Metabotropic Glutamate Receptor Activation Modulates Kainate and Serotonin Calcium Response in Astrocytes

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Although metabotropic glutamate receptor (mGluR) modulation has been studied extensively in neurons, it has not been investigated in astrocytes. We studied modulation of glutamate-evoked calcium rises in primary astrocyte cultures using fura-2 ratiometric digital calcium imaging. Calcium plays a key role as a second messenger system in astrocytes, both in regulation of many subcellular processes and in long distance intercellular signaling. Suprachiasmatic nucleus (SCN) and cortical astrocytes showed striking differences in sensitivity to glutamate and to mGluR agonists, even after several weeks in culture. Kainate-evoked intracellular calcium rises were inhibited by concurrent application of the type I and II mGluR agonists quisqualate (10 μ M), *trans*-($\pm$)-1-amino-1,3-cyclopentanedicarboxylate (100–500 μ M), and (2S-1'S-2'S)-2-(carboxycyclopropyl)glycine (L-CCG-I) (10 μ M). Inhibition mediated by L-CCG-I had long-lasting effects (>45 min) in ~30% of the SCN astrocytes tested. The inhibition could be mimicked by the L-type calcium channel blocker nimodipine (1 μ M) as well as by protein kinase C (PKC) activators phorbol 12,13-dibutyrate (10 μ M)

and phorbol 12-myristate 13-acetate (500 nM), and blocked by the PKC inactivator ($\pm$)-1-(5-isoquinolinesulfonyl)-2-methylpiperazine (200 μ M), suggesting a mechanism involving PKC modulation of L-type calcium channels. In contrast, mGluRs modulated serotonin (5HT)-evoked calcium rises through a different mechanism. The type III mGluR agonist L-2-amino-4-phosphonobutyrate consistently inhibited 5HT-evoked calcium rises, whereas in a smaller number of cells quisqualate and L-CCG-I showed both inhibitory and additive effects. Unlike the mGluR-kainate interaction, which required a pretreatment with an mGluR agonist and was insensitive to pertussis toxin (PTx), the mGluR modulation of 5HT actions was rapid and was blocked by PTx. These data suggest that glutamate, acting at several metabotropic receptors expressed by astrocytes, could modulate glial activity evoked by neurotransmitters and thereby influence the ongoing modulation of neurons by astrocytes.

Key words: astrocytes; suprachiasmatic nucleus; calcium; serotonin; kainate; metabotropic glutamate receptors; ionotropic glutamate receptor; digital imaging; cortex

Astrocytes are an integral part of signal processing in the nervous system. They express functional receptors for several neurotransmitters and modulators and show stereotyped calcium responses from internal or external sources (Murphy and Pearce, 1987; Barres et al., 1988; Barres et al. 1990; McCarthy and Salm, 1991; Hösl and Hösl, 1993; Sontheimer, 1994). Changes in intracellular calcium not only modify glial physiology, but they also seem to be involved in complex intercellular communication networks (for review, see Finkbeiner, 1993). Astrocytes can control neuronal excitability directly (Nedergaard, 1994; Parpura et al., 1994) or indirectly through modulation of extracellular potassium (Barres, 1991) or glutamate levels (Shao and McCarthy, 1994; Gallo and Russell, 1995).

Astrocytes have been studied in regard to their function in various physiological states, including development (LaMantia, 1995), glucocorticoid neurotoxicity (Horner et al., 1990; Virgin et al., 1991), and ischemia (Tombaugh and Sapolsky, 1990; Dugan et al., 1995;

Papadopoulos et al., 1996), and in psychiatric disorders, including anxiety and depression (Whitaker-Azmitia et al., 1993). Glutamate and serotonin (5HT) play critical roles in several of these states (LaMantia, 1995; Saxena, 1995). In addition, results from several labs have suggested that astrocytes may play a role in the control of circadian rhythmicity (Ewer et al., 1992; van den Pol et al., 1992; Lavialle and Servire, 1993, 1995; van den Pol and Dudek, 1993; Bennett and Schwartz, 1994; Prosser et al., 1994).

Glutamate is the major excitatory neurotransmitter in the CNS, including the cortex and the suprachiasmatic nucleus (SCN). In the SCN it acts as the primary excitatory transmitter conveying the photic signal from the retina that entrains the circadian clock. Modulation of glutamate transmission has been studied extensively in neurons in the CNS. In hypothalamic neurons, modulators such as neuropeptide Y (NPY) and adenosine depress glutamatergic transmission by either a pertussis toxin (PTx)-sensitive G-protein-coupled pathway or by inhibition of voltage-activated calcium channels (VACCs). In neurons, glutamate-evoked calcium rises were depressed by adenosine, whereas in astrocytes calcium responses were potentiated by adenosine (Obrietan et al., 1995).

Interactions between ionotropic and metabotropic receptors have been shown in several neuronal systems (Glaum and Miller, 1993; Nakanishi, 1994; Schoepp, 1994; Gereau and Conn, 1995; Gorman et al., 1995). Modulation may occur by downregulation of ion channel receptor sensitivity directly or by cross-regulation

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through another transmitter system or second messenger pathway. mGluRs have been shown in neurons to modulate potassium channel function (Gerber and Gähwiler, 1994) and calcium channel currents (Sahara and Westbrook, 1993; Chavis et al., 1995). This interaction is sometimes sensitive to G-protein inhibitors such as PTx but not to second messenger blockade, raising the possibility of a direct action of the G-protein in neurons (Lester and Jahr, 1990; Swartz and Bean, 1992; Chavis et al., 1994; Ikeda et al., 1995; Choi and Lovinger, 1996).

Metabotropic glutamate receptors (mGluRs) are divided into three groups based on sequence homology of the cloned receptors and on group-selective agonists and second messenger pathways (Nakanishi, 1992, 1994; Nakanishi and Masu, 1994). All three groups of mGluRs are expressed in SCN cells (van den Pol, 1994; van den Pol et al., 1995, 1996a). Modulatory interactions between ionotropic and mGluRs have not been studied in astrocytes.

In this paper, we describe a difference in glutamate sensitivity in astrocytes derived from SCN and cortex. We investigated how agonists for specific classes of mGluRs modulated astrocyte responsiveness to 5HT and to the ionotropic GluR (iGluR) agonist kainate. We show that mGluR agonists not only acutely enhance or depress transmitter-elicited calcium rises, but also may induce a depression in kainate responsiveness that lasts even after the mGluR agonist is gone. We also demonstrate transmitter-specific modulatory transduction pathways within astrocytes.

Some of these data have been published previously in abstract form (Haak et al., 1995).

MATERIALS AND METHODS

Tissue culture

Neonatal (E22–P2) Sprague Dawley rat brains (Simonsen, Gilroy, CA) were washed three times in ice-cold HEPES-buffered saline solution (HBSS) (Life Technologies, Grand Island, NY). Coronal slices (525 μ m) were prepared using a tissue chopper and washed in ice-cold HBSS. A polished flat 20 gauge stainless steel needle was used to punch the SCN (see Fig. 1A) and medial pieces of the neocortex from the same slice. Individual tissue punches were placed into 0.5 ml thin-walled microcentrifuge tubes containing 10 μ l of cold HBSS and washed twice with 100 μ l of HBSS lacking calcium and magnesium (HBSS[−]). Punches were incubated overnight at 4°C in 100 μ l of HBSS[−] containing 0.2% trypsin–EDTA (Life Technologies). After incubation for 30 min at 37°C, trypsin was removed, and 100 μ l of warm growth medium was added. Each punch was triturated gently to create a single-cell suspension and then was plated on a 22 mm² poly-D-lysine-coated glass coverslip in a single well of a 6-well tissue culture plate. Coverslips had been soaked previously overnight in Cleaning Concentrate (Bio-Rad, Richmond, CA), rinsed for 1 hr in deionized water, and then dried and autoclaved. To increase cell density, dispersed punches were plated within a 7-mm-diameter glass cloning ring placed on the coverslip. Two hours after plating, 3 ml of growth medium was added per well, and rings were removed. Cultures were maintained in a Napco 6100 incubator at 37°C and 5% CO₂ in growth medium: glutamate- and glutamine-free MEM (Life Technologies) supplemented with heat-inactivated 10% bovine calf serum (Hyclone, Logan, UT), 100 U/ml penicillin/streptomycin (Life Technologies), and 1 mM kynurenate (RBI, Natick, MA) (Finkbeiner and Stevens, 1988). Growth medium was replaced weekly. Cells were used after at least 14 d *in vitro*.

Histology

Verification of SCN cultures. Slices and punches were made as described above. Chilled slices were fixed overnight at 4°C in 4% paraformaldehyde and 0.3% glutaraldehyde and then incubated for 20 min with 2 μ g/ml propidium iodide (Molecular Probes, Eugene, OR). Staining was visualized using a Molecular Dynamics Multiprobe 2010 confocal microscope. Excitation wavelength (Argon/Krypton laser) was 488 nm; the emission wavelength was attenuated below 590 nm. Laser illumination was attenuated to 10% maximum intensity by neutral density filters. An example of a slice from which the SCN was punched out for culture is seen in Figure 1A.

Immunocytochemistry. Cells growing on coverslips were rinsed once in 100 mM PBS [containing (in gm/l): 8 NaCl, 0.2 KCl, 1.44 Na₂HPO₄, 0.24 KH₂PO₄] and then fixed in ice-cold anhydrous methanol. After cells were fixed, they were washed with four rinses in PBS and then stained, following the protocol included in the Vectastain Elite ABC Kit (Vector Labs, Burlingame, CA). In brief, cells were incubated with 1.5% goat serum diluted in PBS. After 30 min, serum was removed and primary mouse monoclonal antibody diluted in 1.5% goat serum was added as follows: microtubule-associated protein (MAP2) (Sigma, St. Louis, MO) 1:500 dilution, glial fibrillary acidic protein (GFAP) (Novocastra/Vector Labs) 1:100 dilution. Cells were incubated with primary antibody for at least 1–6 hr and then washed with three rinses of PBS, at which time the secondary goat anti-mouse antibody (ABC Kit) was added for 30 min. Then cells were washed with three rinses of PBS, incubated with ABC complex (ABC Kit) for 30 min, and again washed three times with PBS. Finally, staining was visualized with diaminobenzidine intensified with NiCl₂, added to the cells for 1–2 min. To stop the peroxidase reaction, cells were rinsed in PBS.

Calcium digital imaging

Before imaging, cells were incubated for 30 min at 37°C with 5 μ M fura-2 acetoxymethylester (Molecular Probes) in a HEPES-buffered perfusion solution (137 mM NaCl, 25 mM glucose, 5 mM KCl, 1 mM MgCl₂, 3 mM CaCl₂, 10 mM HEPES, pH 7.4). Cells were then washed, loaded into a 180 μ l laminar flow perfusion chamber (Forscher et al., 1987), and perfused with HEPES buffer at a constant rate of 1 ml/min. For experiments using zero-calcium HEPES buffer, we omitted CaCl₂ from the perfusion solution and added 1 mM EGTA and a total of 10 mM MgCl₂. Perfusion solutions were applied from reservoirs using individual capillary lines abutting the laminar flow cell surface. Switching between solutions was accomplished by opening and closing stopcock valves on individual lines. Solutions moved uniformly and rapidly across the perfusion chamber, with complete washout in ~5 sec. All experiments were performed at room temperature. In the course of these experiments and the preliminary work leading to them, we tested 9360 SCN astrocytes and 4700 cortical astrocytes from 35 separate sets of cultures, with 8–18 coverslip cultures per set.

Cells were imaged using a 40 $\times$ Olympus DApo objective with high ultraviolet transmittance on a Nikon Diaphot 300 inverted microscope. Individual cells were tagged with a 5 $\times$ 5 pixel square, and 340/380 nm Ca²⁺ ratio images were captured every 2 sec and recorded using FLUOR software (Universal Imaging, West Chester, PA) running on a 486 PC. Switching between 340 and 380 nm wavelengths was accomplished using a Sutter (Novato, CA) Lambda 10 controller and filter wheel. Excitation light was provided by a 150 W xenon lamp (Optiquip, Highland Mills, NY) and was attenuated by 90% to minimize photo bleaching and photo toxicity and to maximize recording time. Fluorescence emitted by the cells was passed through a 480 nm filter on the microscope and into a Hamamatsu 2400 silicon-intensified-target video camera. Between 10 and 40 cells were recorded simultaneously in a single experiment. Ratiometric fluorescent values collected in this way were converted to Ca²⁺ concentrations with the calibration method described by Grynkiewicz et al. (1985) using fura-2 and calcium standard solutions from Molecular Probes. Calibrated Ca²⁺ data were analyzed on an Apple Macintosh computer using IgorPro Software (WaveMetrics, Lake Oswego, OR). In the figures presented, each trace represents the ratiometric calcium response of one astrocyte.

Data for histograms representing peak heights were calculated by subtracting from the peak Ca²⁺ rise an averaged baseline Ca²⁺ level from the 3 min just before the Ca²⁺ rise. Increases of >60 nM over baseline were pooled and averaged. Data are from two to five experiments on different cultures and are presented as the average nanomolar Ca²⁺ rise $\pm$ SEM. To determine statistical significance we used a Student's *t* test, unless mentioned otherwise.

Chemicals. Sodium glutamate and poly-D-lysine hydrobromide (MW >300,000) were purchased from Sigma; kynurenate, *trans*-($\pm$)-1-amino-1,3-cyclopentanedicarboxylate (t-ACPD), L-2-amino-4-phosphonobutyrate (L-AP4), kainate, quisqualate, serotonin-creatinine complex, PTx, phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-dibutyrate (PDBu), and nimodipine from RBI (Natick, MA); and 2S-1'-S-2'-S)-2-(carboxycyclopropyl)glycine (L-CCG-I) and ($\pm$)-1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7 dihydrochloride) from Tocris-Cookson (St. Louis, MO).

Figure 1. Immunocytochemistry. *A*, Confocal image of a propidium iodide-stained brain slice from a neonatal rat containing the bilateral SCN. The SCN has been punched out of the right side for use in tissue culture, and this micrograph shows the remaining tissue slice. Bar, 50 μ m. *B*, *D*, GFAP immunoreactivity was found in SCN and cortical astrocytes. *C*, *E*, In contrast, both cultures were negative for the neuronal marker MAP2. Bar, 25 μ m.

RESULTS

Glutamate response differs in SCN and cortical astrocytes

Glutamate dose-responses

Astrocytes derived from SCN and cortex showed different calcium responses to glutamate. To determine the dose-response relations, astrocyte cultures were tested with a range of GluR agonist concentrations, applied twice for 30 sec each. Each concentration was tested on at least three cultures. Only cells that responded to both applications with a calcium rise at least twice that of basal fluctuations (noise) were counted as responders. Results are expressed as a percentage of cells responding per total number of cells tested for each concentration or as the mean change in cytosolic calcium values. The morphology and GFAP staining intensity of astrocytes from SCN and cortex appeared similar (Fig. 1*B,D*). The cultures were primarily astrocytes; immunostaining with an antibody against the neuronal antigen MAP2 did not reveal neurons in these cultures (Fig. 1*C,E*). Although the morphology of the astrocyte cultures looked similar, SCN and cortex astrocytes showed marked differences in glutamate sensitivity

(Fig. 2*A,B*, dotted lines). Although only 33% ($n = 211$) of the SCN astrocytes responded to 100 μ M glutamate, almost all cells (95%, $n = 151$) responded in cortical astrocyte cultures.

Specific GluR agonist dose-responses

Because glutamate stimulates several subtypes of glutamate receptors, we tested agonists for specific GluR subtypes to determine whether the reduced responsiveness of SCN cells was a result of comparatively lowered response at all GluRs. By using the same dose-response protocol described above for glutamate, we found that SCN astrocytes were severalfold less sensitive to the ionotropic GluR agonist kainate ($n = 263$) than were cortical astrocytes. The mGluR agonist t-ACPD rarely evoked calcium rises in SCN astrocytes ($n = 241$), even at doses up to 1 mM (Fig. 2*A*). In contrast, cortical astrocytes showed dose-responses to glutamate, kainate ($n = 287$), and t-ACPD ($n = 306$) that were very similar to each other (Fig. 2*B*). Although the response to individual agonists was different between cortex and SCN, >90% of the astrocytes from each region responded to some glutamate receptor agonist.

Figure 2. GluR agonist dose-response curves in astrocytes. *A, B*, Dose-response curves for SCN and cortical astrocytes for glutamate (dotted lines), kainate, and t-ACPD. The number of cells that showed a repeatable calcium rise to each concentration tested was plotted, and a curve was fit to the response using the logistic function (IgorPro). EC_{50} for glutamate, kainate, and t-ACPD in cortical astrocytes was $\sim 30 \mu M$. In contrast, the EC_{50} to kainate in SCN astrocytes was $\sim 100 \mu M$. t-ACPD and glutamate evoked a calcium rise in $<50\%$ of SCN astrocytes.

Modulation of kainate responses by mGluRs

Using doses of kainate to which 50% of the cells responded (EC_{50}), we investigated the modulation of kainate-evoked calcium rises by mGluR agonists. Repeated application of kainate evoked calcium rises with $<15\%$ variation between peaks (Fig. 3*A*). In SCN astrocyte cultures, activation of mGluRs inhibited kainate-evoked calcium rises (Fig. 3*B,C*). We tested four mGluR agonists: t-ACPD, which stimulates both type I and II mGluRs; quisqualate, which stimulates both AMPA iGluRs and type I mGluRs; L-CCG-I, which is fairly specific for type II receptors; and the specific type III mGluR agonist L-AP4 (Schoepp, 1994; Pin and Duvoisin, 1995; Roberts, 1995). t-ACPD ($500 \mu M$, $n = 71/78$, $-42.8 \pm 1.4\%$; $100 \mu M$, $n = 33/72$, $-33.3 \pm 3.1\%$), quisqualate ($10 \mu M$, $n = 123/145$, $-58.3 \pm 2.0\%$), and L-CCG-I ($10 \mu M$, $n = 43/52$, $-43.7 \pm 3.1\%$) inhibited kainate-evoked calcium rises by the percentages given and in the number (n) of astrocytes indicated. The method used to determine percentage change is described in the legend to Figure 3. The type III agonist L-AP4 ($10 \mu M$) was effective in only a few cells ($n = 9/53$, $-40.2 \pm 5.7\%$).

In some cases, quisqualate ($10 \mu M$, $n = 15/45$, $280.6 \pm 84.4\%$) and t-ACPD ($100 \mu M$, $n = 15/72$, $189.4 \pm 51.9\%$) increased the kainate-evoked calcium rise, consistent with their ability to stimulate more than one glutamate receptor subtype. In cortical glial

cultures, $100 \mu M$ t-ACPD induced calcium rises by itself but decreased the maximal amplitude of the kainate-evoked calcium rises ($n = 68/68$, $-74.1 \pm 1.7\%$). Thus, the t-ACPD-mediated depression of the kainate response was greater in cortical than in SCN astrocytes. Dose-response curves to t-ACPD (Fig. 2) suggest that SCN and cortical astrocyte cultures contain different populations of functional mGluR receptors. In addition, AMPA and quisqualate show a differential ability to evoke calcium rises in SCN and cortical cultures. In cortex, most astrocytes show calcium rises in response to AMPA ($10 \mu M$, 81.8% , $n = 154$) and quisqualate ($10 \mu M$, 99.0% , $n = 105$). Fewer SCN astrocytes showed calcium rises: 43.9% responded to quisqualate ($n = 82$). Even at $100 \mu M$, only 43.7% showed a calcium response to AMPA ($n = 137$), whereas $>95\%$ of cortical astrocytes responded to $100 \mu M$ AMPA. Taken together, these results suggest that mGluR modulation in cortical and SCN astrocytes may involve different mGluR subtypes.

mGluR agonist treatment evokes long-lasting effects

In addition to acute effects, t-ACPD and L-CCG-I evoked a long-lasting depression of subsequent kainate-evoked calcium rises in some SCN and cortex astrocytes (Fig. 4*A,B*) but had little detectable effect on others (Fig. 4*C*). Long-lasting depression was defined as a minimum 15% reduction of kainate-evoked calcium rise by the mGluR agonist coupled with subsequent kainate-evoked calcium rises that did not show recovery to pre-mGluR levels. The post-mGluR kainate rise had to be depressed by within 10% or more of the percentage decrease found during mGluR application. Long-lasting depression was seen in 33% (20/60) of SCN astrocytes. L-CCG-I depressed the mean kainate-evoked cytosolic calcium level by $38.3 \pm 3.4\%$, from 707 ± 108 to 466 ± 76 nM ($p < 0.001$; paired t test). Kainate responses 5–20 min after L-CCG-I was removed remained depressed by $36.9 \pm 2.3\%$. In contrast, astrocytes that did not respond to L-CCG-I in the same experiments ($n = 40/60$) showed little decrease in the amplitude of kainate responses over the same period ($-8.7 \pm 3.9\%$; $p < 0.001$ vs cells showing long-lasting depression; t test). In separate experiments, we demonstrated that L-CCG-I treatment evoked long-lasting depression of kainate-evoked calcium rises that extended beyond 45 min ($n = 12/55$, $-58.4 \pm 4.2\%$). This depression was significantly different ($p < 0.001$; t test) from calcium rises in astrocytes in the same experiments not showing an initial depression by L-CCG-I ($n = 43/55$).

Signal transduction pathways: G-proteins

To elucidate how mGluR agonists might decrease responsiveness to kainate, we focused on signal transduction pathways. Because mGluRs act through G-proteins, we asked which G-proteins were necessary for inhibition. Cultures were treated overnight with the G_i/G_o protein inhibitor PTx (200 ng/ml). PTx did not block the inhibitory effects of $10 \mu M$ quisqualate ($n = 85$), $500 \mu M$ t-ACPD ($n = 48$), or $10 \mu M$ L-CCG-I ($n = 48$), indicating that the inhibitory actions of these mGluR agonists could not be accounted for solely by action via a G_i/G_o protein-coupled receptor (Fig. 5). Of interest, PTx treatment dramatically increased the number of cells that showed a calcium rise on application of $500 \mu M$ t-ACPD from $0/143$ to $56/108$ cells, suggesting that there may be specific signal transduction systems that are constitutively inactivated or overruled in SCN astrocytes. PTx may block type II mGluRs and thereby uncover a response to PTx-insensitive type I mGluRs. In parallel, the inhibitory effects of quisqualate were

Figure 3. mGluR-iGluR modulation in astrocytes. *A*, Representative astrocyte showing reproducible amplitude of kainate-evoked calcium rises. Kainate (100 μ M) was applied at all arrows for 30 sec. There is no significant difference between peak heights. Maximum variation between peaks was <15%. *B*, A representative calcium trace demonstrating the inhibition of kainate calcium rises in one SCN astrocyte tested with four mGluR agonists, demonstrating complex modulatory actions in a single astrocyte. mGluR agonists (500 μ M t-ACPD, 10 μ M quisqualate, 10 μ M L-AP4, or 10 μ M L-CCG-I) were applied concurrently (large-ended arrows) with 100 μ M kainate, or astrocytes were pretreated for 2–3 min with the mGluR agonist (bars) before kainate was applied for 30 sec. This particular cell did not show any apparent long-lasting inhibition by L-CCG-I that was seen in other cells (see Fig. 4). *C*, Kainate-evoked calcium rises were depressed by mGluR agonists in astrocytes. For each astrocyte, amplitude of kainate-evoked calcium rises was compared to the calcium rise evoked by kainate plus mGluR agonist. Difference in amplitude was normalized by dividing by the kainate-only amplitude and multiplying by 100 to obtain a percentage change. Only cells that showed a calcium rise to kainate >60 nM over basal levels were included in the analysis. We used a criterion of a 15% change (+ or –) in calcium rise amplitude as the low cutoff indicating a modulatory interaction. Negative numbers indicate an inhibition of the kainate calcium rise by mGluR agonist. Although both quisqualate and t-ACPD usually depressed calcium rises, in a few cases they enhanced kainate calcium rises (see text). The number of responding cells is shown below the bars. The flags represent the SEM.

potentiated by PTx treatment (without PTx, $-50.9 \pm 2.5\%$; with PTx, $-77.6 \pm 2.3\%$; $p < 0.001$; t test).

Signal transduction pathways: protein kinase C (PKC)

Brief exposure (5–30 sec) to 500 μ M t-ACPD or 10 μ M L-CCG-I had no effect on subsequent kainate responses; however, when cells were pretreated for at least 2–3 min, a significant depression of subsequent kainate responses was seen. Modulatory pathways that are limited to membrane components are very rapid (Hille, 1994), so the long time course of the mGluR-kainate depression indicated that a second messenger pathway was involved. One possible inhibitory transduction pathway involves PKC. Incubation with the PKC activators PMA (500 nM, $n = 61$, $-80.9 \pm 2.4\%$) or PDBu (10 μ M, $n = 57$, $-82.8 \pm 1.2\%$) depressed kainate-evoked calcium rises (Fig. 6A–C). The inhibitory effects of L-CCG-I ($n = 37$) and t-ACPD ($n = 68$) could be reduced by 30 min pretreatment with the PKC inactivator H-7 (200 μ M). A

calcium trace from a representative SCN astrocyte is shown in Figure 6D1. Before H-7 treatment, t-ACPD depressed the kainate-mediated calcium rise by $-32.9 \pm 2.4\%$. H-7 significantly eliminated the t-ACPD depression of kainate-evoked calcium rises in 26 of 68 cells (Fig. 6D2) and increased the kainate response in the presence of t-ACPD by $55.9 \pm 16.5\%$ ($p < 0.001$; paired t test). These results implicate PKC in the inhibition of kainate responses by mGluRs.

mGluR agonists modulate VACCs

Part of the calcium rise with kainate stimulation may be attributable to activation of voltage-gated calcium channels. We next tested the hypothesis that mGluR agonists inhibited kainate-evoked calcium rises by blocking a calcium channel. Kainate-evoked calcium responses disappear when cells are incubated in buffer lacking extracellular calcium (Cornell-Bell et al., 1990). In our experiments, kainate-evoked calcium responses were mediated in large part by

Figure 4. Long-lasting depression. L-CCG-I evokes a long-lasting depression of kainate-evoked calcium rises in astrocytes; 10 μ M L-CCG-I was applied continuously throughout time indicated by horizontal bar. *A, B*, In these SCN astrocytes, kainate calcium rises were depressed by L-CCG-I and did not recover to levels seen before L-CCG-I was applied. *C*, In this astrocyte from the same experiment as *A* and *B*, little effect of L-CCG-I was found. Long-lasting depression was seen in 20/60 SCN astrocytes. Depressed cells showed a statistically significant difference ($p < 0.001$) in mean calcium rise when compared with all other cells not showing depression and when compared with controls in the absence of L-CCG-I.

Figure 5. Effects of PTx on mGluR-mediated depression of kainate calcium rises. Overnight treatment of SCN astrocytes with 200 ng/ml PTx induced little significant effect on the percentage decrease of kainate-evoked calcium rises mediated by either t-ACPD or L-CCG-I; however, quisqualate-mediated depression seemed to be enhanced by PTx ($*p < 0.001$). Percentage decrease was calculated as described in Figure 3.

VACCs, because evoked rises were inhibited by the L-type VACC blockers nimodipine or nickel. Nimodipine (1 μ M) inhibited by at least 20% the kainate-evoked calcium rise in 123/146 cells (mean

decrease $63.3 \pm 1.9\%$). Nickel (100 μ M) showed parallel effects in 84/93 cells (mean decrease $58.9 \pm 2.3\%$).

Cell depolarization caused by application of 55 mM potassium also evoked calcium rises via VACCs. Potassium evoked repeatable calcium rises with $<15\%$ variation between peaks ($n = 16$) (Fig. 7*A*). In SCN astrocyte cultures, nimodipine (1 μ M) transiently blocked calcium rises evoked by 55 mM potassium in all cells tested ($n = 51$; pre/post nimodipine, $-13.3 \pm 2.0\%$; during nimodipine, $-79.1 \pm 2.2\%$; $p < 0.001$; paired t test). Most of the potassium-evoked calcium rise was blocked by nimodipine; other calcium channel antagonists were not tested. Calcium rises elicited by potassium were inhibited by the PKC activator PMA (500 nM, $n = 14/18$, $-46.0 \pm 5.0\%$) (Fig. 7*D*) and mGluR agonists (Fig. 7*B*). Quisqualate (10 μ M) evoked long-lasting calcium rises, consistent with its ability to stimulate type I mGluRs. Quisqualate reduced the amplitude of potassium-evoked calcium rises in 44 of 47 cells (Fig. 7*C1*). t-ACPD (500 μ M, $n = 42/58$, $-42.9 \pm 2.5\%$), and L-CCG-I (10 μ M, $n = 42/58$, $-34.8 \pm 1.8\%$) (Fig. 7*C*) inhibited potassium-evoked calcium rises. Furthermore, both t-ACPD and L-CCG-I (Fig. 7*C3*) generated a long-lasting depression of subsequent potassium-evoked calcium rises to an amplitude (t-ACPD: $-40.5 \pm 3.7\%$ from 258.0 ± 33.0 nM to 154.7 ± 22.48 nM, $p < 0.001$, paired t test; L-CCG-I: $-32.0 \pm 4.4\%$ from 211.3 ± 43.5 nM to 139.8 ± 26.8 nM, $p < 0.01$, paired t test) and percentage (t-ACPD: 30.3% of cells; L-CCG-I: 27.6% of cells) similar to those of the mGluR depression of kainate responses resulting in long-lasting depression. Together, these data showing long-lasting effects of t-ACPD and L-CCG-I on kainate- and potassium-evoked calcium rises suggest that activation of type II mGluRs depressed kainate-evoked calcium rises by reducing the conductance of voltage-gated calcium channels.

mGluR-5HT receptor interactions

5HT receptors are found throughout the brain, and high densities are found in the SCN and cortex. Many 5HT receptors seem to be G-protein-coupled (Chilmonczyk, 1995; Saxena, 1995; Sleight et al., 1995). 5HT receptors have been localized in the SCN (Lovenberg et al., 1993), and 5HT agonists have been shown to modulate glutamatergic activity in the circadian system *in vivo* (Rea et al., 1994; Srkalovic et al., 1994). To determine which glutamate receptors may be involved in modulation of 5HT responses, we investigated mGluR modulation of 5HT actions in SCN astrocytes.

Similar 5HT response in SCN and cortex astrocytes

Unlike the differential responsiveness to glutamate described above, SCN and cortical astrocytes showed very similar calcium responsiveness to 5HT (Fig. 8). Almost all SCN (87%, $n = 199$) and cortical (82%, $n = 102$) astrocytes showed a calcium rise to 1 μ M 5HT. The construction of dose-response curves revealed that the EC_{50} was ~ 100 nM in astrocytes from both areas.

mGluR modulation of 5HT calcium responses

Glutamate and 5HT interacted to produce calcium rises different from those produced when each transmitter was applied alone (Fig. 9*A*). In SCN astrocyte cultures, 100 μ M glutamate plus 100 nM 5HT showed synergistic (Fig. 9*A1, A2*) or additive (Fig. 9*A3*) effects in 31% of cells ($n = 128$). Synergism between 5HT and glutamate was found in astrocytes where one of the transmitters had evoked little calcium rise by itself but greatly amplified the response to the other transmitter. Similarly, in 31% of cortical astrocytes there was also an additive effect on calcium levels when cells were perfused concurrently with 10 μ M glutamate and 100 nM

Figure 6. Stimulation of PKC mimics effects of mGluR activation in SCN astrocytes. *A*, PDBu (10 μ M) applied for 3 min before 10 μ M L-CCG-I pretreatment further inhibited 100 μ M kainate calcium rise (arrows) and also evoked a long-lasting depression of subsequent kainate calcium rises. *B1*, *B2*, PDBu mimicked the depression of kainate calcium rises mediated by 500 μ M t-ACPD. PDBu generated a long-lasting depression of kainate-evoked calcium rises that did not recover to pre-PDBu levels. *C*, Both PDBu (10 μ M) and PMA (500 nM) depressed kainate-evoked calcium rises. Astrocytes were tested with 30 sec kainate applications, treated with PDBu or PMA for 3 to 5 min, washed for 30 sec, and then retested with kainate. Percentage decrease was calculated as described in Figure 3. *D1*, The inhibitory effects of t-ACPD could be reduced by the PKC inhibitor H-7. Cells were treated with 200 μ M H-7 for 20 min before cells were retested with t-ACPD. *D2*, Depression of kainate-evoked calcium rises by t-ACPD was reversed significantly by treatment with H-7 in 26/68 astrocytes (pre-H-7, $-32.9 \pm 2.4\%$; post H-7, 55.9 ± 16.5 ; $p < 0.001$; paired t test).

5HT ($n = 84$). This additive effect was not attributable to activation of the kainate-sensitive iGluRs because in SCN ($n = 64$) and cortical ($n = 106$) cultures, 100 μ M kainate did not show significant ($>15\%$ difference in peak amplitude) additive effects when applied with 100 nM 5HT. Kainate seemed to show averaged (Fig. 9*B1*, *B2*) or independent (Fig. 9*B3*) effects when applied with 5HT; 500 μ M t-ACPD (Figs. 9*C*, 10*B*) inhibited the 5HT-evoked calcium rise in SCN glia ($n = 36/51$; $-37.9 \pm 3.9\%$), indicating a modulatory role for mGluRs.

5HT evoked repeatable calcium rises in SCN (Fig. 10*A*) and cortical astrocytes if an interval of at least 5 min occurred between applications. If shorter intervals were used, the amplitude of subsequent responses did not recover fully, resulting in smaller amplitude calcium rises caused by 5HT receptor desensitization. For that reason all tests of mGluR modulation of 5HT were performed at intervals of >5 min. Because the first application of

5HT yielded calcium rise amplitudes with $>15\%$ variability, this initial peak was not included in the analysis. Experiments with specific mGluR agonists showed that 10 μ M quisqualate, which activates both AMPA iGluRs and type I mGluRs, typically enhanced 5HT-evoked calcium rises ($n = 49/97$, $81.2 \pm 9.1\%$) (Fig. 10*B*). The type I/II mGluR agonist L-CCG-I (10 μ M) could enhance ($n = 23/130$, $83.2 \pm 2.6\%$) or inhibit ($n = 49/130$, $-35.7 \pm 2.6\%$) 5HT-mediated calcium rises. Coupled with the t-ACPD inhibitory effects, these results suggest that GluRs insensitive to kainate are responsible for the additive effect seen between glutamate and 5HT.

The type III mGluR agonist L-AP4 (10 μ M) strongly inhibited the 5HT-evoked calcium rise in SCN astrocytes ($n = 104/159$, -51.4 ± 2.6). Cortical glia showed similar effects. Inhibition was rapid, not requiring extended pretreatment with L-AP4 (Fig. 10*C*), and could be blocked completely by overnight incubation with

Figure 7. mGluR activation depresses potassium-evoked calcium rises. **A1**, A representative calcium trace from an SCN astrocyte showing repeatability of 55 mM potassium-evoked calcium rises. **A2**, Mean potassium-evoked calcium rise ($n = 16$). Difference between peaks was not statistically significant and did not exceed 15%. **B**, t-ACPD (500 μ M), quisqualate (10 μ M), and L-CCG-I (10 μ M) all depressed calcium rises evoked by depolarizing SCN astrocytes with 30 sec pulses of 55 mM potassium. Percentage change in evoked calcium rise was calculated as described in Figure 3. **C1**, A representative calcium trace from an SCN astrocyte showing quisqualate could itself evoke calcium rises, and it reduced the maximal response to potassium. **C2**, A calcium trace from an SCN astrocyte showing t-ACPD depressing potassium-evoked calcium rises (arrows). t-ACPD was applied for the duration indicated by the horizontal bar. **C3**, Application of L-CCG-I evoked long-lasting depression of subsequent potassium-evoked calcium rises in this representative SCN astrocyte. **D**, The PKC activator PMA (500 nM) reduced calcium rises evoked by 55 mM potassium. The cell shown here exhibited one of the most dramatic responses to PMA seen in these cells.

PTx (200 ng/ml, $n = 77$, $-0.1 \pm 8.8\%$). This indicated that a rapid, PTx-sensitive pathway was involved. This is in striking contrast to the slower PTx-insensitive interaction described above between mGluRs and kainate.

DISCUSSION

Our results demonstrate that mGluR activation modulates calcium rises evoked by kainate and 5HT in astrocytes. Distinct mGluR subtypes and second messenger pathways were involved in mediating modulatory interactions with kainate and 5HT. In addition, a novel form of long-lasting depression was demonstrated by the modulation of kainate responses by type I/II mGluR agonists L-CCG-I and t-ACPD.

Transmitter sensitivity

SCN neurons have been reported to be relatively insensitive to low concentrations of glutamate (Shibata et al., 1986; Cahill and Menaker, 1989). Indeed, in SCN slices and cultured cells, Meijer et al. (1993) found no toxic effects of 1 mM glutamate applied for 30 min. Our results from dose-response analyses of transmitter action show that SCN astrocytes were significantly less sensitive to glutamate than were cortical astrocytes, suggesting that SCN astrocytes mirror the glutamate sensitivity of neurons in the same area. In contrast, the 5HT sensitivity of astrocytes from SCN and cortex is similar, suggesting that astrocytes from these brain regions differentially regulate which transmitter receptors they ex-

Figure 8. Dose-response curves for 5HT in SCN and cortical astrocytes. SCN ($n = 199$) and cortical ($n = 102$) astrocytes show similar responses to 5HT. Cultures were tested with a range of duplicate doses of 5HT. Only astrocytes that showed calcium rises twice that of basal calcium variation to both 5HT applications were included as responders for that dose. At least three cultures were tested for each dose. Percentage responders were calculated as described earlier for glutamate agonist dose-response curves. Standard error bars are shown on the 5HT dose-response curve in SCN astrocytes for the following doses: 10 nM, 100 nM, 1 μ M, and 10 μ M (four experiments each).

press, and these differences are detectable *in vitro* even after several weeks in culture.

mGluR-iGluR inhibitory interactions

Our findings are the first to demonstrate in astrocytes the modulation of glutamate and 5HT calcium responses by mGluR agonists. Glutamate mGluR-iGluR modulation has been studied in neurons. Previous studies using patch-clamp techniques in brain slices and cultured cells have demonstrated an inhibition of kainate actions by mGluR activation in neurons. Schoepp (1994) suggested that type II mGluR activation could decrease postsynaptic sensitivity to kainate. t-ACPD and the type II-specific mGluR agonist DCG-IV have been shown to inhibit AMPA/kainate neural transmission in cortical-striatal cocultures (Tyler and Lovinger, 1995). Type I and III mGluRs have also been implicated in depression of excitatory synaptic transmission (Gereau and Conn, 1995; Wan and Cahusac, 1995). Type I agonists such as t-ACPD and quisqualate are generally thought to exert their actions by stimulating phosphoinositide hydrolysis, with a consequent rise in cytoplasmic calcium. Activation of type II and III mGluRs inhibits cAMP hydrolysis and thus might not be expected to result in an intracellular calcium rise.

In our experiments in SCN astrocytes, quisqualate sometimes stimulated an intracellular calcium rise but often evoked no calcium rise. Similar to t-ACPD and L-CCG-I, quisqualate was effective at inhibiting kainate-evoked calcium rises. This may indicate that type I and II mGluRs are both involved in mediating the mGluR-iGluR interaction, or that a novel mGluR is involved. Alternatively, quisqualate may not be acting on a type I mGluR but instead may alter a glutamate uptake mechanism (Littman et al., 1995) or modulate calcium channels through a pathway not involving phosphoinositide hydrolysis (for review, see Pin and Duvoisin, 1995). We found that mGluR activation could generate either a rise or a fall in calcium, depending in part on which brain region the astrocytes were derived from. Rises in cytoplasmic calcium may come from intracellular stores in the endoplasmic reticulum. On the other hand, inhibition of kainate-induced cal-

cium rises indicates an influence of mGluRs on calcium influx from extracellular space. The differential response in different cells could be mediated either by different combinations of mGluRs or from complex interactions between different mGluRs and their second messenger pathways. In this light, it is interesting that mRNA for all eight cloned mGluRs is expressed by SCN cells (Obreitan and van den Pol, 1996; van den Pol et al., 1996a).

In neurons, mGluR agonists may inhibit kainate-induced excitotoxicity (Bruno et al., 1994; Pin and Duvoisin, 1995). Synaptic long-term depression (LTD) may also involve mGluR modulation of iGluR-evoked calcium influx. Specifically, induction of cerebellar LTD seems to require the coactivation of mGluRs and kainate/AMPA receptors (for review, see Linden, 1994). LTD may act to protect cells from high levels of glutamate present perisynaptically, or it may act as a kind of gate providing an increased stimulus threshold. NPY-mediated LTD has been demonstrated in SCN neurons using calcium imaging and whole-cell recording (van den Pol et al., 1996b). In the present study, we show for the first time that t-ACPD and L-CCG-I can elicit a long-lasting depression of the amplitude of subsequent kainate-evoked calcium rises in SCN astrocytes. Other researchers have shown that gliotoxins seem to block cerebellar LTD (Winder et al., 1996), and mice deficient for the astrocyte-specific intermediate filament GFAP show deficient LTD (Shibuki et al., 1996). Our results suggest that one mechanism for long-lasting effects may involve mGluR-mediated depression of astrocyte responses to fast transmitters.

Second messenger pathways

mGluRs depressed not only kainate-evoked calcium rises but also high potassium-evoked calcium rises. L-type VACC blockers nifedipine and nickel blocked this. Taken together, these results suggest that mGluR activation depressed kainate-induced calcium rises by blocking VACCs in astrocytes. Parallel studies in neurons have reported similar mechanisms (Sayer et al., 1992; Chavis et al., 1995; Glaum and Miller, 1995; Choi and Lovinger, 1996). It is also possible that mGluR activation modulates kainate function by changing ligand binding or gating properties at the kainate-sensitive receptor.

Depression of kainate calcium rises by mGluR activation could reflect direct modulation of calcium channels (Swartz, 1993; Chavis et al., 1994) by a G-protein-coupled receptor kinase (Diversé-Pierluissi et al., 1996) or by modulation via a slower pathway involving second messengers (for review, see Anwyl, 1991; Hille, 1992, 1994). The mGluR inhibitory modulation of 5HT we described was rapid, required no pretreatment with the mGluR agonist L-AP4, and was blocked by PTx. The rapid time course of inhibition suggests that a membrane-delimited, G_i/G_o protein-coupled pathway may be involved. In contrast, because PTx did not block the t-ACPD or L-CCG-I inhibitory interaction with kainate, a G_i/G_o protein may not be involved in the mGluR-iGluR modulatory pathway. Also, astrocytes had to be pretreated with t-ACPD or L-CCG-I to inhibit kainate calcium rises, suggesting that a slow-acting second messenger pathway was involved.

The type II mGluR has been shown to couple to its intracellular transduction system via the PTx-sensitive G_i/G_o protein (Tanabe et al., 1992, 1993). Our findings indicate that the type II mGluR receptor in astrocytes may be coupled with a different G-protein, perhaps G_q , which is insensitive to PTx but coupled to the same second messenger pathway as G_i/G_o . Splice variants of mGluRs have been described (Nakanishi and

Figure 9. Glutamate-5HT interactions. *A*, Glutamate (100 μ M) and 5HT (10 μ M) showed additive or synergistic interactions in SCN (shown) and cortical astrocytes. Calcium traces are shown from three representative cells from the same experiment. In all three cells, the 5HT + glutamate calcium rise is larger than the calcium rise evoked by either 5HT or glutamate alone. *A1* and *A2* show examples of synergy, whereas the cell in *A3* shows an example of an additive interaction. *B*, Kainate (100 μ M) showed no additive interaction with 5HT (10 μ M). *B1* and *B2* show interactions in two astrocytes in which the combined application of 5HT and kainate seems to yield an averaged calcium response. *B3* shows a cell in which kainate and 5HT calcium effects seem to be independent. *C1*, t-ACPD (500 μ M), although not evoking a calcium rise alone, completely blocked the 5HT-evoked calcium rise in this astrocyte. *C2*, A cell from the same experiment showed no effect of t-ACPD on 5HT-evoked calcium responses.

Masu, 1994) and may be one explanation for this transduction difference. Another possibility is that L-CCG-I, along with t-ACPD and quisqualate, activated a type I mGluR and thereby inhibited kainate-evoked calcium rises.

mGluR-mediated inhibition did not require pretreatment of cells with quisqualate. This is consistent with work on neurons by Lester and Jahr (1990), which showed that quisqualate rapidly and directly inhibited calcium currents through a pathway not involving second messengers. Taken together, our results implicate both type I and II mGluRs in the inhibition of kainate-evoked calcium rises. The mGluRs could be coupled to the same (Sahara and Westbrook, 1993) or distinct (Ikeda et al., 1995) calcium channels.

Experiments with PKC activators and inhibitors support the hypothesis that the mGluR inhibition of the kainate-evoked cal-

cium response involved a second messenger pathway. The PKC activators PMA and PDBu mimicked the inhibition, and the PKC inactivator H-7 blocked the effects of t-ACPD. Although its substrate is not known in our system, PKC has been shown to inhibit coupling between G_q proteins and downstream second messengers (Willars et al., 1996). It is also possible that PKC acts to modify an astrocyte VACC or phosphorylates some other protein that facilitates VACC action. PKC has been shown to modulate transmitter inhibition of VACCs in neurons (Rich et al., 1984; Swartz, 1993; Swartz et al., 1993), and our results suggest that PKC activators may act to inhibit astrocyte VACCs.

Functional considerations

Our data demonstrate that mGluRs can exert a profound effect on kainate- and 5HT-evoked changes in cytosolic calcium in astro-

Figure 10. mGluR-5HT interactions. SCN and cortex astrocytes showed similar interactions between mGluR agonists and 5HT-evoked calcium rises. *A*, 5HT evoked repeatable calcium rises in SCN (shown) and cortex astrocytes. Here a typical control astrocyte is shown that responds to repeated applications of 5HT with similar amplitude calcium rises. *B*, Quisqualate (10 μ M), L-AP4 (10 μ M), and L-CCG-I (10 μ M) were applied with 5HT (100 nM), and the resultant calcium rise amplitude was compared with the rise evoked by 5HT alone. Percentage change in calcium rise was calculated as described in Figure 3. Negative values indicate depression, and positive values indicate enhancement of the 5HT-evoked calcium rise. Unlike the mGluR-iGluR interaction, quisqualate generally enhanced 5HT calcium rises, L-CCG-I showed mixed results, and L-AP4 showed a very strong and consistent PTx-sensitive depression of 5HT-evoked calcium rises. This indicated that different mGluR subtypes can independently and oppositely modulate 5HT-evoked calcium rises. *C1*, L-AP4 (10 μ M) showed rapid and repeatable depression of a 5HT-evoked sustained calcium rise. *C2*, L-AP4 effects were blocked by overnight application of PTx (200 ng/ml).

cytes. This could have a number of physiological ramifications. Long-distance communication between astrocytes, found in the SCN (van den Pol et al., 1992) and elsewhere in the brain (Cornell-Bell et al., 1990), could be altered in terms of both the spatial spread of a calcium wave and the probability of the initiation of calcium waves. 5HT may be more likely than glutamate to initiate a calcium wave in SCN astrocytes, whereas cortical astrocytes may show a greater relative sensitivity to glutamate. One might also consider transmitter signaling in astrocytes in a spatial context. Steady-state levels of glutamate, for instance, could activate mGluRs that might then act to modulate the responses to glutamate or other transmitters rapidly and discretely released at high concentrations from specific axonal terminals.

The complex modulation of astrocyte calcium levels by mGluRs could play a significant role in intercellular signaling. Increases in glial calcium could result in the opening of calcium-activated potassium channels (Quandt and MacVicar, 1986; Barres et al.,

1988; MacVicar et al., 1988), leading to potassium release and increased excitability in nearby neurons. Rises in astrocyte calcium could also increase phospholipase A2. The resulting increase in arachidonic acid would reduce glutamate uptake, resulting in an increased extracellular glutamate level that would alter neuronal activity (Axelrod et al., 1988; Barbour et al., 1989; Marin et al., 1991). In the SCN, as in many other areas of the brain, astrocytes can surround axodendritic contacts (van den Pol et al., 1992). The rapid movement of extracellular calcium into an astrocyte from the extracellular space between neuron and astrocyte might alter calcium-dependent transmitter release from an axonal bouton that the astrocyte surrounds.

Glutamate is the primary excitatory transmitter of the cortex. It is the transmitter released by optic nerve axons in the SCN that play an important role in phase-shifting the circadian clock. We demonstrate several novel aspects of mGluR modulation in astrocytes. These reveal a level of complexity of transmitter interactions, not only between different transmit-

ters but even in the response of different receptors for the same transmitter. mGluRs, acting on astrocytes, may play a pivotal role in setting the gain for the response of these cells to transmitters, dependent on both spatially and temporally relevant cues. The long-lasting effect of brief exposure of astrocytes to specific mGluR agonists suggests that they may serve as a temporal integrator recording and integrating transmitter activation over a considerable period of time.

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