Physiological Activation of Presynaptic Metabotropic Glutamate Receptors Increases Intracellular Calcium and Glutamate Release

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Schwartz, Neil E. and Simon Alford. Physiological activation of presynaptic metabotropic glutamate receptors increases intracellular calcium and glutamate release. J Neurophysiol 84: 415–427, 2000. Activation of metabotropic glutamate receptors (mGluRs) has diverse effects on the functioning of vertebrate synapses. The cellular mechanisms that underlie these changes, however, are largely unknown. The role of presynaptic mGluRs in modulating Ca2+ dynamics and regulating neurotransmitter release was investigated at the vestibulospinal-reticulospinal (VS-RS) synapse in the lamprey brain stem. Application of the specific Group I mGluRs antagonist 7-(hydroxyimino)cyclopenta[b]chromene-1a-carboxylate ethyl ester (CPCCOEt) reduced the amplitude of consecutive high-frequency evoked excitatory postsynaptic currents (EPSCs). A series of experiments using techniques of electrophysiology and calcium imaging were carried out to determine the cellular mechanisms by which this phenomenon occurs. Concentration-dependent increases in the pre- and postsynaptic [Ca2+]i were seen with the application of mGluR agonists. Similarly, high-frequency stimulation of axons caused a Group I mGluR-dependent enhancement in presynaptic Ca2+ transients. Application of mGluR agonist caused a depolarization of the presynaptic elements, while thapsigargin decreased the high-frequency stimulus- and agonist-induced rises in [Ca2+]i. These data suggest that both membrane depolarization and the release of Ca2+ from intracellular stores potentially play a role in mGluR-induced Ca2+ signaling. To determine the effect of this modulation of Ca2+ dynamics on spontaneous glutamate release, miniature EPSCs were recorded from postsynaptic reticulospinal neurons. A potent Group I mGluR agonist, (S)-homosquissalic acid, caused a large increase in the frequency of events. These results demonstrate the presence of presynaptic Group I mGluRs at the VS-RS synapse. Activation of these receptors leads to a rise in [Ca2+]i, and enhances the spontaneous and evoked release of glutamate. Taken together, these studies highlight the importance of synaptic activation of these facilitatory autoreceptors in both short-term plasticity and synaptic transmission.

INTRODUCTION

Synaptic strength may be altered by receptor activation at both pre- and postsynaptic elements. Clearly modulation of neurotransmitter release is an important mechanism for altering synaptic efficacy. The small size of the mammalian terminal, however, has made the direct study of presynaptic cellular mechanisms difficult in intact preparations. Modulation of release has implications for the rapid fluctuations in neuronal output that occur with short-term plasticity (Zucker 1989) as well as for adaptive changes that take place over longer time scales (Bliss and Collingridge 1993). Experiments at the vertebrate and invertebrate neuromuscular junction have shown that Ca2+ entry from prior action potentials can affect the amount of neurotransmitter released with subsequent spikes (Kamiya and Zucker 1994; Katz and Miledi 1968). Less is known, however, about the effect that receptor-mediated processes (e.g., autoreceptors) have on Ca2+ dynamics and transmitter release particularly at central glutamatergic synapses. Until recently, autoreceptors were thought to function in only a negative feedback capacity; it now seems clear, however, that various presynaptic receptors are able to facilitate transmitter release as well (Cochilla and Alford 1998, 1999; Langer 1997; Liou et al. 1996; Miller 1998). Synaptosomal studies have demonstrated that activation of metabotropic glutamate receptors (mGluRs) can lead to an enhancement of transmitter exocytosis (Herrero et al. 1998).

The amino acid glutamate is the neurotransmitter at the majority of excitatory synapses in the vertebrate CNS (Mayer and Westbrook 1987). Glutamate mediates fast synaptic transmission by binding to ionotropic receptors of both N-methyl-D-aspartic acid (NMDA) and non-NMDA subtypes (Collingridge and Lester 1989). In addition, glutamate acts on metabotropic glutamate receptors that are linked to various intracellular effector systems. At least eight distinct mGluR subtypes have been characterized to date (Pin and Duvoisin 1995; Schoepp and Conn 1993); these have been separated into three groups based on sequence homology, transduction mechanisms, and to some extent, their pharmacological profiles. Activation of Group I mGluRs (mGluR1 and mGluR5) results in the production of inositol 1,4,5-trisphosphate (InsP3), which, by binding to its receptor on the endoplasmic reticulum, causes the liberation of Ca2+ from internal stores (Anwyl 1995; Murphy and Miller 1988). This mechanism has been demonstrated to play a physiological role at the terminals of giant axons in the lamprey spinal cord (Cochilla and Alford 1998).

Lamprey reticulospinal neurons of the posterior and medial rhombencephalic reticular nuclei (PRRN and MRRN, respectively) provide descending excitatory inputs to the spinal cord (Buchanan et al. 1987; Ohta and Grillner 1989). Vestibulospinal axons from two vestibular nuclei, the nucleus octavomotor intermediate (nOMI) and posterior (nOMP), make en
passant synapses onto the dendrites of reticulospinal neurons and contain a chain of presynaptic elements along their length (Rovainen 1979; Shupliakov et al. 1992). The vestibulospinal-reticulospinal (VS-RS) synapse is glutamatergic, and the fast synaptic response comprises AMPA, NMDA, and electrical components (Alford and Dubuc 1993). The unmyelinated axons of the vertebrate lamprey are large, and the tissue is optically clear, providing experimental access to presynaptic elements for both electrophysiological recordings and imaging experiments with Ca$^{2+}$-sensitive dyes. Here we demonstrate the presence, properties, and likely mechanisms used by presynaptic Group I mGluRs in regulating Ca$^{2+}$ dynamics and glutamate release. The physiological activation of these receptors has implications for both short- and long-term plasticity.

Parts of this paper have been published in abstract form (Schwartz and Alford 1997).

**Methods**

*In vitro lamprey brain stem preparation*

Experiments were performed on the isolated brains of larval (amnocoete) lampreys, *Petromyzon marinus*, in accordance with all local and national guidelines. The animals were anesthetized with 3-amino-benzoic acid ethyl ester methanesulfonate salt (MS-222; 100 mg/l; Sigma Chemical, St. Louis, MO), decapitated and dissected in a cold saline solution (Ringer) of the following composition (in mM): 100.0 NaCl, 26.0 NaHCO$_3$, 4.0 glucose, 2.6 CaCl$_2$, 2.1 KCl, 1.8 MgCl$_2$, bubbled with 95% O$_2$–5% CO$_2$ to a pH of 7.4 (modified from Wickelgren 1977). The skin, musculature, braincase, and choroid plexus were removed; the obex was opened and the optic tectum/cerebellum cut, fully exposing the basal and alar plates of the fourth ventricle. For experiments that required imaging, the tissue was placed dorsal surface upward in a cooled ~500-μl chamber with a glass coverslip floor that was inserted onto the stage of an inverted Nikon Diaphot microscope. For experiments that required only electrophysiology, the tissue was pinned dorsal surface upward in a cooled ~1-ml recording chamber with a silicone elastomer (Sylgard) floor. In both cases, the preparations were continuously superfused with cold oxygenated Ringer solution (8–10°C) or solutions of pharmacological agents bath-applied at a rate of approximately 1 ml/min.

**Microfluorimetry**

Dye filling of the brain stem was achieved by the application of the dextran-amine-conjugates of Ca$^{2+}$-sensitive dyes (McClellan et al. 1994) [3.000 or 10.000 MΩ Calcium Green-1 or 10,000 MΩ Oregon Green 488 BAPTA-1; 5 mM; Molecular Probes, Eugene, OR]. Segregation of vestibulospinal and reticulospinal tracts in the spinal cord enabled the selective labeling of either presynaptic axons or postsynaptic somata and dendrites. This technique was used to avoid contamination of presynaptic Ca$^{2+}$ transients by postsynaptic Ca$^{2+}$ signals and vice versa. The details of this have been previously described (Schwartz and Alford 1998). Preparations were superfused with cold oxygenated Ringer solution throughout the 16- to 22-h labeling period.

Imaging was accomplished with a confocal laser scanning microscope (Bio-Rad MRC-600, Bio-Rad, Hercules, CA) using the 488-nm line of an argon ion laser as the excitor source. A fluorescein filter set was used with a λ<20 (0.75 NA) objective. For experiments in which agonists were washed into the bath on retrogradely filled preparations, images were acquired once per 15 s (at 0.067 Hz) for control periods, once per 30 s (at 0.033 Hz) for washout periods, and once per 5 s (at 0.2 Hz) during the drug application. For similar experiments on single patch-clamped cells, images were acquired at 0.067 Hz during control and 0.2 Hz during drug application and washout. For those studies that explored the modulation of synaptically activated Ca$^{2+}$ transients,

image of axons and/or dendrites was carried out with a series of 16 frames (at 2 Hz) acquired before, during, and after a stimulus. Extracellular stimulations (0.5–20.0 μA) were given with tungsten electrodes (impedance = 0.5–2.0 MΩ) activated via a stimulus isolation unit. Three-dimensional reconstruction of image slices from multiple z planes (at 1-μm intervals) was performed with a Silicon Graphics Indigo 2 workstation using VoxelView software (Vital Images, Minneapolis, MN).

Image analysis was performed on a Macintosh computer using NIH Image software. Identifiable axons or dendrites were selected and an automated search macro was used to analyze the identically sized region in all image frames of the trial; the macro optimized the mean intensity within the defined region of interest within 11 pixels of the initial location in either (x-y) dimension. For experiments with tetanic stimuli, increases in fluorescence intensity above background (ΔF) were normalized to the first three or four frames (prestimulus), giving a baseline value of ΔF/ΔF = 1.00. For time-series experiments, responses were normalized to the control period (i.e., pixel intensity before the addition of drug). All levels of significance are from Student’s t-tests expressed as a probability, P, that the results are from the same population. Errors are expressed as standard errors of the mean (SE), which were normalized to the control (predrug) response for pharmacology experiments.

**Electrophysiology**

Whole cell patch-clamp recordings were made from reticulospinal neurons and vestibulospinal axons using a modified “blind” technique (Alford and Dubuc 1993). An intracellular solution of the following composition was used (in mM): 102.5 potassium gluconate or methanesulphonate, 5.0 HEpes, 3.0 ATP, 1.0 GTP, 1.0 MgCl$_2$, 1.0 NaCl, and 0.05–5.0 EGTA (osmolarity = 230 mosmol; pH = 7.20). For some experiments, cesium fluoride was substituted for potassium gluconate or methanesulphonate or 1–10 mM guanosine-5’-O-(2-thiodiphosphate) (GDP-β-S) was substituted for GTP. For such experiments, the recording was not started until whole cell access was maintained for at least 20 min, ensuring adequate time to disable G-protein-mediated signal transduction systems. Pipettes were pulled from thick-walled borosilicate glass and had an open-tip resistance of 5–10 MΩ. Most axons were recorded within the midline cleft just rostral to the PRRN, where there is a dense plexus of crossing fibers from the nOMP just under the epithelial surface (see Fig. 5). All cells were held at ~70 mV. Extracellular stimulations (0.5–20.0 μA) were achieved with tungsten electrodes (impedance = 0.5–2.0 MΩ) activated via a stimulus isolation unit and controlled with a WPI PulseMaster A300 stimulator (World Precision Instruments, Sarasota, FL).

Electrophysiological recordings of spontaneous miniature excitatory postsynaptic currents (mEPSCs) were digitized at 5 kHz and low-pass filtered at 1 kHz. This level of filtering did not affect the peak amplitude of the events. The average root mean squared (RMS) noise (taken from 33 randomly chosen event-free epochs culled from all the cells used in the mEPSC analysis) was 0.72 pA. Frequency and amplitude analysis of mEPSCs was done with a Macintosh computer using Igor Pro software. A macro was written that automated the detection of mEPSCs, returning the time of occurrence and the amplitude. Raw data traces were smoothed with a 21-point (±2 ms) box filter, differentiated, and smoothed again with the same filter. This technique corrected for any DC shift in baseline. A threshold level was manually determined for the differentiated control (predrug) data set, and this threshold was maintained for all files taken from a given cell. All events that crossed the threshold were detected and local minima (maximum inward current) were searched for within a window of 2 ms before and 10 ms after the detected event. This data set was mapped back onto a smoothed (5-point box filter) set of the raw data for the visual comparison of detected events with mEPSCs. Cumulative and raw histograms were constructed, and a two-population Kolmogorov-Smirnov goodness-of-fit test reduced to a modified...
\( \chi^2 \) variable (Hays 1988) was used to determine statistical significance between control and drug conditions. Evoked EPSCs were analyzed with pClamp software (Clampfit). Only the fast chemical (monosynaptic non-NMDA-mediated) component was of interest, and cursor placement during the analysis was chosen to reflect this bias. The amplitude of the current at a given time (3–8 ms) after the start of the rising edge of the response (inward current) was measured for each of four responses. The same time point was chosen for all responses recorded from a given cell.

**Combined microfluorimetry and electrophysiology**

Calcium signals in reticulospinal dendrites were monitored with high-affinity Ca\(^{2+}\)-sensitive dyes introduced into the postsynaptic cell via the patch electrode. An internal pipette perfusion system was used to inject Ca\(^{2+}\)-sensitive dye after obtaining whole cell access, thus preventing dye leakage into the calcium-rich extracellular space (Alford et al. 1993). For these experiments 3 \( \mu \)l of 50 \( \mu \)M Oregon Green 488 BAPTA-1 hexapotassium salt was injected into the pipette tip with a 10-\( \mu \)l syringe after breaking into the cell. A complete dye-fill of reticulospinal neurons out to the terminal dendrites was usually obtained within 20 min of stable whole cell access. Electrophysiological recordings were made in the whole-cell patch clamp configuration under voltage clamp as described in the following text.

**Drugs**

All drugs were bath-applied in Ringer solution at a superfusion rate of ~1 ml/min. The compounds 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 2-amino-5-phosphonopentanoic acid (AP5), 7-(hydroxyimino) cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt), L-quinoisquamic acid (QA), (S)-homosquinsquamic acid (HQA), (1S,3R)-1-amino-ciclopentane-1,3-dicarboxylic acid [(1S,3R)-ACPD], and (2S,1’S,2’S)-2-(carboxycyclopropyl)glycine (L-CCG-I) were obtained from Tocris Cookson (Ballwin, MO). Tetrodotoxin (TTX), GDP-\( \beta \)-S, and bicuculline methobromide were obtained from Sigma Chemical. Hexamethonium dichloride was obtained from Research Biochemicals International (Natick, MA). Thapsigargin was obtained from Alomone Labs (Jerusalem, Israel).

**RESULTS**

**Effect of Group I mGluR antagonism on the evoked release of neurotransmitter**

The synaptic activation of presynaptic Group I mGluRs leads to an enhancement of glutamate release with repetitive stimulation. This phenomenon is most easily demonstrated by the use of the specific Group I mGluR antagonist CPCCOEt (Annoura et al. 1996). The postsynaptic elements of the VS-RR synapse (reticulospinal neurons of the PRRN) were voltage clamped under whole cell patch-clamp conditions while presynaptic vestibulospinal axons were stimulated extracellularly. Monosynaptic glutamate-mediated EPSCs were recorded. The patch solution contained GDP-\( \beta \)-S (1–2 mM) to prevent the activation of postsynaptic mGluRs; we demonstrate that the inclusion of this GDP analogue effectively eliminates transduction through postsynaptic G proteins (Fig. 7). Three stimuli were applied to the presynaptic axons at an inter-pulse interval of 20 ms (i.e., 50 Hz). The average amplitudes of the third EPSCs were smaller than those of the first. [i.e., mean amplitude ratio (1st EPSC/3rd EPSC) = 1.65 ± 0.10; \( n = 5 \); Fig. 1]. When CPCCOEt (500 \( \mu \)M) was added to the superfusate, the amplitude ratio was significantly enhanced (to 2.39 ± 0.16; \( P < 0.001; n = 5 \); Fig. 1), reflecting a reduction in the relative amplitude of the third EPSC to the first EPSC. There was not a significant effect on the amplitude of the first response with the addition of CPCCOEt. It has been demonstrated in the lamprey spinal cord that this compound does not alter the amplitude of single-fiber-evoked EPSCs (Cochilla and Alford 1998).

**Pre- and postsynaptic calcium responses to the application of mGluR agonists**

We wished to demonstrate that Group I mGluRs are present on presynaptic elements at this synapse. We show that activation of mGluRs by exogenous agonists at the VS-RR synapse raises the postsynaptic Ca\(^{2+}\) concentration. Vestibulospinal axons and reticulospinal dendrites and somata were retrogradely labeled in isolation (see METHODS) with high-affinity Ca\(^{2+}\)-sensitive dyes. Vestibulospinal axons are easily identified as linear processes coursing toward the spinal cord from the ipsilateral nOMI (Fig. 2A); reticulospinal dendrites are either

![Image](http://jn.physiology.org/ Downloaded from by 10.220.33.6 on November 7, 2017)
punctate or short linear structures found at the level of the PRRN and aligned perpendicularly to the axons in confocal sections (Fig. 2C) (Schwartz and Alford 1998).

To test for the presence of presynaptic mGluRs capable of altering \([Ca^{2+}]_i\), various mGluR agonists were applied to preparations with dye-filled vestibulospinal axons. Figure 2A shows an example of such an experiment in which the potent nonselective mGluR agonist l-quisqualic acid (QA; 25 \(\mu\)M) was added to the bathing medium. As QA is also an agonist at AMPA and kainate-sensitive ionotropic glutamate receptors, CNQX (10 \(\mu\)M) was included in the superfusate; this concentration of CNQX has been shown to effectively block AMPA receptor-mediated transmission in the lamprey brain stem (Alford and Dubuc 1993). Additionally it has been demonstrated that agonist application of neither AMPA (Schwartz and Alford 1998) nor kainate (Cochilla and Alford 1997, 1999) causes a rise in presynaptic \([Ca^{2+}]_i\). Furthermore TTX (1 \(\mu\)M) was applied during drug applications to prevent synaptic transmission and functionally isolate the individual neuronal processes. The \(Ca^{2+}\) rise began immediately on superfusion; declines were recorded with washout on a slower time-scale (Fig. 2B). The variability in the time that the tissue was exposed to agonist (approximately 8 min), the precise superfusion rate, the fluid level in the recording chamber, and the depth within the tissue of the scanned region of interest all preclude the extraction of meaningful kinetic parameters following agonist application. As such, only peak amplitudes, which usually occurred at the end of agonist wash-in or within 1 min of washout, are reported here. QA (1–100 \(\mu\)M) was applied to 40 preparations, and a total of 88 axons were analyzed. For most experiments (\(n = 72\) axons), 25 \(\mu\)M QA was used. This concentration resulted in a substantial increases in \([Ca^{2+}]_i\) (mean peak \(\Delta F/F = 2.23 \pm 0.16\)). The Group I mGluR agonist HQA (Porter et al. 1992) (10–100 \(\mu\)M) [in the presence of CNQX (10 \(\mu\)M)] also resulted in an increase in axonal fluorescence (\(n = 9\)). Twenty-five micromolar HQA was used for the majority of experiments (\(n = 5\)) and gave a mean peak \(\Delta F/F\) of 2.45 \(\pm 0.66\). Likewise application of 1S,3R-ACPD (5–10 \(\mu\)M; \(n = 3\))
caused a presynaptic calcium transient (mean peak ΔF/F of 2.48 ± 1.01). The Group II mGluR agonist L-CCG-I (50–100 μM) did not cause a detectable increase in presynaptic [Ca2+]i (n = 2). We conclude that Group I mGluRs are present on the vestibulospinal axons and that their activation leads to a rise in presynaptic [Ca2+]i.

Clearly postsynaptic activation of mGluRs could modulate the fast synaptic response. To test for the presence of such mGluRs that are capable of altering Ca2+ levels, reticulospinal dendrites of the PRRN were studied in the same manner as in the preceding text. These protocols provided similar results. Calcium transients were recorded in the postsynaptic compartment of retrogradely labeled neurons in response to mGluR activation. QA (5–250 μM) was applied to 18 preparations, and analysis was performed on 32 dendritic processes. A mean peak ΔF/F of 3.17 ± 0.24 (n = 26) was measured in reticulospinal dendrites during wash-in of 25 μM QA. The application of HQA (25–100 μM; n = 22) also resulted in large increases in the postsynaptic Ca2+ signal (mean peak ΔF/F of 1.88–3.29). Figure 2C shows an example of dendritic calcium transients in response to 50 μM HQA in CNQX (10 μM) and TTX (1 μM).

Concentration-response relationships for QA (in 10 μM CNQX and 1 μM TTX) were determined for both the axonal (n = 7) and dendritic (n = 3) calcium responses. Sequential series of drug applications (1–250 μM) were applied to retrogradely labeled preparations, as described in the preceding text. Figure 3 shows examples of concentration-response relationships for a vestibulospinal axon (Fig. 3A) and reticulospinal dendrites (Fig. 3B). A reversible concentration-dependent increase in [Ca2+]i was seen on both sides of the synapse in response to mGluR activation.

Effect of metabotropic glutamate receptor antagonists on tetanus-induced calcium transients

It has previously been shown that repetitive stimulation of vestibulospinal axons leads to a large rise in [Ca2+]i in both pre- and postsynaptic elements of the VS-RS synapse, a signal that is due in part to the activation of ionotropic glutamate receptors (Schwartz and Alford 1998). To test if the activation of mGluRs by the physiological release of glutamate might be responsible for a component of the presynaptically-evoked Ca2+ signal, imaging experiments were performed with selectively-labeled vestibulospinal axons filled with high-affinity Ca2+-sensitive dyes (Schwartz and Alford 1998). Repetitive stimulation (50 Hz for 1 s; 1.0 ms/stimulus) applied to axons of the ipsilateral nOMI resulted in large prolonged increases in [Ca2+]i, both pre- and postsynaptically. The “peak” response during the tetanus (which occurred at its termination) and the “tail” response at the end of the trial were used for analysis. The presynaptic tail response represents a measure of the Ca2+ signal after termination of the stimulus that will not be dominated by the large transients that result from presynaptic action potentials (i.e., Ca2+ flux through voltage-operated channels). To eliminate the ionotropic glutamatergic component of the presynaptically evoked Ca2+ signal, we obtained control responses in the presence of CNQX (10 μM) and AP5 (100 μM). Application of CPCCOEt (500 μM) in the presence of the ionotropic glutamate receptor antagonists caused a significant reversible reduction in the peak and tail amplitudes of the evoked presynaptic Ca2+ transient [decreased by 25.7% (P < 0.01) and 31.9% (P < 0.01) from the previous response in CNQX/AP5, respectively; n = 20; Fig. 4]. These findings indicate that a component of the Ca2+ signal recorded at the presynaptic element during repetitive stimulation results from the activation of presynaptic Group I mGluRs.

Activation of presynaptic mGluRs leads to a depolarization of the presynaptic element

Metabotropic glutamate receptor-mediated rises in presynaptic [Ca2+]i, could be caused by either the depolarization of the presynaptic element and subsequent Ca2+ influx through voltage-operated Ca2+ channels (VOCCs) or via the release of Ca2+ from intracellular stores. Electrophysiological recordings were made from vestibulospinal presynaptic elements using both intracellular and patch electrodes. These axons form a dense plexus as they cross the midline just under the epithelium of the fourth ventricle (Fig. 5B). A stimulating electrode was placed high on the alar plate in the region of the nOMP to identify putative axons as vestibulospinal; those axons that responded to low intensity (less than ~3.0 μA) stimulation with an immediate (<1.0 ms) action potential were deemed vestibulospinal. As some preparations were also retrogradely labeled with Ca2+-sensitive dyes, it was possible to target VS axons visually using their confocal image. Experiments were performed in cur-
Activation of presynaptic mGluRs causes a release of Ca\(^{2+}\) from intracellular stores

Although bath-application of QA leads to the depolarization of the presynaptic element, the coupling of Group I mGluRs in mammalian systems to phosphoinositide metabolism (Pin and Duvoisin 1995) suggests that InsP\(_3\)-sensitive internal stores may play a role in the Group I mGluR-induced Ca\(^{2+}\) signal. Bath-application of the endoplasmic reticulum Ca\(^{2+}\)-ATPase inhibitor thapsigargin (Jackson et al. 1988) was utilized to test this point directly. The wash-in of thapsigargin caused a small fast transient rise in intracellular Ca\(^{2+}\) (in 5 of 7 axons; peak \(\Delta F/F = 1.57 \pm 0.19\)). Experiments were performed to determine the role of these stores in the agonist (QA)-induced Ca\(^{2+}\) transient. Dye-labeled VS axons were challenged with 25 \(\mu\)M QA (in 10 \(\mu\)M CNQX and 1 \(\mu\)M TTX) before and after thapsigargin (10 \(\mu\)M) was added to the tissue (e.g., Fig. 6A.). Incubation with thapsigargin caused a 36.5% reduction in the mean peak Ca\(^{2+}\) response to QA. This was not, however significant reflecting the large variability in response amplitude to these agonist applications (from \(\Delta F/F = 2.39 \pm 0.43\) to 1.88 \(\pm\) 0.28; \(n = 6\); \(P < 0.10\); Fig. 6Aii.).

A more consistent mechanism to activate presynaptic Ca\(^{2+}\) signals that are significantly reduced by blockade of group I mGluRs is repetitive stimulation of the axons. It is also of interest to know if a Ca\(^{2+}\) release from internal stores can be synaptically activated during repetitive stimulation of the presynaptic element. Experiments were performed in an analogous manner to the mGluR antagonist experiments. CNQX (10 \(\mu\)M) and AP5 (100 \(\mu\)M) were included in the superfusate to eliminate that portion of the presynaptic Ca\(^{2+}\) transient that is due to the activation of ionotropic glutamate receptors. Addition of thapsigargin (10 \(\mu\)M) significantly reduced the tetanically induced peak presynaptic Ca\(^{2+}\) signal from \(\Delta F/F = 2.57 \pm 0.21\) to 2.13 \(\pm\) 0.25 (a decrease of 36.5%; \(n = 6\); \(P < 0.05\)). Likewise, the tail of the Ca\(^{2+}\) transient was decreased from \(\Delta F/F = 1.32 \pm 0.05\) to 1.22 \(\pm\) 0.03 (a 32.8% reduction; \(n = 6\); \(P < 0.05\); Fig. 6C).

We have demonstrated the presence of Group I mGluRs on both presynaptic vestibulospinal axons and postsynaptic reticulospinal dendrites. Activation of these receptors by either exogenous agonist or endogenous glutamate release leads to a rise in [Ca\(^{2+}\)]\(_i\), on both sides of the synapse. Presynaptically, this Ca\(^{2+}\) transient may be the result of more than one process, as activation of mGluRs leads to both a depolarization of the presynaptic element as well as a thapsigargin-sensitive Ca\(^{2+}\) signal. In the following sections, we will investigate what effect activation of these presynaptic Group I mGluRs has on synaptic transmission, namely the release of glutamate.

Blockade of postsynaptic G proteins inhibits the mGluR-induced rise in dendritic calcium

In the following electrophysiological experiments, we use postsynaptic reticulospinal neurons as detectors of glutamate release from vestibulospinal axons. It was demonstrated above that these cells contain Group I mGluRs, and so it was necessary to eliminate the effect of their activation to study mGluR-mediated presynaptic mechanisms in isolation. As mGluRs mediate their signal via heterotrimeric G proteins, the selective inactivation of
these proteins in the reticulospinal cells should isolate the effect of mGluR activation to the presynaptic element of the synapse. Control experiments were carried out to demonstrate the feasibility of such a protocol. Simultaneous electrophysiology and microfluorimetry was used to visualize the dendritic Ca\textsuperscript{2+} signal while monitoring the postsynaptic current in reticulospinal neurons during the application of mGluR agonist. Further, this technique allows for the selective loading of GDP-β-S into these cells.

Reticulospinal neurons were voltage clamped under whole-cell patch-clamp conditions; the patch solution (and the injected dye

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**FIG. 5.** Quisqualic acid depolarizes presynaptic vestibulospinal (VS) axons. Fibers from the nucleus octomotorii posterior (nOMP) vestibular nucleus cross the midline between the medial rhombencephalic reticular nucleus (MRRN) and PRRN reticulospinal cells before they descend into the spinal cord. These VS axons make excitatory synapses onto the proximal dendrites of the PRRN cells. A: a diagram showing a patch electrode recording from the VS axons in the midline cleft between the halves of the brain stem. A stimulating electrode (not shown) was normally placed within the nOMP to confirm that the recorded fibers were vestibulospinal. B: a reconstruction of a retrograde fill with dextran-conjugated fluorescein of the midline (—) region of the brain stem where VS axonal recording were routinely made. The VS axons from the nOMP have a diameter of ~4–8 μm. C: an example electrophysiological trace taken from a whole cell patch-clamp recording of a VS axon. The 1st half of the trace starts with the wash-in of 25 μM QA (in 10 μM CNQX and 1 μM TTX); ↑, beginning of the washout. A peak depolarization (···) of ~20 mV occurred in this experiment.

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**FIG. 6.** A component of the synaptically activated presynaptic Ca\textsuperscript{2+} signal is due to the liberation of Ca\textsuperscript{2+} from inositol 1,4,5-trisphosphate (InsP\textsubscript{3})-sensitive stores. Vestibulospinal axons were retrogradely labeled with Ca\textsuperscript{2+}-sensitive dyes. QA (25 μM), in the presence of CNQX (10 μM) and TTX (1 μM), was bath-applied before and after the tissue was incubated in the Ca\textsuperscript{2+}-ATPase inhibitor thapsigargin (10 μM). A: example of a presynaptic QA-induced rise in [Ca\textsuperscript{2+}], before (control) and after the addition of thapsigargin. A: summary graph shows a 36.5% decrease in the mean peak response to QA after thapsigargin treatment (n = 6). Error bars are SE. B: tetanic stimulation (50 Hz for 1 s) was applied to vestibulospinal axons to assess the role of InsP\textsubscript{3}-sensitive internal stores in shaping the presynaptic Ca\textsuperscript{2+} signal. Tetani were given to VS axons before and after the tissue was bathed in 10 μM thapsigargin [with CNQX (10 μM) and AP5 (100 μM)]. B: summary graph (n = 6) showing the comparison of the tetanus (+)-induced Ca\textsuperscript{2+} signal in control (○) and thapsigargin (●) conditions. Error bars are SE normalized to the mean control response. B: summary bar graph (n = 6) representing the peak and tail Ca\textsuperscript{2+} signals in control and thapsigargin conditions. Error bars are SE; *, P < 0.05.
solution) contained GDP-β-S to inactivate postsynaptic G proteins. Figure 7A shows an example of a live dye-filled reticulospinal cell. Note that the cell body is just under the dorsal surface of the brain and that the extensive dendritic tree passes through its entire extent (200–300 μm). Twenty-five micromolar HQA (in the presence of 10 μM CNQX, 100 μM AP5, and 1 μM TTX) was bath-applied soon after obtaining whole cell access, while still allowing adequate time for dye filling. This protocol evoked a reversible increase in dendritic calcium (mean ΔF/F = 2.00 ± 0.23; n = 7; e.g., Fig. 7B), and this transient was accompanied by an inward current [mean current = 381.8 ± 82.9 pA (relative to baseline); n = 3]. When more time was allowed for the inactivation of G proteins, the same application of mGluR agonist resulted in a significantly smaller Ca²⁺ signal [mean ΔF/F = 1.39 ± 0.08 (with G proteins inactivated) Fig. 7C, left; n = 6; Fig. 7C, right]; the rise in Ca²⁺ was accompanied by an inward current [mean = 272.8 ± 159.5 pA (relative to baseline)]. These control experiments show that the inclusion of GDP-β-S in the postsynaptic cell is able to disrupt G-protein-mediated mGluR

FIG. 7. Postsynaptic G proteins are required for the mGluR-induced rise in dendritic calcium. Simultaneous whole cell patch-clamp electrophysiology and confocal Ca²⁺ imaging were performed on reticulospinal neurons. Patch pipettes were filled with 50 μM Oregon Green 488 BAPTA-1 hexapotassium salt and 10 mM GDP-β-S. Cells were voltage clamped at −70 mV. A: reconstruction from multiple z-plane images of a live MRRN cell filled with dye through the patch electrode (arrowhead). Note that the dendritic tree defines the boundaries of the brain. Midline is toward the right. Data for B–D is taken from the same reticulospinal neuron. B: calcium response in dendrites to the wash-in (●) of the Group I mGluR agonist HQA (25 μM; in 10 μM CNQX, 100 μM AP5 and 1 μM TTX) shortly after obtaining whole cell access. C: confocal images of Ca²⁺ transients in dye-filled dendrites. Sample control (i), drug (ii), and washout (iii) frames (each an average of 3) are shown for the application of HQA and N-methyl-D-aspartate (NMDA; 100 μM; in 10 μM CNQX and 1 μM TTX). The wash-ins were begun after 85 min of whole cell access. The midline is toward the right. The color look-up table is shown in Fig. 2. D: graphical representation of data in C showing sequential wash-ins of HQA (blue) and NMDA (red) as indicated by the ● (points i–iii corresponds to image frames shown in C). The steady background increase in the Ca²⁺ signal reflects an increase in dendritic dye concentration with time.
signaling, thereby allowing the postsynaptic reticulospinal cell to serve as a detector for neurotransmitter release in experiments that use bath applications of mGluR agonists.

Whole cell patch-clamp recording of spontaneous mEPSCs from reticulospinal neurons

To examine the effect of the activation of presynaptic Group I mGluRs on the spontaneous release of glutamate at the VS-RS synapse, spontaneous mEPSCs were recorded in reticulospinal neurons of the PRRN held at −70 mV under whole cell patch-clamp conditions \((n = 18)\). The electrode contained either 1–2 mM GDP-β-S or cesium fluoride (CsF; 102.5 mM) to effectively inactivate G proteins; thus signal transduction through postsynaptic mGluRs was prevented. An example of a typical experiment is shown in Fig. 8. Excitatory events of variable amplitude were readily seen under control conditions (data not shown). The application of TTX (1 μM) and strychnine (5 μM) decreased the frequency of, but did not eliminate, the EPSCs. The bath-application of a low concentration (5 μM) of the mGluR agonist HQA (in TTX and strychnine) resulted in a marked increase in the frequency of events (Fig. 8, A–C). There was no change in the holding current with the wash-in of HQA. The effect of the mGluR agonist was reversible. Addition of 10 μM CNQX to the superfusate (in TTX and strychnine) decreased the frequency of, but did not abolish, the mEPSCs. The events that remained were insensitive to AP5 (≤200 μM), bicuculline (5 μM), and hexamethonium (≤20 μM; data not shown).

Histograms of mEPSCs were constructed to quantify the
FIG. 9. A high concentration of mGluR agonist causes a large synaptically induced inward current. Whole cell patch-clamp recordings of reticulospinal PRRN cells (V_e = −70 mV) were made using an intracellular solution which contained 1 mM GDP-β-S. Whole cell access was maintained for ≥20 min prior to drug applications. A: plot of mean holding current recorded in a single PRRN cell. Each point represents the average of a 32.77-s period. At arrow, 1 μM TTX and 5 μM strychnine was added to the superfusate. At times indicated by the black bars (H), 25 μM HQA was added to the superfusate. At the time marked by the gray bar (CNQX), 10 μM CNQX was added to the superfusate. B: individual current traces taken from the points marked i–iii in A. The mean inward current (I_in) is indicated for each trace. Traces were smoothed with a box filter (9-point running average). The scale bars apply to all traces.

effect of HQA on the frequency and amplitude of spontaneous events. Application of 5 μM HQA (in TTX and strychnine) resulted in an increased number of events per time. Figure 8D shows an interval histogram for the example cell comparing the interval between mEPSCs for a total 8.192 min in both HQA and control conditions, 5,665 and 4,460 events, respectively. Of all 18 cells tested, the average interval decreased by 21.16% [from 109.72 ms (range 10.00 to −1457.86 ms) to 86.50 ms (range 10.10−1,187.66 ms)] when the Group I mGluR agonist was added to the preparation. Similarly each cell recorded showed a significant increase in mEPSC frequency. A cumulative interval histogram from an example cell (Fig. 8D, inset) shows a shift toward higher frequency events in the presence of drug (1,392 and 978 events in a period of 228 s in HQA and control conditions, respectively; P < 0.001). There was a small but significant decrease in the amplitudes of mEPSCs in the presence of 5 μM HQA. The average mEPSC amplitude decreased from −5.71 to −5.40 pA (a 5.43% reduction) when Group I mGluRs were activated.

The application of a higher dose of HQA (25 μM; in 1 μM TTX and 5 μM strychnine) resulted in a large inward current (I_in) recorded in reticulospinal cells that was insensitive to G-protein inactivation with 1 mM GDP-β-S loaded intracellularly. The mean peak I_in was 383.4 ± 81.2 pA (relative to baseline; n = 10 cells). This current was eliminated by the addition of CNQX (10 μM; n = 6/6 cells), and this effect was usually reversible (Fig. 9A; n = 3/4 cells). The inward current was accompanied by a large increase in noise (Fig. 9B_i). This increase in noise that occurs with the addition of 25 μM HQA is eliminated by the prior addition of 10 μM CNQX (Fig. 9B_m); however, some residual events remain.

We have demonstrated that Group I mGluRs are present both pre- and postsynaptically at the VS-RS synapse and that activation of these receptors, both endogenously and exogenously, leads to a rise in [Ca^{2+}]_i. Further, we have shown that the increase in presynaptic Ca^{2+} in response to either agonist or repetitive stimulation of these axons is due in part to the release of Ca^{2+} from intracellular storage organelles. The activation of these receptors with agonist leads to an enhancement in the frequency of mEPSCs. Taken together, these results suggest that a presynaptic rise in Ca^{2+} secondary to the activation of Group I mGluRs augments the release of glutamate. Indeed it is likely that the observation that CPCCOEt reduces the amplitude of subsequent EPSCs delivered at high-frequency (Fig. 1) results from the inhibition of this process.

DISCUSSION

Effects of mGluR activation on synaptic transmission are multiple

We have demonstrated that the synaptic activation of presynaptic Group I mGluRs leads to both an increase in intracellular Ca^{2+} and an enhancement of spontaneous and evoked transmitter release. The most parsimonious explanation for the effect of CPCCOEt on synaptic transmission is that it prevents a rise in [Ca^{2+}]_i, and that this in turn inhibits the enhancement in glutamate release that occurs when these presynaptic facilitatory autoreceptors are activated. In many preparations, mGluRs modulate both synaptic transmission and spontaneous neurotransmitter release. These effects on release, which have been variously reported as inhibitory (Baskys and Malenka 1991; Burke and Hablitz 1994; Dong et al. 1996; Krieger et al. 1996; Takahashi et al. 1996) and facilitatory (Budd and Nicholls 1995; Herrero et al. 1992; Zhang and Dorman 1993), could be due to a variety of mechanisms. The relationship between mGluR activation and Ca^{2+} dynamics shown here, however, makes the modulation of [Ca^{2+}]_i, in the presynaptic element a likely target for affecting release. Increased presynaptic Ca^{2+} entry subsequent to synaptic mGluR activation can result from either InsP_3-triggered liberation of Ca^{2+} from internal stores, direct mGluR-mediated modulation of VOCCs (Rothe et al. 1994; Stefani et al. 1996), uncoupling of a presynaptic inhibitory receptor (Budd and Nicholls 1995; Swartz et al. 1993), or some combination thereof. At the VS-RS synapse, activation of presynaptic mGluRs results in depolarization as well as the liberation of Ca^{2+} from intracellular storage organelles. These phenomena allow for a role of mGluRs in the short-term modulation of glutamate release.
Pre- and postsynaptic mGluR activation leads to an increase in intracellular Ca\(^{2+}\)

Bath-application of mGluR agonists evokes Ca\(^{2+}\) transients in pre- and postsynaptic elements of the VS-RS synapse. These signals are insensitive to the blockade of synaptic transmission by TTX. The most robust response was generated by the nonselective excitatory amino acid agonist QA (in the presence of CNQX); this agonist has been reported to be the most potent activator of Group I mGluRs (Anwyl 1995; Tanabe et al. 1992). The specific Group I mGluR agonist HQA (Porter et al. 1992) also produced a large Ca\(^{2+}\) transient both pre- and postsynaptically. Given the essential role of calcium in neurotransmitter exocytosis, the presence of prejunctional mGluRs that are capable of affecting [Ca\(^{2+}\)]\(_i\) is an important variable to consider for the modulation of synaptic transmission.

Activation of presynaptic mGluRs enhances spontaneous neurotransmitter release

Activation of presynaptic mGluRs affects transmitter release; however, little is understood of the cellular mechanisms by which this occurs, and there is marked variability depending on the particular synapse chosen for study. For example, Group I mGluRs have been shown to both inhibit Ca\(^{2+}\) channel gating and to open Ca\(^{2+}\) channels (Chavis et al. 1994; Lester and Jahr 1990). In the current study, we have provided evidence that suggests a causal relationship between the mGluR-dependent rise in presynaptic Ca\(^{2+}\) and an increase in glutamate release. Here the postsynaptic reticulospinal neurons were used as detectors of presynaptic activity. The frequency of mEPSCs is dependent on transmitter release probability (Fatt and Katz 1952; Redman 1990); this in turn, at the neuromuscular junction, has been demonstrated to be dependent on the [Ca\(^{2+}\)]\(_i\) in the presynaptic element. We have shown that the Group I mGluR agonist HQA significantly increases the frequency of mEPSCs. Although a change in the behavior of “silent synapses” could account for such a shift in mEPSC frequency (Isaac et al. 1995; Liao et al. 1995), the inactivation of postsynaptic G proteins in these experiments precludes this as a likely mechanism. The coincidence of this increase in mEPSC frequency with a rise in presynaptic [Ca\(^{2+}\)] makes the presynaptic Ca\(^{2+}\) signal an attractive candidate for this mechanism of action of mGluRs. The spontaneous activity was antagonized by the application of the non-NMDA receptor antagonist CNQX. There was, however, a component that was insensitive to CNQX, AP5, strychnine, bicuculline, and hexamethonium; the transmitter(s)/receptor(s) mediating these events remain to be determined.

Activation of presynaptic mGluRs by the exogenous application of HQA (5 µM) results in an increase in the spontaneous release of neurotransmitter. With a higher concentration (25 µM) of the same agonist, a large CNQX-sensitive inward current was recorded in the postsynaptic cell. We propose that this is due to a sizable release of glutamate from presynaptic vestibulospinal axons. Several lines of evidence converge on this conclusion. Firstly, postsynaptic G proteins were effectively inactivated by GDP-β-S or CsF; this was confirmed by the selective elimination of the HQA-mediated postsynaptic Ca\(^{2+}\) transient in these neurons. In hippocampal CA1 cells, 500 µM of GDP-β-S included in the patch solution eliminated the (1S,3R)-ACPD-induced calcium-activated nonspecific cat-ion current (Congar et al. 1997). Second, it is reasonable to assume that the increase in spontaneous release seen at low concentrations of HQA would be enhanced by higher concentrations. Indeed, Ca\(^{2+}\) transients of increasing magnitude were recorded with application of progressively higher concentrations of mGluR agonists. As a whole, this result suggests that activation of Group I mGluRs leads to the action potential-independent increase in glutamate release from the presynaptic element.

Sources of the presynaptic Group I mGluR-induced Ca\(^{2+}\) transient are several

Activation of presynaptic Group I mGluRs results in a large increase in intracellular Ca\(^{2+}\) at the VS-RS synapse. The Ca\(^{2+}\) signal can potentially arise from one or more of several different sources. First, phosphoinositide hydrolysis secondary to phospholipase C (PLC) activation results in an increase in the formation of InsP\(_3\); activation of InsP\(_3\) receptors mobilizes Ca\(^{2+}\) from distinct intracellular storage pools. Ca\(^{2+}\) released from intracellular stores may diffuse throughout the axon within 10 ms (Cochilla and Alford 1998, Schwartz and Alford 1998). Second, protein kinase C (PKC) is stimulated by diacylglycerol (DAG) (with or without Ca\(^{2+}\) as a cofactor) subsequent to PLC activation. The phosphorylation of ion channels by protein kinases is thought to play an important role in the regulation of various ionic conductances. At the neuromuscular junction in Drosophila, activation of presynaptic mGluRs has been shown to increase glutamate release via a cAMP-dependent mechanism (Zhang et al. 1999). In synaptosomes, it is known that activation of Group I mGluRs facilitates glutamate release by closing K\(^+\) channels secondary to PKC activation; this facilitation is enhanced by arachidonic acid and desensitizes rapidly as PKC acts through negative feedback on the mGluR itself (Coffey et al. 1994; Herrero et al. 1992). In this regard, the second potential source of Ca\(^{2+}\) is extracellular, given its entrance through VOCCs on membrane depolarization. Such a Group I mGluR-mediated presynaptic depolarization was seen in our studies; however, the precise mechanism that underlies it is currently unknown at this synapse.

Release of endogenous glutamate activates presynaptic mGluRs

The activation of presynaptic Group I mGluRs by the application of an exogenous agonist can evoke a Ca\(^{2+}\) transient in the presynaptic element, and this in turn alters the frequency of spontaneous release. We investigated the possibility that the evoked release of endogenous glutamate might alter presynaptic [Ca\(^{2+}\)] by acting on these receptors. A high-frequency input to vestibulospinal axons results in a long-lasting (>6.0 s) Ca\(^{2+}\) transient on both sides of the synapse that far outlasts the duration of the stimulus. The sources of the calcium are several. First, Ca\(^{2+}\) flux through VOCCs is expected to contribute significantly to the presynaptic signal during the stimulus. Second, NMDA and non-NMDA receptor-activation results in an increase in [Ca\(^{2+}\)] in both compartments of the synapse (Schwartz and Alford 1998). Activation of presynaptic NMDA receptors have been shown to alter glutamate release in both mammalian (Glitsch and Marty 1999) and lamprey (Cochilla...
and Alford 1998) preparations. Finally, axonal and dendritic mGluRs are synthaptically activated and appear to be capable of altering Ca\textsuperscript{2+} levels on either side of the synapse. Application of the Group I mGluR antagonist CPCCOEt reduces the amplitude of Ca\textsuperscript{2+} transients evoked by high-frequency stimulation of vestibulospinal axons. This result implies that physiologic release of glutamate can alter presynaptic [Ca\textsuperscript{2+}], by acting at these mGluRs. Further, this activation of presynaptic receptors and subsequent Ca\textsuperscript{2+} entry causes an enhancement of evoked EPSC amplitudes resulting from repetitive stimulation of the vestibulospinal terminal. Previously we have shown the recruitment of ionotropic glutamate receptor-mediated axo-axonic inputs from vestibulospinal axons onto other vestibulospinal axons during tetanic stimulus; activation of these receptors can alter the level of Ca\textsuperscript{2+} in the presynaptic element (Schwartz and Alford 1998). The current study has not distinguished between mGluR activation by glutamate that is released from axo-axonic synapses and that released by the axon that is being recorded from (i.e., autoreceptor activation of mGluRs). Metabotropic glutamate receptors serve a modular role and do not result in a fast electrophysiologic response like their ionotropic counterparts. These results highlight the importance of these receptor-mediated processes for synaptic transmission.

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