Astrocytic responses to activation of metabotropic glutamate receptors group I (mGluRs I) and α₁-adrenoceptors in cultured cells have been assessed using spectral analyzes and calcium imaging. Concentration-dependent changes were observed after stimulation with the mGluR I agonist (S)-3,5-dihydroxyphenylglycine (DHPG). These responses changed from a regular low frequency signal with sharp peaks at 1 μM to pronounced stage of irregularity at 10 μM. After stimulation with 100 μM the signal was again homogenous in shape and regularity but occurred at a higher frequency. In contrast, the spectral properties after stimulation with the α₁-adrenergoreceptor agonist phenylephrine, exhibited considerable variation for all investigated concentrations. DHPG-induced increases in [Ca²⁺]ᵢ were also associated with astroglial glutamate release, whereas no release was observed after noradrenergic stimulation. Both DHPG-mediated calcium signaling and glutamate release were inhibited by preincubation with 10 or 100 μM phenylephrine. Collectively, the present investigation provides new information about the spatial-temporal characteristics of astroglial intracellular calcium responses and demonstrates distinct differences between noradrenergic and glutamatergic receptors regarding intracellular calcium signaling and coupling to glutamate release. The noradrenergic modulation of DHPG-induced responses indicates that intracellular astroglial processes can be regulated in a bi-directional feedback loop between closely connected astrocytes and neurons in the central nervous system.

Astrocytes, the main population of glial cells, form gap junction-coupled multicellular networks and communicate among themselves through variations in intracellular calcium concentration ([Ca²⁺]ᵢ). These cells express a calcium-based excitability in which receptor-mediated calcium elevations can propagate as intracellular waves over considerable distances (1, 2). A diverse range of [Ca²⁺]ᵢ changes are induced in astrocytes after exposure to various neurotransmitters (2–6) and the cells display [Ca²⁺]ᵢ oscillations and intracellular waves in response to neuronal activity (7–9). Previous results demonstrate the importance of glutamate for the mediation of these neuronal-glial interactions. However, the potential contribution of other important neurotransmitters such as those belonging to the noradrenergic system has not yet been fully investigated.

Variations in astrocytic [Ca²⁺]ᵢ, mediate the uptake and release of different neuroactive substances (10) such as arachidonic acid, neurotrophins, and neurotransmitters, and via this mechanism possibly modulate synaptic transmission (11–13). Consistent with this, previous studies have shown that activation of glial metabotropic glutamate receptors in concert with β-adrenoreceptors (β-AdRs) can result in depression of synaptic activity in which the release of CAMP from astrocytes is central (14). Furthermore, adrenergic and glutamatergic interactions can also greatly affect both CAMP- and calcium-related signaling in astroglial cells (15). Both α₁-AdRs (3, 16) and mGluRs (1) (17–19) are expressed on astrocytes and stimulate phospholipase C (PLC) to hydrolyze phosphoinositides. The nature of astrocytic mGluRs is still somewhat unclear. Studies of the distribution of mGluRs in various brain regions have detected only mGluR₃ and mGluR₅ immunoreactivity and, in Northern blot studies, only mRNA for the mGluR₃ and mGluR₅ have been found (20, 21). Calcium transients after mGluR activation have been detected in most astroglial preparations and the oscillatory behavior in mGluR I-induced calcium changes is mediated through the activation of mGluR₅ (2, 22, 23). Their activation elicits oscillatory changes in [Ca²⁺]ᵢ that are distinct from the simple non-oscillatory transients evoked by other subtypes of glutamate and adrenergic receptors.

Oscillations in [Ca²⁺]ᵢ, are important components in the regulation of several diverse intracellular functions in different

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cell types, including astrocytes (24–26). It has been suggested that the repetitive fluctuations in [Ca\(^{2+}\)](aq), constitute frequency-encoded signals (27) that transform the original stimuli into defined biological messages. These can in turn initiate multiple cellular processes such as the differentiation of gene transcription (28) and the secreting of insulin in pancreatic beta cells (29). The multifaceted pattern of receptor-mediated [Ca\(^{2+}\)](aq) changes might also be utilized in the bi-directional interplay between astrocytes and neurons. For example, the characteristics of calcium oscillations are probably crucial for events such as the recently described astrogial glutamate release (30). Experiments performed, both in cell cultures (31, 32) and acute brain slices (5), have demonstrated that glutamate is released from astrocytes through a pulsatile calcium-dependent process resembling the synaptic release of neurotransmitters. In addition, the mechanisms behind this release do not involve swelling (33) or the reversal of transporters (34). Together, these studies indicate a close connection between [Ca\(^{2+}\)](aq) oscillations and glutamate release in astrocytes, where the periodical changes in [Ca\(^{2+}\)](aq) represent the signal that controls the frequency of the pulsatile release (30).

An increasing amount of evidence suggests that astrocytic calcium signaling plays a crucial role in information processing in the brain (35). However, in this context, a more detailed investigation of the nature of the oscillatory responses is required. In the present study, we have used spectral analyses to characterize and compare calcium oscillations from single astroglial cells after mGluR I and \(\alpha_1\)-adrenoceptor stimulation. Adrenergic effects on mGluR I-mediated glutamate release were also evaluated. The results demonstrate the diverse nature of the oscillatory responses with respect to the receptor system activated. The activation of group I mGluRs but not \(\alpha_1\)-adrenoceptor stimulation, elicits glutamate release from cultured astrocytes. We further show that \(\alpha_1\)-AdRs and mGluRs I are co-localized on cultured astrocytes and that \(\alpha_1\)-adrenergic activation inhibits DHPG-elicited [Ca\(^{2+}\)](aq) oscillations and the subsequent glutamate release from astroglial cells.

**EXPERIMENTAL PROCEDURES**

**Model Systems**

**Astroglial Cell Cultures**—Primary astroglial cell cultures were prepared from P1 or P2 Sprague-Dawley rat pups (Charles River, Uppsala, Sweden) as previously described (4, 36). In brief, the brains were removed and cerebral cortices were dissected and cleaned from meninges. The tissues were then passed through an 80-μm nylon mesh net into Eagle’s minimum essential medium supplemented with Earle’s salts (Life Technologies Inc., Paisley, UK). Additional substances were added to make up the following final composition: double concentrations of amino acids and quadruple concentrations of vitamins (Life Technologies), 2 mM l-glutamine (Life Technologies) 7.5 mM glucose (Sigma Chemical Co., St. Louis, MO), double concentrations of NaHCO\(_3\) (Merck, Darmstadt, Germany), 1% penicillin-streptomycin (Life Technologies), and 20% (v/v) fetal calf serum (Harlan Sera-Lab, Loughborough, LE, UK), all at pH 7.3. The medium was changed after 3 days of cultivation, and thereafter three times a week. Cultures were grown to confluence in a humidified atmosphere of 5% CO\(_2\) in air at 37 °C and were used for experiments within 14–16 days.

**Brain Slices**—Sprague-Dawley rats (P15 and P30) were transcardially perfused for 10 min (infusion pressure, 200–220 mm Hg at room temperature) through a silicon catheter with 4% paraformaldehyde in a 0.1% phosphate buffer. The brains were removed, sections were taken, dehydrated, and paraffin-embedded. Slices (6 μm) were then prepared using a DTK 1500e Microslicer.

**Methods**

**Immunocytochemistry**—Primary cortical astroglial cultures were stained for glial fibrillary acidic protein (GFAP) or mGluR5, by incubating the cultures with a rabbit affinity-purified IgG anti-mGluR5 (1:400; Upstate Biotechnology Inc., Lake Placid, NY) or a rabbit affinity IgG anti-GFAP (1:100) (Dako Co., Glostrup, Denmark). Secondary antibodies used were affinity-purified biotinylated goat anti-rabbit IgG diluted 1:200 (Vector Laboratories, Burlingame, CA), followed by an avidin biotin complex (ABC) detection system (Vectastain Elite, Vector Laboratories). Immunoreaction was visualized with 3,3’-diaminobenzidine (DAB). Coverslips were mounted on microscope slides using fluorescent mounting medium (Dako Co.) and viewed in a Nikon Optiphot-2. The tissues were then passed through an 80-μm nylon net into Eagle’s minimum essential medium supplemented with Earle’s salts (Life Technologies Inc., Paisley, UK), all at pH 7.3. The medium was changed after 3 days of cultivation, and thereafter three times a week. Cultures were grown to confluence in a humidified atmosphere of 5% CO\(_2\) in air at 37 °C and were used for experiments within 14–16 days.

**Local Application of Drugs**—When appropriate, drugs were delivered directly to individual cells with the aid of a pressure-driven pico-injector (Nikon PLI-188). Microprobes were pulled on a Narishige PP-83 pipette puller from borosilicate capillaries (Clark Electromedical Instruments, Pangbourne, UK). Applications were preformed using a Narishige IMS micromanipulator connected to the injector.

**Frequency Analysis**—Frequency analyses of each oscillatory fluorescence signal were performed using fast Fourier transformation (FFT). Signals were collected at 1–10 frames/s for 1000 s and low-pass-filtered at 0.3 Hz. The analysis was made according to normal signal processing standards described in digital signal processing textbooks (e.g. Ref. 39). In brief, the oscillogram windows having sequences varied in length, the sequences were padded with zero samples to get an equal sequence length of an even power of two. The mean value and first order trend was subtracted from the original signal, and the resulting signal was connected front to end to form a cycle of the signal to be transformed. A window, Hamming, which dampens the signal amplitudes at the beginning and the end, was also applied to the sequence to eliminate a signal jump at the connection point. The information of this cyclic sequence and its Fourier transform are equivalent.

Mean averages of the power spectra were compared with regard to frequency range and amplitude. The spectra were used to determine characteristics of the oscillations as well as the shape of the individual deflections; a stable frequency during oscillation will generate narrow peaks, and sharp deflections will generate many harmonics. Properties of each individual peak in the power spectra were expressed as the ratio of the frequency at the peak amplitude divided by the half power width, \(k\), (the width at 1/2 of the peak value in the signal spectrum), where \(i\) indicates the peak number. Logarithmic values of the \(k\), \(i = 1\) to 3, are plotted to illustrate these properties; high absolute values will indicate stable frequency during the oscillation, whereas a steep slope will indicate rounded and/or variable waveforms.

**Glutamate Measurement**—Glutamate release was measured using glutamate dehydrogenase (GDH) oxidation of glutamate to \(\alpha\)-keto-gluutarate. In this reaction \(\beta\)-nicotinamide adenine dinucleotide (NAD\(^+\)) is reduced to NADH. Because the NADH produced in the reaction is fluorescent when excited by UV light, the reaction can be followed as changes in NADH fluorescence and as an indirect indicator of the glutamate released (40, 41). The experiments were performed at room temperature (20–22 °C), using an upright Nikon Eclipse E600-FN microscope equipped with a PTI imaging system. Light from a xenon lamp was filtered at 335 nm, and emissions were collected at 470 nm. Background subtraction was performed, and data was normalized to baseline and expressed as \(\Delta F/F_0\), where \(\Delta F\) is the fluorescence deviation from \(F_0\) to \(F_0\) (651.3 μm) for the reaction was determined using known
concentrations of glutamate added to a solution of GDH and NAD$^+$, and where $C_{ev}$ was plotted against $C$ using a Hanes plot. Standard buffer was used with addition of GDH (50 units/ml) and NAD$^+$ (1 mM). All experiments were ended with the application of glutamate (100 $\mu$m) to verify the method.

High Pressure Liquid Chromatography—The concentration of extra-cellular amino acids was determined using reverse-phase high pressure liquid chromatography (HPLC) employing automated precolumn fluorogenic labeling with O-phthalaldehyde/$\beta$-mercaptoethanol (42). The column (300 $\times$ 4.6 mm) was packed with Nucleosil 100–5C$_18$ (Macherey-Nagel, Germany). Derivatives were eluted with a gradient from 0 to 90% methanol (containing 1.25% tetrahydrofuran (v/v)) in a sodium phosphate buffer, 50 mM, set at pH 5.40 with 1 M NaOH and containing 2.5% tetrahydrofuran. The autoinjector (Water Wisp 717 plus) was programmed to add 25 $\mu$L of OPA/$\beta$-mercaptoethanol to 50 $\mu$L of incubation medium. Controls were collected at 30, 20, and 10 min prior to application of 10–100 $\mu$L noradrenaline, and samples for determining the efflux of amino acids were taken <1, 3, 10, and 20 min after the stimulation. Results are presented as relative increases or decreases compared with control value.

Statistics—The level of significance between groups was calculated using variance analysis (ANOVA) followed by post hoc range tests and pairwise multiple comparisons (Bonferroni), or Student’s double-tailed $t$ test.

Chemicals—Glutamate, DHPG, S-carboxyphenyl glycine, kainic acid, and AMPA were obtained from Tocris-Cookson (St. Louis, MO). Fluronic acid was supplied by Calbiochem (La Jolla, CA). All other chemicals of analytical grade were manufactured by Sigma Chemical Co. (St. Louis, MO).

RESULTS

Receptor Expression: Astrocytes in Primary Cultures and in Brain Slices Express mGlu$_R_5$ Receptors

In the astroglial cultures, the majority of the cells were GFAP-positive (Fig. 1A) and shown to express immunoreactivity for the mGlu$_R_5$ marker (Fig. 1B). Fig. 1C illustrates the expression of mGlu$_R_5$ on GFAP-positive cells in the cortical brain slice. GFAP-positive cells appear red; the mGlu$_R_5$ is green.

The presence of the $\alpha_1$-adrenoreceptor is previously well defined on astrocytes in cultures as well as in different in situ preparations (43–46). In addition, they are previously functionally characterized within our culture system (3, 4).

mGluR I-induced Calcium Signaling

mGluR Activation Induces Periodic Changes of $[Ca^{2+}]_i$, with Distinct Properties in CULTURED Astrocytes—The kinetics of $[Ca^{2+}]_i$ changes were characterized after both continuous as well as transient exposure to glutamate and the mGluR group I agonist (S)-3,5-dihydroxyphenylglycine (DHPG). The agonists elicited calcium transients with different spatial-temporal patterns and varying frequencies in the majority of the cells (40 trials; $>$500 cells) (Fig. 2A and B). No or sparse oscillatory behavior were seen after the ionotropic receptor agonists $\alpha$-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) or kainic acid.

The individual calcium responses were characterized, and the response patterns were classified into three separate groups (Fig. 3, A–C). The first group displayed monophasic responses consisting of one single peak during 300 s of observation time. The second group was characterized by biphasic responses displaying two distinct separated peaks, and the third group demonstrated oscillatory responses exhibiting three or more transients during the initial 300 s. In astrocytes displaying oscillations, a second or third stimulation repeatedly elicited responses with similar frequency and duration (Fig. 4). As a common criterion, an increase in $\Delta F$ of 20% or above was regarded as a response. Spontaneous calcium transients or oscillations were seen in less than 5% of the cell population. Oscillatory changes in $[Ca^{2+}]_i$, were seen in 16.7 ± 8.3 and 32.6 ± 6.0% (mean ± S.D.) of the responding cells after 10 $\mu$m glutamate and DHPG, respectively. No oscillatory responses were seen after low concentrations of glutamate ($\leq$1 $\mu$m), whereas 15.1% of the cells responding to 1 $\mu$m DHPG exhibited periodic changes in $[Ca^{2+}]_i$. Glutamate or DHPG concentrations exceeding 50 $\mu$m did not increase the proportion of oscillatory responses (Fig. 2B).

Uncoupling of gap junctions demonstrated the independence of intracellular communication for single cell responses. Responses and oscillation rates were non-significantly altered by exposure to the gap junctional uncoupler 18-o-glycyrrhetinic acid, 10 $\mu$m for 10 min ($n$ = 10 trials; 200 cells).

Changes in PKC Activity by PMA and Ro 31-8220 Inhibit DHPG-induced Oscillations—The sensitivity for changes in protein kinase C (PKC) activity were evaluated after DHPG stimulation. Data showed that the PKC activator phorbol 12-myristate-13-acetate (PMA, 150 $\mu$m) reduced the oscillatory responses to a single peak response or, in less than 10%, no response at all (Fig. 5A). This indicates a rapid PKC-mediated negative feedback. Exposure to the PKC inhibitor Ro 31-8220 (10 $\mu$m, 5 or 10 min) totally inhibited the DHPG-induced oscillations (Fig. 5B), further verifying the dependence of a well-regulated PKC activity for the initiation and maintenance of mGluR-coupled $[Ca^{2+}]_i$, oscillations ($n$ = 20 trials; 400 cells).

Frequency Analyses Demonstrate Concentration-dependent Changes in the Properties of Frequency-encoded Calcium Signals—Individual oscillating signals (40 cells/agonist and concentration) were randomly chosen from a total number of DHPG-induced responses exceeding 500 cells/agonist/concentration. Both individual cells as well as groups of different cells were sequentially stimulated with the appropriate agonist, and average power spectra obtained from the FFT analyses of single cell oscillations were compared and analyzed. No significant differences between these approaches were detected.

After exposure to 1 $\mu$m DHPG, the spectra demonstrated a range of oscillatory frequencies between 5 and 21 MHz, with $>$90% occurring at 6.8–14.0 MHz and an average at 10.1 ± 5.7 MHz (mean ± S.D.). Mean frequencies after 10 and 100 $\mu$m DHPG were 15.9 ± 12.5 and 22.0 ± 5.9 MHz, respectively (Fig. 6A). At increasing concentrations, the frequency domain exhibited a rightward shift, revealing a pronounced concentration dependence. As described in Fig. 6B, the regularity and shape of the original calcium transients displayed significant differences (ANOVA, $p < 0.001$) after exposure for 1 and 10 $\mu$m but not after 1 and 100 $\mu$m DHPG. The application of 1 $\mu$m DHPG induced slow oscillations with sharp peaks and low frequencies in 15.1% of the responding cells. In contrast, 10 $\mu$m displaced a higher average frequency, 15.88 ± 12.51 MHz, and a more pronounced irregularity in both rhythm and shape. After the exposure to 100 $\mu$m DHPG, the regularity of the oscillations was regained and the shape of the individual peaks was sharpened, although at a higher frequency (21.98 ± 5.93 MHz). Interestingly, no significant differences in the duration of the oscillations were seen between the different concentrations.
Taken together, the above results directly demonstrate that the oscillatory nature of calcium transients after mGluR I activation can be controlled between, at least, two distinct different signaling states.

**Comparison between Adrenoreceptor- and mGluR-induced Calcium Signaling**

The results obtained from the DHPG-stimulated cells were compared with the kinetics of the \([Ca^{2+}]_i\) changes and spectra obtained after adrenergic stimulation. Noradrenaline and phenylephrine (PE, \(\alpha_1\)-AdrR) were shown to elicit similar patterns of \([Ca^{2+}]_i\) changes (Fig. 7, A and B) as DHPG. Either none or sparse oscillations were seen after \(\alpha_2\)- or \(\beta\)-adrenoreceptor activation (\(n = 10\) trials; 200 cells). The sensitivity for changes in PKC activity was confirmed for \(\alpha_1\)-AdrR. Both the activation of PKC (PMA; 150 nM, 15 min) and the inhibition (Ro 31-8220; 10 \(\mu\)M, 5 or 10 min) abolished the oscillatory nature of the PE-induced calcium responses. The inhibition of PKC also sig-

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**Fig. 2.** A, dose-response relationship for glutamate and DHPG in cultured astrocytes. B, spatial-temporal appearance of the \([Ca^{2+}]_i\) changes after the two agonists. The graph demonstrates the differences in concentration dependence for response rates and oscillatory tendency (40 trials; number of cells >500).

**Fig. 3.** Individual calcium responses were characterized, and the response patterns were classified into three separate groups. The first group displayed monophasic responses consisting of one single peak during 300 s of observation time (A). The second group was characterized by biphasic responses displaying two distinct separated peaks (B), and the third group demonstrated oscillatory responses exhibiting three or more transients during the initial 300 s (C).
significant lowered the response rate (n = 15 trials; 300 cells), whereas the incubation with PMA totally inhibited PE-induced calcium responses (n = 10 trials; 200 cells). Considering the oscillatory behavior, it was surprising that neither concentration dependences nor different oscillatory states were seen after PE application (Fig. 7B). It is also noteworthy that the individual oscillation frequency (millihertz) did not increase with higher agonist concentration (Fig. 8A), although the number of responding cells did (Fig. 7A). Additional experiments were made at the concentrations of 0.1 to 1 mM PE to rule out the possibility of differences in concentration dependence. Although a tendency toward concentration dependence was found, the resemblance to DHPG-induced responses was never observed. The graphical presentation of the shape and regularity parameter k (described under “Methods”) clearly shows that no particular consistency was found in the original signal after PE stimulation (Fig. 8B), as seen after the activation of the group I mGluRs (Fig. 6B). However, the higher absolute k values after PE stimulation indicate that these oscillations are more stable in frequency than the DHPG-induced oscillations. These results demonstrate an obvious diversity between the two agonists with respect to both concentration and agonist-dependent changes in shape and regularity of the individual calcium signal.

Interactions between mGluR and PE Receptor Systems

The sensitivity for PKC activity indicates that pre-activation of PKC could be a physiological method of regulating the oscillatory response and that the modulation of PKC activity could alter or modulate the oscillatory behavior. Experiments were therefore performed to evaluate possible interactions between adrenergic and glutamatergic receptor agonists.

Local Application of DHPG and PE Confirms Functional mGluRs and α1-AdrRs on Single Astroglial Cells—Co-localization of mGlu group I receptors and α1-AdrR on individual astrocytes was confirmed after sequential stimulation with DHPG and PE. The drugs were applied directly to the individual cell in the presence of 18-glycyrhretinic acid (10 μM, 10 min) for gap junctional uncoupling. Agonist specificity was verified using S-4-carboxyphenyl glycine (10–100 μM) and prazosin (10–100 μM), the selective antagonists for DHPG and PE, respectively. From these experiments, it was concluded that 12 out of 20 cells displaying elevations in [Ca2+]i, after DHPG stimulation also displayed functional α1-AdrRs (n = 20 trials).
α₂-Adrenergic Stimulation Regulates DHPG-induced [Ca²⁺]i Oscillations—Pre-application of noradrenaline (10–100 μM) 3–5 min prior to DHPG exposure reduced the oscillatory responses after DHPG stimulation to single peaks or no transients at all in 88% of the performed experiments (n = 10 trials; 200 cells). The effects were mimicked by PE and reversed by the antagonist prazosin (10–100 μM) (Fig. 9, A and B). Application of PE during ongoing oscillations also abruptly extinguished the periodic changes in [Ca²⁺]i. Previous or simultaneous applications of α₁- or β-adrenergic agonists (10–100 μM) were without effects (n = 10 trials; 200 cells).

Receptor-mediated Glutamate Release

One potential physiological role for the observed calcium signaling is that it mediates astroglial glutamate release. A series of experiments were performed to ascertain the existence of transmitter release in the model system and to evaluate possible adrenergic effects on mGluR-induced glutamate release.

DHPG-induced Glutamate Release—By measuring the extracellular accumulation of the NADH produced in the GDH-driven oxidation of glutamate to α-ketoglutarate, glutamate release was seen after DHPG stimulation in 60% of the cells examined (Fig. 10A) (n = 10 trials, 40 cells). Depletion of intracellular calcium stores by pre-application with thapsigargin (0.1 μM, 30 min) completely inhibited both the DHPG-induced [Ca²⁺]i elevation and the subsequent glutamate release (Fig. 10B), confirming its calcium dependence (n = 5 trials, 20 cells). Application of the mGluR antagonist S-4-carboxyphenyl glycine (10–100 μM) verified the agonist specificity (n = 5 trials; 20 cells).

No Detectable Glutamate Release after Adrenergic Stimulation—The effects of different noradrenergic agonists on glutamate release were evaluated. In the concentration range of 1–100 μM, no accumulation of extracellular NADH was detectable after noradrenaline or after the different adrenoreceptor agonists (n = 10 trials; 40 cells). The absence of extracellular glutamate after adrenergic stimulation was verified with HPLC (n = 5). The results demonstrate that no glutamate or other amino acids involved in the synthesis of glutamate were released after 10 or 100 μM noradrenaline (Fig. 11).

3- to 5-min Pre-activation of α₂-AdRs Inhibits mGluR-induced Glutamate Release—Effects of the pre-application of noradrenaline were assessed with respect to glutamate release.
In 85% \((n = 10\text{ trials}; 40\text{ cells})\), the DHPG-mediated accumulation of NADH was inhibited by noradrenaline or PE \((10^{-6}\text{ M})\) \((n = 5\text{ trials}; 20\text{ cells})\) and reversed by prazosin \((10^{-6}\text{ M})\) \((n = 5\text{ trials}; 20\text{ cells})\) (Fig. 12, A and B). As shown with HPLC, no amino acids involved in the synthesis of neurotransmitters or other amino acids that could mediate the observed effects were released after noradrenaline (Fig. 11).

**DISCUSSION**

There is a considerable body of evidence indicating that receptors for multiple neurotransmitters coexist on astrocytes (47) and that their simultaneous activation can influence calcium-related signaling (15). The present study demonstrates the expression of the subtype mGluR5 on cortical astrocytes \textit{in situ} and shows that the expression is preserved in cultures. To our knowledge, the only mGlu group I receptor expressed by astrocytes is the mGluR5 (20, 21), and thus it is likely that the responses observed using the mGluR group I agonist DHPG are mediated primarily by this receptor. However, subtypes or splice variants of mGluR1 that have not been detected in previous studies, might account for some of the non-oscillatory responses given in this report, whereas the oscillatory responses are probably mediated through mGluR5 activation. Furthermore, the functional co-localization of mGluRs group I and \(\alpha_7\)-AdRs on single cortical astrocytes was also observed using calcium imaging. Their presence indicated that synergistic as well as antagonistic effects could be expected if they were activated simultaneously and their concurrent involvement in oscillatory \([\text{Ca}^{2+}]_i\), changes and glutamate release were evaluated.

**Spectral Analyses and Different Signaling States in Astrocytes**—Spectral analysis is a standard procedure in signal processing. Such analysis has been applied to numerous biological processes, including mitochondrial contributions to calcium signaling and the examination of amplification sites for calcium waves in astrocytes (48, 49). In this report, the investigation was focused on two spectral properties: peak sharpness and harmonic content. Narrow peaks and a high harmonic content were noted in three experimental situations: 1 and 100 \(\mu\text{M}\) DHPG and 1 \(\mu\text{M}\) PE. At 100 \(\mu\text{M}\) DHPG the oscillating frequency was twice that at 1 \(\mu\text{M}\). This can be interpreted as two stable states. If calcium variations were to be governed by basic processes such as diffusion or the chemical reaction rate, the mean spectrum from a collection of individual spectra would reflect these properties. This is what our results indicate in the above-mentioned experimental situations. The recorded calcium variations might be looked upon as a mean of numerous active sites within the cell with pulse-shaped output. In such case, the recorded calcium level not only reflects the basic chemical process but also the timing of the activity of each individual site. If the activities were totally random, a constant somewhat noisy level would be recorded, and if a significant number of sites were acting almost simultaneously, calcium variations would be observed. As the jitter decreases in the synchronous action, the closer the output resembles the shape of the pulse of an individual site action. The spectra then have high harmonic contents, and the oscillating frequency should be similar in each cell. Not all sites need to act simultaneously. If we consider the sites to be weakly coupled stochastic processes, theory and numerous real world observations suggest that some states are more likely to occur than others. Phase locking, in particular, is a common feature. The fact that we
observe oscillation frequencies (at 100 μM DHPG) of twice the basic frequency (at 1 μM DHPG) further supports a suggested model for calcium variations based on synchronization of individual sites in which several release sites could synchronize through weak coupling.

Consistent with this finding, power spectrum analyses were performed and concentration-dependent variations in shape and regularity of DHPG-mediated [Ca²⁺]ᵢ responses were demonstrated. The mean calcium signal could be altered from an initial phase of low frequency and sharp peaks to a pronounced phase of irregularity by increasing the concentration of DHPG in the medium from 1 to 10 μM. At the highest concentration of DHPG investigated (100 μM), the signal was again homogenous both in shape and regularity but displayed a higher frequency. These results demonstrate a concentration-dependent transformation from one signaling condition to another. A possible explanation for this is that consistent frequencies might be a result of saturation effects, which are common phenomena in several biological systems. In that case, stable oscillations with little or no variability would appear at high stress and no-control situations, while considerable variability is observed within the normal working range. Consistent with this, earlier studies have shown that only astrocytes displaying slow regular oscillations can increase their frequency and exhibit long term changes in oscillatory behavior after enhanced neuronal activity (50).

Synchronization of Intracellular Release Sites Could Model Components in the Oscillatory Behavior—Oscillatory behaviors in clusters of “weakly coupled processes” are a common phenomenon that has been observed in biological systems. These systems range from cardiorespiratory dynamics at the organ level (51) to microscopic interactions within cell populations (52, 53). There is no reason to expect such behavior not to be important among intracellular processes, as well. The synchronization represents a general mechanism of self-organization in complex systems, resulting in a significant decrease of the effective degree of freedom.

The suggested underlying synchronization process could occur if the spatial distributions of calcium release sites are heterogeneous, or if a proportion of the sites don’t respond to...
the stimulation. The idea of localized release sites in astrocytes has been presented after observations that noradrenaline-mediated intracellular wave propagation is supported by specialized regenerative calcium release loci distributed along the length of the cell (54). In accordance with the present findings, Yagodin et al. (54) discovered that subcellular oscillation sites differ from each other with regard to the period of oscillation, amplitude, and rate of $[Ca^{2+}]_i$ increase. The presence of local invariant intracellular sites with inherent kinetic properties provides the possibility for a high degree of specialization of the patterns of response seen in a given cell or cell population. By a selective activation of subsets of these loci, different patterns of agonist-mediated calcium responses can be elicited. Consequently, the degree of synchronization of the release sites can reflect the properties of the obtained oscillatory signals and might explain the differences in oscillatory properties seen after increased DHPG concentrations.

Specificity in Receptor-mediated Calcium Signaling—The marked concentration dependence of the frequency of the oscillations seen after DHPG stimulation is consistent with the common features of periodic $[Ca^{2+}]_i$ changes (55). In accordance with the previously described diversity between glutamatergic- and adrenergic-induced oscillations (50), this was restricted to group I mGluRs, and not $\alpha_1$-AdRs stimulation. Even if the initiation and the maintenance of the oscillations are dependent on PKC activity for both receptors, the diversity in oscillatory behavior demonstrates agonist specificity in the produced signal, further verifying astroglial selectivity for neuro-

![Fig. 9](image1.png)

*Fig. 9.* A, the inhibition of DHPG-induced $[Ca^{2+}]_i$ oscillations by 10 μM PE ($n = 10$ trials; 200 cells). B, this effect was reversed by the $\alpha_1$-Ad antagonist prazosin (100 μM) demonstrating the receptor specificity. $n = 10$ trials; 200 cells.

![Fig. 10](image2.png)

*Fig. 10.* A, 10 μM DHPG induced glutamate release from a single astrocyte in cortical primary culture ($n = 10$ trials, 40 cells). B, the release is inhibited by the depletion of intracellular calcium stores by 0.1 μM thapsigargin ($n = 5$ trials; 20 cells).

![Fig. 11](image3.png)

*Fig. 11.* The absence of extracellular glutamate or glutamate-related amino acids after noradrenaline (10 μM in cultured astrocytes as shown with HPLC. The graph shows average values of four consecutive experiments as relative changes (%) at <1, 3, and 10 min after stimulation. Control values are the average of samples taken 30, 20, and 10 min prior application of noradrenaline ($n = 5$).

the stimulation. The idea of localized release sites in astrocytes has been presented after observations that noradrenaline-mediated intracellular wave propagation is supported by specialized regenerative calcium release loci distributed along the length of the cell (54). In accordance with the present findings, Yagodin et al. (54) discovered that subcellular oscillation sites differ from each other with regard to the period of oscillation, amplitude, and rate of $[Ca^{2+}]_i$ increase. The presence of local invariant intracellular sites with inherent kinetic properties provides the possibility for a high degree of specialization of the patterns of response seen in a given cell or cell population. By a selective activation of subsets of these loci, different patterns of agonist-mediated calcium responses can be elicited. Consequently, the degree of synchronization of the release sites can reflect the properties of the obtained oscillatory signals and might explain the differences in oscillatory properties seen after increased DHPG concentrations.

Specificity in Receptor-mediated Calcium Signaling—The marked concentration dependence of the frequency of the oscillations seen after DHPG stimulation is consistent with the common features of periodic $[Ca^{2+}]_i$ changes (55). In accordance with the previously described diversity between glutamatergic- and adrenergic-induced oscillations (50), this was restricted to group I mGluRs, and not $\alpha_1$-AdRs stimulation. Even if the initiation and the maintenance of the oscillations are dependent on PKC activity for both receptors, the diversity in oscillatory behavior demonstrates agonist specificity in the produced signal, further verifying astroglial selectivity for neuro-
transmitter stimulation. PE-mediated calcium responses displayed considerable variation in the elicited response patterns. This is consistent with the notion of specialized release sites where the significant variations in the [Ca\(^{2+}\)] oscillations could reflect a receptor-mediated specificity in the activation of the respective sites. It may also explain the close relationship between the density of receptor expression and the elicited calcium signal, indicating that the spatial distribution of receptors over the cell body could be a determining factor for the activation of different subcellular components, including release mechanisms. Thus, the probability of an astrocyte to respond to any given neuroligand is related to the density of receptors expressed and the concentration of the neuroligand applied. In addition, the pronounced variations in response patterns after receptor stimulation suggest that the biorhythmicity of the individual cells may contribute to the different calcium responses. Moreover, for oscillatory-encoded biological processes that demand specific properties of the received signal, the initiating and maintenance of these processes might increase stepwise, because the intermediate signaling condition would not be able to produce the required signal pattern.

Inhibitory Action by Adrenergic Stimulation upon mGluR-mediated Calcium Signaling—The oscillatory frequencies of receptor-mediated [Ca\(^{2+}\)] changes in astrocytes have been shown to be modified long term by enhanced neuronal activity. These changes persist for several hours after repetitive glutamatergic stimulation. The effects are mediated through mGluRs and occur without alterations in the amplitude of the calcium elevations. Interestingly, this is not observed for noradrenergic stimulation, which further indicates the presence of a dynamic and diversified control of astrocytic calcium homeostasis that could be achieved by specific neuronal activity. In line with the previous studies, we evaluated the effects of pre- or simultaneous stimulation of adrenergic receptors on DHPG-induced [Ca\(^{2+}\)] oscillations. The results demonstrated that pre-application of noradrenaline largely inhibited these oscillations, and that the effect was mediated by \(\alpha_1\)-AdR activation.

The present results also demonstrated that both inhibition and activation of PKC result in the absence of oscillatory behavior after DHPG and PE stimulation, consistent with previous studies. Furthermore, they point out that the modulation of PKC activity might be a central physiological pathway for the control of receptor-mediated calcium signaling. Because both the glutamatergic and adrenergic transmitter systems involve the activation of PLC and PKC, experiments blocking one part of the second messenger pathway will consequently inhibit the effects of all agonists utilizing this pathway. This may limit the experimental possibilities to reveal such an interaction. However, it is possible that the dual action of the agonists on the IP\(_3\)-sensitive stores attenuates the activation of calcium release channels and inhibits the process of oscillatory calcium changes. Thus, the refractory period for IP\(_3\)-dependent calcium is relatively short and appears to be determined primarily by the activity of calcium pumps responsible for refilling the intracellular stores (56). It is also possible that the observed effects could reflect the inactivation of the IP\(_3\)R rather than the exhaustion of calcium stores. In this scenario, the inhibitory action of ongoing oscillations could indicate that the signaling cascades of both group I mGluRs and \(\alpha_1\)-AdRs act at either a common pool of [Ca\(^{2+}\)] or subsets of stores that are tightly functionally coupled to different biological responses. Regardless of the primary mechanisms, the specific interactions between glutamatergic and adrenergic receptors could play an important role in the control of neuronal function by glial cells.

Astrocytic Glutamate Release and Adrenergic Receptors—The next part of the study verified that astrocytes release glutamate in a calcium-dependent way after mGluR stimulation. Thus, the astrocytes might receive information via this mechanism from the surrounding and transmit messages to adjacent cells. The mechanisms behind the release have been described previously and probably involve the actions of prostaglandins. In light of this, the observations that DHPG but not adrenoreceptor agonists induced glutamate release were surprising. Because both increases in [Ca\(^{2+}\)], and the activation of \(\alpha_1\)-AdRs induce the synthesis and release of arachidonic acid, as well as increasing the production of prostaglandin \(E_2\) in different types of cells, it could be expected that adrenergic stimulation would induce glutamate release in a similar way to activation of mGluRs. With respect to the diverse properties of the observed PE- and DHPG-induced calcium responses, it is possible that the processes underlying the release of glutamate will require specific biochemical reactions encoded by the properties of the calcium signal. It is also possible that the compartmentalization of calcium signals or the distribution of release sites activated by \(\alpha_1\)-AdRs could be different from the signals activated by mGluRs. This might indicate that some degree of specificity in the Ca\(^{2+}\)
signal is required to trigger the release and could reflect a fundamental principle in the specificity of receptor mediated subcellular processes.

Regarding the α₂-adrenergic suppression of the DHPG-mediated glutamate release, an obvious hypothesis would be that the inhibition of the oscillatory calcium response was sufficient to inhibit the release of glutamate. Consistent with such a hypothesis are the results presented by Parpura and Hayden (61) demonstrating a strict calcium dependence of the astroglial glutamate release. However, interactions between second messenger-mediated production and release of arachidonic acid (59), and changes in cAMP production (15) could provide an additional pathway for interactions between the receptor systems. Provided that a direct effect on PKC and the absence of calcium transients would not be enough to inhibit the release, this may help explain the mechanisms behind the inhibited glutamate release.

Is There a Biological Relevance of Astrocytic Calcium Signaling?—Several suggestions about the physiological importance of astroglial calcium signaling have emerged, but the biological relevance of [Ca²⁺]oscillations in astrocytes remains to be revealed. Their appearance as a response to receptor activation and the proposed plasticity of astroglial calcium signaling (50) demonstrates a dynamic and diversified control of astrocytic calcium homeostasis that can be achieved as a consequence of a diversified neuronal activity. Enhanced neuronal firing leading to repetitive exposure to glutamate induces long lasting changes of the calcium oscillatory frequencies in the astrocytes, which further support previous theories that the code mediated changes of the calcium oscillatory frequencies in the astrocytes, which further support previous theories that the code mediated changes of the calcium oscillatory frequencies in the astrocytes, which further support previous theories that the code mediated changes of the calcium oscillatory frequencies in the astrocytes, which further support previous theories that the code mediated changes of the calcium oscillatory frequencies in the astrocytes, which further support previous theories that the code mediated changes of the calcium oscillatory frequencies in the astrocytes, which further support previous theories that the code mediated changes of the calcium oscillatory frequencies in the astrocytes, which further support previous theories that the code mediated changes of the calcium oscillatory frequencies in the astrocytes, which further support previous theories that the code mediated changes of the calcium oscillatory frequencies in the astrocytes, which further support previous theories that...
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