the stratum oriens region of the CA1 area, which receives abundant cholinergic input, respond to ACh released by synaptic terminals. The synaptically released ACh acts on mAChRs, releasing Ca^{2+} from the internal stores.

Glutamate-mediated Ca^{2+} increases in hippocampal astrocytes located in the stratum radiatum have been shown previously to be responsible for neuron–glia signaling in hippocampal slices

(Porter and McCarthy, 1996; Pasti et al., 1997). However, our data indicate that neuron-evoked Ca^{2+} increases in stratum oriens astrocytes are mediated by cholinergic rather than by glutamatergic receptors. The insensitivity of the astrocytic Ca^{2+} signaling to glutamate was surprising, because afferent fibers in the stratum oriens/alveus also included glutamatergic axons (Ramón y Cajal, 1904; Klishin et al., 1995; Maccaferri and McBain, 1995). The lack of a glutamate effect on the neuron-evoked astrocytic Ca^{2+} signaling was not attributable to the absence of functional glutamate receptors because ionophoretically applied glutamate increased the astrocytic Ca^{2+} levels (cf. Shelton and McCarthy, 1999). Furthermore, the recorded glutamate transporter currents demonstrate that astrocytes were able to sense synaptically released glutamate. A possible explanation for the absence of glutamate receptor activation could be a moderate rise in the extracellular concentration of glutamate, sufficient to activate glutamate transporters but not glutamate receptors. However, this seems unlikely, because the affinity of mGluRs and transporters for glutamate is similar (in the micromolar range) (Conn and Pin, 1997; Anderson and Swanson, 2000). Although the affinity of AMPA receptors for glutamate is relatively lower (EC_{50} ~0.5 mM; Patneau and Mayer, 1990), glutamate-induced Ca^{2+} elevations in hippocampal astrocytes are primarily mediated by mGluRs (Porter and McCarthy, 1996; Pasti et al., 1997). Alternatively, a spatially restricted localization of glutamate and cholinergic receptors may be the simplest explanation for these results. This interpretation is also supported by the different Ca^{2+} signal responses observed in different astrocytic regions after nerve stimulation. Furthermore, although glutamate receptors do not contribute to the neuron-evoked Ca^{2+} elevations, our results show simultaneous glutamate-mediated uptake currents and cholinergic-mediated Ca^{2+} elevations. Therefore, together these results suggest the existence of functional astrocytic subcellular domains. This suggestion is in agreement with a recent report that demonstrated that Bergmann glial cells show subcellular microdomains that may respond independently to synaptic activity (Grosche et al., 1999).

Nicotinic responses have been reported recently in cultured astrocytes (Sharma and Vijayaraghavan, 2001). However, our results indicate that the cholinergic signaling found in the stratum oriens is mediated by mAChRs, which is in agreement with many studies that report that the types of cholinergic receptors expressed by glial cells *in situ* are muscarinic (Rochon et al., 2001).

Cholinergic signaling in the nervous system is highly complex; it is mediated by different types of nAChRs and mAChRs that may act presynaptically or postsynaptically. Additional complexity arises from the recently demonstrated ACh-mediated neuron–glia interaction in molluscs, in which synaptically released ACh induces the release of an ACh-binding protein from glial cells that modulate cholinergic transmission (Smit et al., 2001). In addition to the cholinergic neuronal transmission, we show an additional cholinergic communication between neurons and astrocytes, which adds more complexity to the highly complex cholinergic signaling in the CNS.

Because Ca^{2+} elevations in astrocytes have been shown to evoke the release of glutamate (Araque et al., 1998a,b, 2000; Bezzi et al., 1998; Parpura and Haydon, 2000), which can modulate the neuronal activity and the glutamatergic and GABAergic transmission (Araque et al., 1998a,b; Kang et al., 1998; Newman and Zahs, 1998), the cholinergic-mediated astrocytic Ca^{2+} increases may lead to glutamate release that would modulate synaptic transmission in the hippocampus.

The importance of cholinergic transmission in the physiology of the hippocampus is well established. Several studies suggest that cholinergic inputs play a key role in the generation of the hippocampal theta rhythm, which is extremely relevant in different behavioral states (Vertes and Kocsis, 1997; Leung, 1998). Deficits in cholinergic transmission have been associated with pathophysiological conditions such as Alzheimer's disease (Kasa et al., 1997). Furthermore, hippocampal cholinergic transmission has been proposed to be involved in some forms of synaptic plasticity, such as long-term potentiation, a cellular mechanism thought to underlie processes of learning and memory (Auerbach and Segal, 1996; Yun et al., 2000). Considering the active role of glia in modulating neuronal excitability and synaptic transmission (Araque et al., 1999, 2001; Haydon, 2001), the present demonstration of cholinergic-mediated neuron–astrocyte signaling suggests that astrocytes might participate in such phenomena. Finally, several groups have demonstrated previously the existence of bidirectional communication between astrocytes and neurons, in which astrocytes respond to the activity of synaptic terminals of local circuit neurons (Araque et al., 1999). Here we show that hippocampal astrocytes respond to the activity of axons arising from the septum and diagonal band of Broca, suggesting that astrocytes are a target of axonal inputs between different brain areas, which adds additional complexity to the functional communication pathways in the nervous system.

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