The Role of Metabotropic Glutamate Receptors for the Generation of Calcium Oscillations in Rat Hippocampal Astrocytes

Ca\textsuperscript{2+} oscillations are part of the intra-and intercellular signalling in many cell types. We have studied Ca\textsuperscript{2+} oscillations in astrocytes in acute brain slices of the hippocampus of juvenile rats (postnatal 8–14 days old), using confocal laser scanning microscopy and bulk-loading of the Ca\textsuperscript{2+}-sensitive dye Fluo-4. Astrocytes were identified morphologically in the stratum radiatum, and by their Ca\textsuperscript{2+} response in the absence of external K\textsuperscript{+}. Thirty-five per cent of astrocytes (43 slices) showed spontaneous Ca\textsuperscript{2+} oscillations, with a frequency of 1.26 ± 0.11 transients/min (n = 366). These Ca\textsuperscript{2+} signals were unaffected by tetrodotoxin (0.5 μM) and Ni\textsuperscript{2+} (2 mM), but were sensitive to interference with the phospholipase C-mediated Ca\textsuperscript{2+} release from intracellular stores. Spontaneous Ca\textsuperscript{2+} oscillations were reduced or suppressed by antagonists of metabotropic glutamate receptors (mGluRs) of groups I and II, but not affected by antagonists of group III. Glutamate (1–100 μM) and specific agonists of mGluR groups I and II evoked concentration-dependent Ca\textsuperscript{2+} signals, which were oscillatory at intermediate concentrations (e.g. at 10 μM glutamate). Our results indicate that mGluRs of both groups I and II are involved in mediating Ca\textsuperscript{2+} oscillations in astrocytes, which might be glial responses to micromolar changes of glutamate in the extracellular spaces.

Keywords: APDC, confocal microscopy, DHPG, Fluo-4, MCPG

Introduction

Single and repetitive Ca\textsuperscript{2+} transients, which can occur in various cell types, spontaneously or as evoked signals, can initiate or change cell activity (for review, see Bertridge et al., 2003). These may be short-term changes due to the activation of Ca\textsuperscript{2+}/Calmodulin-dependent proteins or long-term effects as a result of gene expression. Our knowledge about the function and mechanisms of Ca\textsuperscript{2+} oscillations in cells is, however, still rather limited. In electrically non-excitable cells, Ca\textsuperscript{2+} oscillations may be of particular importance for generating or initiating specific cellular functions (Dolmetsch et al., 1998; Li et al., 1998; Rose and Konnerth, 2001; Morita et al., 2003).

In the nervous system, spontaneous Ca\textsuperscript{2+} transients have been reported in both neurons (Woodward et al., 1999) and glial cells, in culture and in situ (Cornell-Bell et al., 1990; Charles, 1998; Strahonja-Packard and Sanderson, 1999; Aguado et al., 2002; Nett et al., 2002). Neurons appear to show spontaneous Ca\textsuperscript{2+} oscillations primarily at early developmental stages, which might be linked to the formation of neuronal circuits in the developing brain (Garaschuk et al., 2000). In neurons, Ca\textsuperscript{2+} release is associated with transmitter exocytosis, synaptic plasticity and electrical excitability (Rose and Konnerth, 2001). Astrocytes are electrically non-excitable cells, which often employ Ca\textsuperscript{2+} signalling in response to chemical or mechanical stimuli (Chen et al., 1997; Kimelberg et al., 1997; Deitmer et al., 1998), but have also been found spontaneously, i.e. in the absence of given stimuli (Parri et al., 2001; Aguado et al., 2002; Beck et al., 2004). Ca\textsuperscript{2+} signals in astrocytes often occur as oscillations (repetitive transients), and can propagate along cell processes and possibly even beyond cell boundaries to neighbouring cells (Parri et al., 2001; Nett et al., 2002; Schipke et al., 2002). They have also been associated with Ca\textsuperscript{2+}-dependent exocytosis of transmitters (Bezzi et al., 1998; Innocenti et al., 2000; Angulo et al., 2004; Fiacco and McCarthy, 2004; Zhang et al., 2004) and modulation of regional blood flow (Zonta et al., 2003; Mulligan and MacVicar, 2004). The mechanisms, however, that initiate and maintain Ca\textsuperscript{2+} oscillations in astrocytes in situ are not yet known.

The aim of the present study was to elucidate the role of neurotransmitters, in particular glutamate, in generating Ca\textsuperscript{2+} oscillations in astrocytes. We have therefore investigated Ca\textsuperscript{2+} transients and Ca\textsuperscript{2+} oscillations in rat hippocampal slices using confocal fluorescent Ca\textsuperscript{2+} imaging. We have recorded spontaneous Ca\textsuperscript{2+} oscillations in cells identified as astrocytes, and used a number of transmitter receptor ligands to elucidate the mechanism involved in initiating these Ca\textsuperscript{2+} signals. Our results suggest that metabotropic glutamate receptors (mGluRs) of groups I and II play a major role in mediating Ca\textsuperscript{2+} oscillations in astrocytes in situ. We discuss whether the initiation of different types of Ca\textsuperscript{2+} signals in astrocytes might reflect the level of extracellular, non-synaptic glutamate in the hippocampal tissue. Some of the results have been reported in preliminary form (Zur Nieden and Deitmer, 2002, 2004).

Material and Methods

Slice Preparation

Hippocampal slices were prepared from juvenile rats (postnatal 8–14 days old). In brief, rats were decapitated and their brain were quickly transferred into a chilled (4°C) calcium-reduced (0.5 mM) artificial cerebrospinal fluid (aCSF) following the method of Edwards et al. (1989). The saline was continuously gassed with carbogen (95% O\textsubscript{2}/5% CO\textsubscript{2}) and buffered to pH 7.4 by bicarbonate throughout the entire preparation. Frontal slices of the forebrain containing the hippocampal formation were cut using a Vibratome (Leica VTS1000, Bensheim, Germany). The brain slices (250–300 μm thick) were immediately transferred into a second chamber, where they were stored for at least 1 h in Ca\textsuperscript{2+}-reduced aCSF (0.5 mM Ca\textsuperscript{2+}) at 30°C before dye-loading.

Identification of Cell Types

Cells types were identified by their change in intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]i) in response to application of K\textsuperscript{-}-free and high-K\textsuperscript{+} (50 mM) aCSF, and by their stellate morphology. In brief, hippocampal astrocytes in rat and mice show a rise in [Ca\textsuperscript{2+}]i, when perfused with nominal K\textsuperscript{-}-free saline for 3–5 min (Dallwig et al., 2000; Dallwig and Deitmer, 2002). More than 80% of the fluo-4-AM-stained cells in the stratum radiatum, as well as those in the stratum lacunosum-moleculare

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of the CA1 region of the rat hippocampus, responded to K+-free aCSF with an increase in [Ca2+]i. These cells often displayed a stellate morphology and were presumed to be astrocytes, as indicated by immunocytochemical markers (Beck et al., 2004). Cells showing only a weak (<10% change in fluorescence F/F0) or strongly delayed onset (>1 min) in their response to K+-free saline were not considered to be viable astrocytes. Cells showing a fast onset in their response to high-K+ containing aCSF but poor fluo-4-AM staining with no change in intracellular Ca2+ concentration during the application of K+-free saline were presumed to be neurons.

Solutions

The standard aCSF for acute brain slices contained (in mM): NaCl 125, KCl 2.5, CaCl2 2, MgCl2 1, 2-glucose 25, NaHCO3 26, NaH2PO4 1.25, l-lactate 0.5, gassed during the entire experiment by carboxygen to adjust the pH to 7.4. In Ca2+-reduced saline (0.5 mM), 1.5 mM CaCl2 was replaced by 1.5 mM MgCl2. In nominally Ca2+-free aCSF, CaCl2 was substituted by equimolar amounts of MgCl2, and 1 mM EGTA was added to reduce the remaining free Ca2+ to <10 nM. In K+-free saline (0K) or high-K+ solution (50 mM K+, 50K), KCl was exchanged by/for NaCl.

Metabotropic glutamate receptor antagonists, (RS)-3,5-dihydroxyphenylglycine (DHPG), (2R,4R)-2-carboxy-y-phenylglycine (CPPG), (±)-1-amino-6-carboxy-nicotinic acid (6-CINA), (±)-2-amino-2-(1S,2R)-3-carboxy-cyclopropylglycine (DOP IV) and (2S,2R)-2-amino-2-(1S,2R)-3-carboxy-cyclopropylglycine ((S)-APICA) were purchased from Tocris Cookson (Bristol, UK). Tetrodotoxin (TTX) was purchased from Alomone Labs (Jerusalem, Israel). ATP and the purinergic P2 antagonist pyridoxalphosphate-6-azophenyl-2 and the SERCA-pump inhibitor cyclopiazonic acid (CPA) were obtained from ALEXIS Corp., Lausen, Switzerland, (RS)-apo-5-carboxy-2-naphthoic acid (LY 341495), (RS)-2-amino-2-(1S,2R)-3-carboxy-cyclopropylglycine and the SERCA-pump inhibitor cyclopiazonic acid (CPA) were purchased from ALEXIS Corp., Lausen, Switzerland. Solutions

Dye-loading

Acute brain slices were incubated in the dark at room temperature (21–24°C) in Ca2+-reduced aCSF containing 2 μM fluo-4-AM (Molecular Probes, Eugene, Oregon). The dye was dissolved in dimethylsulfoxide (DMSO) and added to the dye-loading solution, to make a final concentration of 4 μM and incubated for at least 90 min. The final concentration of DMSO never exceeded 0.1% in the saline. All stock solutions were stored at -20°C. The pH of aCSF was readjusted to pH 7.4 with HCl after adding all drugs. All saline for acute brain slices was gassed throughout the entire experiment with carbogen to maintain pH and oxygen at constant levels.

Laser Scanning Microscopy

Measurements of Ca2+ changes in cells of an acute brain slice were performed with a confocal laser scanning microscope (Zeiss LSM 510, Oberkochen, Germany). The Ca2+-sensitive dye fluo-4 was excited using the 488 nm line of an argon laser. To separate the excitation from the emission pathway, a dichroic mirror was used. The emission signal was truncated by a optical bandpass filter at wavelengths of 505 and 550 nm. To measure Ca2+ dynamics, images were acquired in one focal plane with a frequency of 0.5–1.0 Hz. The regions of interest (ROIs) were defined, and fluorescence changes were normalized to the average baseline level (F0) of the first 20 time points. All changes are expressed as the relative change of the fluorescence intensity F/F0 normalized to F0, as F/F0. A deflection of the baseline of >10% F/F0 (the noise was usually <5% F/F0) could be clearly identified as a Ca2+ signal. Laser-, optical filter- and microscope settings as well as data acquisition were controlled by PC software (AIM 3.0, Zeiss).

Patch-clamp Recording

Hippocampal slices from juvenile rats (10–16 days old) were fixed in a recording chamber with a U-shaped platinum wire and nylon grid on the stage of an upright microscope (Axioscope, Zeiss, Oberkochen, Germany). The chamber was continuously perfused with carbon-gassed aCSF (see above). Pipettes were pulled with a horizontal puller and heat-polished to a tip resistance of 2–3 MΩ filled with an ‘intracellular solution’ containing (in mM): CsCl, 120; tetraethylammonium chloride, 20; MgCl2, 2; Na-ATP, 2; EGTA, 0.5; HEPES, 10; pH adjusted to 7.4 with NaOH. This pipette solution strongly reduced background K+ currents and allowed a better observation of spontaneous postsynaptic currents (Konnerth et al., 1990). Series resistance was 4–15 MΩ and was compensated. Recordings were controlled with pClAMP software (Axon Instruments, Forster City, CA) with a digidata 1200 interface and an Axopatch-1D amplifier. For further details, see Brockhaus and Deitmer (2002).

Statistics

Measurements are given as mean values ± SEM, with n as the number of cells or the number of slices as indicated. Significance of statistical differences were calculated under the assumption that the results show a normal distribution using Student's t-test and performed by Origin 7.0 (OriginLab Corp., Northampton, MA). Figures were prepared using Corel Draw (Corel Corp., Toronto, Canada).

Results

Identification of Spontaneous Ca2+ Oscillations

Acutely dissected brain slices were continuously superfused in the experimental chamber with saline for at least 5 min before recording of intracellular Ca2+ were begun. Over the next 10 min, spontaneous Ca2+ signals were recorded in normal saline as control. The experimental protocol included the 10–15 min after different agonists and antagonists were applied. At the end of each experiment, the slices were superfused with saline containing 50 mM K+ (50K) and/or 0 mM K+ (0K), for identification of the cell type (Fig. 1.4; see also Dallwig and Deitmer, 2002; Beck et al., 2004). While both neurons and astrocytes responded to 50K (see Fig. 2C for a Ca2+ response in an astrocyte) with a prominent Ca2+ transient, only astrocytes showed Ca2+ rises in 0K. Ca2+ oscillations were defined as two or more repetitive Ca2+ transients, and they occurred spontaneously, i.e. without exogenous stimulus, in presumed astrocytes (Fig. 1B). The amplitude of these Ca2+ oscillations was variable, amounting to a mean increase of the relative fluorescence by 4±12% F/F0 in 156 cells of 17 brain slices (n = 156/17). Often, the Ca2+ transients, especially those evoked by added agonists, declined in amplitude over the course of an experiment, and the amplitude could vary from slice to slice. Therefore, we focused on analysing the number of cells with Ca2+ responses and the frequency of the Ca2+ responses.

Spontaneous Ca2+ oscillations could be recorded in 35 ± 2% (n = 43 slices) of the cells identified as astrocytes due to their response to 0K, in a given brain slice over a time period of 10 min, while only 7 ± 3% (n = 23 slices) of cells, identified as neurons by their morphology and due to the lack of a Ca2+ response to 0K, showed Ca2+ oscillations during this time (Fig. 1C). The overall frequency of spontaneous Ca2+ transients averaged over 10 min was 1.26 ± 0.11 transients/min (n = 366 cells) in astrocytes and 0.15 ± 0.06 transients/min in neurons (n = 31; Fig. 1D). Thus, spontaneous Ca2+ oscillations were much more frequent in astrocytes than in neurons in these hippocampal slices.

We also looked at coincident Ca2+ responses in neighbouring astrocytes in a brain slice, or propagation of Ca2+ transients from one astrocyte to another. We found poor correlation between the Ca2+ responses in different cells, however, and rarely
observed Ca\(^{2+}\) responses crossing cell borders in form of waves. The lack of coincident and propagated Ca\(^{2+}\) responses might be due to the two-dimensional analysis of the Ca\(^{2+}\) responses, which misses all events that might occur in the z-axis.

**Spontaneous Ca\(^{2+}\) Oscillations Dependent on Functional Intracellular Ca\(^{2+}\) Stores and Independent of Neuronal Activity**

After addition of cyclopiazonic acid (CPA, 30 \(\mu\)M; Fig. 2A), which depletes Ca\(^{2+}\) stores by inhibiting the Ca\(^{2+}\)-ATPase of the endoplasmic reticulum, almost no Ca\(^{2+}\) transients could be observed and the percentage of astrocytes displaying spontaneous Ca\(^{2+}\) oscillations dropped to 5 \(\pm\) 2\% \((n = 5\) slices\). ATP was applied in some of these experiments to check that Ca\(^{2+}\) release from functional Ca\(^{2+}\) stores was still working (Fig. 2AB). Bath-applied ATP (100 \(\mu\)M, 1 min), which evoked a single Ca\(^{2+}\) transient under control conditions \((n = 27/5)\) but no Ca\(^{2+}\) oscillations, also failed to elicit a Ca\(^{2+}\) response in CPA, suggesting that Ca\(^{2+}\) release from intracellular stores was involved in the generation of spontaneous Ca\(^{2+}\) oscillations. When extracellular Ca\(^{2+}\) was removed (0 mM Ca\(^{2+}\)), one or few Ca\(^{2+}\) transients could still be recorded in the initial period of application (1-2 min) before they stopped altogether (Fig. 2B).

ATP was applied in some of these experiments to check that Ca\(^{2+}\) release from functional Ca\(^{2+}\) stores was still working (Fig. 2AB). Also, under these conditions ATP no longer elicited a significant Ca\(^{2+}\) response, indicating that the Ca\(^{2+}\) stores had been emptied. The Ca\(^{2+}\) oscillations were also suppressed after preincubation of the brain slices with the phospholipase inhibitor U73122 (10 \(\mu\)M for 30–60 min) containing saline. This also suppressed all Ca\(^{2+}\) responses to ATP and glutamate in these astrocytes (Fig. 2EF). The spontaneous Ca\(^{2+}\) oscillations during the first 10 min of recording (Control, 100\%) and the second 10 min of recording under the same control conditions were very similar with respect to the number of Ca\(^{2+}\) oscillating cells \((95 \pm 10\%, n = 17; \text{Fig. 2E})\) and the frequency of Ca\(^{2+}\) transients \((99 \pm 8\%, n = 106; \text{Fig. 2F})\). These results indicate that the Ca\(^{2+}\) oscillations in astrocytes were likely due to phospholipase C-mediated Ca\(^{2+}\) release from intracellular stores.

In order to suppress the electrical and synaptic activity of neurons, we used 0.5 \(\mu\)M TTX or 2 mM Ni\(^{2+}\) to inhibit the generation of action potentials and calcium-dependent transmitter release, respectively (Fig. 2CD). Both TTX and Ni\(^{2+}\) had no significant effect on the spontaneous Ca\(^{2+}\) oscillations in astrocytes (Fig. 2EF). In some experiments, a smaller amplitude of the Ca\(^{2+}\) responses was observed (Fig. 2D). These experiments suggest that the Ca\(^{2+}\) oscillations in astrocytes were not evoked or maintained by spontaneous neuronal activity.

**Ca\(^{2+}\) Oscillations after Inhibition of mGluRs**

Different antagonists of mGluRs were applied to evaluate the role of these receptors for initiating the spontaneous Ca\(^{2+}\) oscillations in astrocytes (Fig. 3). Both MCPG, an antagonist of group I and possibly group II mGluRs (1 mM; Fig. 3A) and LY341495, a potent antagonist of group II/III mGluRs at the concentration of 2 \(\mu\)M as used here (Fig. 3B), led to a reduction or termination of spontaneous Ca\(^{2+}\) oscillations within the first two min of application. The group II mGluR antagonist APICA also reduced the Ca\(^{2+}\) oscillations, though less so than LY341495 (Fig. 3C). The fraction of oscillating cells were
reduced to 10 ± 7% \( (n = 6 \text{ slices}) \) by MCPG, to 3 ± 1% \( (n = 5) \) by LY341495 and to 41 ± 25% \( (n = 7) \) by APICA (Fig. 3D; Control without antagonist, 100%), while the frequency of remaining Ca\(^{2+}\) transient decreased to 6 ± 4% \( (n = 127) \), 9 ± 5% \( (n = 67) \) and 61 ± 10\% \( (n = 114) \) by these antagonists, respectively (Fig. 3E). The effects of MCPG and LY341495 were irreversible over the period of wash-out (≤15 min), while Ca\(^{2+}\) transients were resumed after the removal of APICA (Fig. 3C). These results strongly suggest that activation of mGluRs is involved in generating the spontaneous Ca\(^{2+}\) oscillations in hippocampal astrocytes.

### Ca\(^{2+}\) Oscillations Can Be Evoked by Activating mGluRs

In order to check the involvement of different types of mGluR in mediating Ca\(^{2+}\) oscillations, we applied different agonists of mGluRs to test their ability to initiate Ca\(^{2+}\) oscillations. Interestingly, trans-ACPD, at 2 µM, evoked a single Ca\(^{2+}\) transient, while it elicited Ca\(^{2+}\) oscillations at 10 µM (Fig. 4A). At 20 µM, trans-ACPD evoked a biphasic response consisting of a fast transient response followed by a shoulder. Since trans-ACPD is known to activate group I as well as group II mGluRs, and group II antagonists also inhibited spontaneous Ca\(^{2+}\) oscillations, we tested different concentrations of DHPG, a potent and specific group I mGluR agonist, and APDC, a specific group II mGluR agonist. Both agonists exhibited a concentration-dependent profile similar to trans-ACPD, evoking Ca\(^{2+}\) oscillations at a concentration of 2 µM for DHPG (Fig. 4B) and at concentrations of 5–10 µM for APDC (Fig. 4C), respectively. At concentrations of 1 and 50 µM, APDC elicited different Ca\(^{2+}\) responses, but no oscillations. L-AP4 (50 µM), a group III-specific agonist, failed to elicit Ca\(^{2+}\) changes in the same astrocytes, in which APDC evoked Ca\(^{2+}\) oscillations (Fig. 4D).

Another group I mGluR agonist, CHPG (100 µM), also increased highly significantly the number of Ca\(^{2+}\) oscillating cells and increased the rate of transients (Fig. 4E). The highly selective group II mGluR agonist DCG IV elicited Ca\(^{2+}\) oscillations at 0.5 µM, while 5 µM DCG IV evoked a tonic Ca\(^{2+}\) rise onto which Ca\(^{2+}\) oscillations were superimposed (Fig. 4F). When agonists of groups I and II were applied together, e.g. DHPG and APDC, at concentrations at which they alone evoked Ca\(^{2+}\) oscillations, a sustained Ca\(^{2+}\) response was observed, as elicited by one of these agonists at a higher concentration (Fig. 4F).

The influence of different group-specific mGluR agonists is summarized in Figure 4G,H, showing that all agonists of
groups I and II mGluRs, but not those of group III, significantly increased the number of oscillating cells and the frequency of Ca$^{2+}$ transients as compared with the control without agonists (set to 100%).

In order to evaluate the involvement of mGluRs in initiating Ca$^{2+}$ oscillations, we compared the responses to glutamate with those to other metabotropic transmitter receptor agonists that are known to elicit Ca$^{2+}$ responses in astrocytes, such as ATP and phenylephrine (Shao and McCarthy, 1995). Whereas application of glutamate at a concentration of 10 µM elicited Ca$^{2+}$ oscillations (Fig. 5A), higher concentrations (100 µM, Fig. 5B) induced biphasic Ca$^{2+}$ changes (peak with shoulder) or a sustained Ca$^{2+}$ rise, and lower concentrations (1 µM, data not shown) elicited only a single Ca$^{2+}$ transient. Perfusion of the acute brain slices with ATP (5, 10 and 30 µM; Fig. 5C,D) evoked Ca$^{2+}$ signals, but rarely Ca$^{2+}$ oscillations, when applied at different concentrations (5-100 µM). At the lowest concentration used (5 µM ATP, 1 µM glutamate) both agonists predominantly evoked a single calcium transient (ATP: 76% in three slices; glutamate: 57% in four slices; Fig. 5E). At a concentration of 10 µM, glutamate evoked Ca$^{2+}$ oscillations in 63% of all astrocytes (seven slices, Fig. 5E), while application of 10 µM ATP predominantly (69% in seven slices) led to a biphasic Ca$^{2+}$ response (a peak with shoulder). Only a small portion (4%) of astrocytes exposed to 10 µM ATP displayed Ca$^{2+}$ oscillations. Application of a higher agonist concentration (e.g. 100 µM) resulted in the case of ATP in 86% (four slices) and in the case of glutamate in 90% (three slices) of all astrocytes in a Ca$^{2+}$
transient followed by a shoulder or sustained Ca\(^{2+}\) rise (Fig. 5B,D). Since ADP (10–100 µM) could also evoke Ca\(^{2+}\) responses, but not Ca\(^{2+}\) oscillations, the responses to ATP were likely mediated by activation of P2Y receptors in the astrocytes. The Ca\(^{2+}\) responses evoked by ATP were reduced by PPADS (10 µM), a P2 receptor antagonist, but PPADS alone had no effect on either the frequency of spontaneous nor of glutamate-evoked Ca\(^{2+}\) oscillations in these cells.

Similar to ATP, phenylephrine (5–100 µM) evoked Ca\(^{2+}\) oscillations only in a minority of cells (<10%; data not shown). While ATP and phenylephrine did not increase the frequency of spontaneous Ca\(^{2+}\) transients, glutamate (10 µM) and DHPG (2 µM) increased the frequency by 50 and 100%, respectively (Fig. 5F).

**The Effect of Agonists of Group II mGluRs**

In order to further confirm the role of both group I and group II mGluRs in directly mediating Ca\(^{2+}\) oscillations in astrocytes, we applied different agonists and antagonists of these receptor classes under different experimental conditions. First, we tested the group II-specific mGluR agonist APDC in the presence of TTX (Fig. 6A,C) and in the absence of external Ca\(^{2+}\) (Fig. 6B,D). The results show that the action of APDC is not dependent on either neuronal activity or Ca\(^{2+}\) influx. In the same cells, this was confirmed also for the group I mGluR agonist DHPG (2 µM; Fig. 6C,D). As stated before, in Ca\(^{2+}\)-free saline Ca\(^{2+}\) responses could only be evoked once by the mGluR agonists, presumably because this one response depleted the intracellular Ca\(^{2+}\) stores when Ca\(^{2+}\) influx was suppressed due to the absence of

![Figure 4](https://academic.oup.com/cercor/article-abstract/16/5/676/276944)
Figure 5. Ca$^{2+}$ responses to metabotropic receptor agonists in astrocytes. (A–D) Glutamate applied at an intermediate concentration (10 µM; A) evoked Ca$^{2+}$ oscillations and at higher concentrations (100 µM; B) a sustained Ca$^{2+}$ response, while ATP evoked single Ca$^{2+}$ transients without shoulder at lower concentrations (5 µM; C) and with shoulder at higher concentrations (10–30 µM; D). (E) Relative number of cells responding to different concentrations of ATP or glutamate with Ca$^{2+}$ transients and Ca$^{2+}$ oscillations. Each column represents the analysis from at least 78 astrocytes responding with a Ca$^{2+}$ signal to either ATP or glutamate. (F) Frequency of Ca$^{2+}$ transients as elicited by ATP, phenylephrine (PHE), glutamate (Glu) and DHPG.

Figure 6. APDC- and DHPG-evoked Ca$^{2+}$ oscillations. (A, B) Ca$^{2+}$ oscillations evoked by 10 µM APDC in the absence and presence of TTX (0.5 µM; A), and in the absence and presence of external Ca$^{2+}$ (B). Summary of the DHPG- and the APDC-evoked responses with respect to the number of oscillating astrocytes (C) and to the frequency of Ca$^{2+}$ transients in the oscillating astrocytes (D).
extracellular Ca\(^{2+}\). After readdition of Ca\(^{2+}\) to the bathing solution, APDC could again evoke Ca\(^{2+}\) oscillations (Fig. 6B).

The agonists DHPG and APDC were applied with various antagonists of group I and II mGluRs to the same brain slices to get a differential pharmacological profile of the evoked Ca\(^{2+}\) oscillations in astrocytes (Fig. 7). The group II antagonist APICA significantly reduced the Ca\(^{2+}\) oscillations evoked by APDC (Fig. 7A), and MCPG stopped the Ca\(^{2+}\) oscillations evoked by DHPG (Fig. 7B). While MCPG terminated the response to the group I mGluR agonist DHPG, the group II-specific mGluR antagonists, LY341495 and APICA, had no effect on the DHPG-evoked responses, but reduced the number of cells and Ca\(^{2+}\) transients evoked by APDC to 20–45% (Fig. 7C,D).

Since activation of group II mGluRs has been linked primarily to the cAMP-mediated pathway (see Discussion), we performed experiments with inhibitors of either the cAMP or the IP\(_3\) pathway. Adenylyl cyclase inhibition with SQ22.536 (200 µM) had no effect on APDC-evoked Ca\(^{2+}\) oscillations, while superfusion of the brain slices with the phospholipase C blocker U73122 (10 µM, 60 min) suppressed the APDC-evoked Ca\(^{2+}\) oscillations (data not shown). This indicates that the Ca\(^{2+}\) oscillations are elicited by APDC presumably via the IP\(_3\)-mediated pathway.

Both APDC and DHPG could elicit Ca\(^{2+}\) oscillations even after preincubation of the brain slices in 4 µM bafilomycin A1, which blocks vesicular transmitter release (Fig. 8). The efficacy of bafilomycin A1 was tested using patch-clamp recordings from pyramidal neurons in acute hippocampal brain slices. Spontaneous postsynaptic currents were recorded in aCSF and at elevated extracellular k\(^+\) concentration (10 mM), which greatly increased the frequency of the postsynaptic currents. After incubation of the slices in 4 µM bafilomycin A1, the frequency of postsynaptic events was reduced and the frequency increase in 10 mM K\(^+\) was abolished (Fig. 8A–C). Brain slices which were treated the same way with bafilomycin A1 still showed Ca\(^{2+}\) oscillations in response to 10 µM APDC and 2 µM DHPG (Fig. 8D). The frequency of spontaneous Ca\(^{2+}\) oscillations was not affected by preincubation with bafilomycin A1. This result was obtained in 102 cells from five brain slices, indicating that Ca\(^{2+}\) oscillations evoked by group I- and II-specific mGluR agonists are not induced by increased vesicular release of neurotransmitters activated by these agonists.

**Discussion**

The present study suggests that activation of mGluRs is needed for the generation of spontaneous Ca\(^{2+}\) transients in rat hippocampal astrocytes. Gial Ca\(^{2+}\) oscillations occur independently of neuronal transmitter release, and are presumably due to tonic activation of group I and group II metabotropic receptors by basal levels of extracellular glutamate. The Ca\(^{2+}\) oscillations could be inhibited by antagonists of mGluR of both group I and group II. Agonists of mGluR groups I and II, but not mGluR group III, elicited dose-dependent Ca\(^{2+}\) oscillations in astrocytes. Like the Ca\(^{2+}\) responses mediated by mGluR group I (Porter and McCarthy, 1995; Nakahara et al., 1997; this study), activation of mGluR group II also appeared to result in Ca\(^{2+}\) release from CPA-sensitive intracellular stores. The dose-dependent activation of mGluR led, at low concentration (e.g., 10 µM for glutamate), to Ca\(^{2+}\) oscillations, while activation with higher agonist concentrations (e.g., 100 µM for glutamate) resulted in responses consisting of a fast transient Ca\(^{2+}\) increase followed by a shoulder, presumably reflecting in part capacitive and non-capacitive calcium influx (Shuttleworth and Thompson, 1999; Jung et al., 2000).

**Spontaneous Ca\(^{2+}\) Oscillations in Rat Astrocytes**

Spontaneous Ca\(^{2+}\) oscillations in astrocytes have been described for cultured (Fatatis and Russel, 1992; Harris-White et al., 1998) and acutely dissociated cells (Charles, 1994), as well as for acute
brain slices (Parri et al., 2001; Nett et al., 2002). Since action potential firing and synaptic transmitter release are not needed to sustain astrocytic Ca\textsuperscript{2+} oscillations in astrocytes, it was proposed that these Ca\textsuperscript{2+} oscillations are due to ‘intrinsic factors’ (Nett et al., 2002). We could confirm that the Ca\textsuperscript{2+} oscillations are not dependent on TTX-sensitive electrical activity and Ni\textsuperscript{2+}-sensitive transmitter release. In contrast to mouse hippocampal slices, where inhibitors of mGluRs did not affect spontaneous Ca\textsuperscript{2+} oscillations in astrocytes (Nett et al., 2002; Aguado et al., 2002), we show here that Ca\textsuperscript{2+} oscillations in rat astrocytes were initiated and maintained by activation of mGluR groups I and II, but not group III. On the other hand, blocking Ca\textsuperscript{2+} channels with Ni\textsuperscript{2+} had no effect on the Ca\textsuperscript{2+} oscillations, while spontaneous glial Ca\textsuperscript{2+} oscillations in mouse preparations were reduced by Co\textsuperscript{2+} (Aguado et al., 2002).

The frequency of spontaneously active astrocytes over at least 10 min was 35%, with 1.26 Ca\textsuperscript{2+} transients/min on average in the juvenile rats. Both these parameters may vary with the stage of development and the type of preparation. In mouse hippocampal brain slices we found that a higher percentage of astrocytes were spontaneously active than in rat hippocampal slices under the same conditions (unpublished observations). An \textit{in vitro} study on rat cortical astrocytes of similar age as in the present study showed that >50% of astrocytes showed a Ca\textsuperscript{2+} response within 10 min, but with a much lower frequency of occurrence (0.12/min; Hirase et al., 2004). The reasons for these variations are still unclear, but indicate that influences from the whole system, as present under \textit{in vivo} conditions, may alter the spontaneous activity.

Our results show that antagonists of either group I or group II mGluRs are able to abolish the Ca\textsuperscript{2+} oscillations in hippocampal astrocytes, but addition of an agonist of either group I or group II mGluRs is sufficient to evoke Ca\textsuperscript{2+} oscillations. The effect of blocking either group I or group II receptors suggests that mGluRs of both groups need to be activated to allow Ca\textsuperscript{2+} oscillations, while the initiation of Ca\textsuperscript{2+} oscillations by the addition of a single, specific agonist for either group I or group II mGluRs suggests that activation of either group I or group II mGluRs is sufficient to evoke Ca\textsuperscript{2+} oscillations. It must be noted here that the degree of specificity of mGluR agonists and antagonists for either group I and/or group II varies; therefore our results might be explained by the lack of group-specificity of the ligands used here. This may explain the action of MCPG, which is believed to block group I and group II mGluRs but did not inhibit the APDC-evoked Ca\textsuperscript{2+} oscillations in our experiments. On the other hand, even when adding a group-specific agonist for mGluRs which alone is able to elicit Ca\textsuperscript{2+} oscillations, both types of mGluR might be activated due to the presence of endogenous, extracellular glutamate in the slice (probably in the low micromolar range; see below). Assuming different affinities and desensitisation properties of the different mGluRs, the background glutamate level in the tissue might be sufficient to activate one, and the added group-specific agonist the other, mGluR, leading to the initiation of Ca\textsuperscript{2+} oscillations. Experimental evidence, however, is yet needed to prove either of these possibilities.

Biophysical models simulating oscillating cytosolic Ca\textsuperscript{2+} either employ oscillating protein kinase C activity and IP\textsubscript{3} production (Hofer et al., 2004) or are based on oscillating Ca\textsuperscript{2+} membrane fluxes into and out of the cytosol (Sneyd et al., 2004). Application of U73122, a blocker of phospholipase C, and CPA, a potent inhibitor of sarcoendoplasmatic Ca\textsuperscript{2+}-ATPase,
abolished spontaneous Ca\textsuperscript{2+} oscillations in rat astrocytes. From this, and the result that Ni\textsuperscript{2+} does not influence Ca\textsuperscript{2+} oscillations, we conclude that oscillating Ca\textsuperscript{2+} plasma membrane fluxes are not responsible for the Ca\textsuperscript{2+} oscillations in rat astrocytes. Furthermore, it is known that evoked neuronal activity elicits Ca\textsuperscript{2+} rises in astrocytes (Porter and McCarthy, 1996) and, in turn, glial Ca\textsuperscript{2+} rises are able to release glutamate ( Fellin et al., 2004) and increase synaptic activity in hippocampal interneurons through activation of kainate receptors ( Liu et al., 2004), or enhance currents through NMDA receptors in interneurons of the thalamus ( Parri et al., 2001). The findings that astrocytes are able to respond even to small changes in the extracellular glutamate concentration and release glutamate in response to an increase in intracellular Ca\textsuperscript{2+} could mean that a positive feedback mechanism operates inducing 'glutamate-induced glutamate release' from astrocytes. This would not only be functional during neuronal activation, but might also be caused by tonic activation of glial mGluR.

**Group II Metabotropic Glutamate Receptor-mediated Ca\textsuperscript{2+} Release in Astrocytes**

Group II mGluRs have been shown to be located mainly on presynaptic terminals ( Petralia et al., 1996). They have been suggested to be involved in the negative feedback on glutamatergic and GABAergic transmitter release ( Moldrich et al., 2001; Smolders et al., 2004). However, *in situ* hybridization and antibody staining revealed that group II mGluRs are found not only in neurons, but also in astrocytes of the hippocampus ( Petralia et al., 1996; Schools and Kimmelberg, 1999). In addition to the negative coupling of group II mGluRs to the activity of the adenyl cyclase via G\textsubscript{i/o}-proteins ( Schoepp et al., 1995; Anwyl, 1999), there is evidence that group II mGluRs might also stimulate phospholipase C activity ( Otani et al., 2002). The specific agonist DCG IV induced long-term depression in the prefrontal cortex, which was shown to be sensitive to U73122 and the IP\textsubscript{3} receptor antagonist heparin. Furthermore, phospholipase D activation by mGluR group II agonists in hippocampal slices was reported by Klein et al. (1997), and application of DCG IV increased PTD and PLC activity with an EC\textsubscript{50} of 22 nM. Our results suggest that activation of glial group II mGluRs leads to a phospholipase C-mediated Ca\textsuperscript{2+} release from intracellular stores. An indirect effect of mGluR group II via modulation of external glutamate levels is unlikely, since inhibition of synaptic transmitter release by Ni\textsuperscript{2+} or Ca\textsuperscript{2+}-free saline as well as incubation with V-ATPase inhibitor bafilomycin A1 failed to inhibit mGluR group II-mediated Ca\textsuperscript{2+} oscillations. In addition, mGluR\textsubscript{2/3} (group II) stimulation via DCG IV or APDC can decrease extracellular glutamate levels in the hippocampus *in situ* ( Moldrich et al., 2001). Release of glutamate by the glutamate-cystine exchanger of astrocytes, as described by Tomi et al. (2003), could be a Ca\textsuperscript{2+}-independent source for external glutamate. Since it has been reported for the nucleus accumbens that activation of group II mGluRs results in an inhibition of the glutamate-cystine exchanger ( Xi et al., 2002), and therefore in a decrease of glutamate release, an involvement of X(c') transporters seems unlikely.

Furthermore, the inhibition of the adenyl cyclase by SQ22.536 did not inhibit DCG IV- or APDC-triggered Ca\textsuperscript{2+} responses, and supports our notion that the cAMP-pathway is not involved in the mGluR\textsubscript{2/3} (group II)-mediated Ca\textsuperscript{2+} responses. These findings cannot exclude the possibility that NMDA receptors and group II mGluR interact as described by Mistry et al. (1998), who reported that activation of group II mGluRs by DCG IV (<1 \mu M) leads to an increase in phosphoinositide turnover. This mechanism cannot be excluded here, though NMDA receptors are generally believed to be absent in hippocampal astrocytes (Seifert and Steinhaüser, 2001; Matthias et al., 2003).

**The Role of Extracellular Glutamate for Spontaneous Ca\textsuperscript{2+} Oscillations in Astrocytes**

Extracellular glutamate levels are derived from vesicular and non-vesicular sources. While glutamate is released during synaptic transmission, it is taken up by glial cells via the excitatory amino acid transporter. Under pathophysiological conditions like stroke and ischaemia, increased levels of glutamate lead to cell death. Although group II mGluRs are known to act as autoreceptors at presynaptic sites, or as heteroreceptors at neuronal cell somata, and are able to tune down synaptic transmitter release, the role of group II mGluRs expressed in astrocytes is not yet clear. Our findings show that astrocytes respond to different concentrations of glutamate with different Ca\textsuperscript{2+} responses. This may enable astrocytes to sense different levels of extracellular glutamate levels in the micromolar range (normal ~3 \mu M). Activation of purinergic, histaminergic and adrenergic metabotropic receptors failed to show this response profile. Prolonged application of low agonist concentrations (e.g. PhE, 3 \mu M or ATP, 10 \mu M) resulted in a single Ca\textsuperscript{2+} transient, which was followed at a reduced Ca\textsuperscript{2+} level by a 'shoulder' at higher agonist concentrations. While the transient change in intracellular Ca\textsuperscript{2+} seems to reflect receptor response and IP\textsubscript{3} production, the subsequent shoulder is presumably due to receptor inactivation, store depletion and/or store-operated Ca\textsuperscript{2+} influx ( Shuttleworth and Thompson, 1999; Jung et al., 2000). This also infers that glial cells may sense extracellular glutamate, and respond with different types of Ca\textsuperscript{2+} transients and frequencies of Ca\textsuperscript{2+} oscillations to micromolar glutamate concentrations or to small step changes of the glutamate concentration in the extracellular spaces.

**Notes**

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