Glutamate Uptake Controls Expression of a Slow Postsynaptic Current Mediated by mGluRs in Cerebellar Purkinje Cells

W. REICHELT AND T. KNÖPFEL
Laboratory for Neuronal Circuit Dynamics, Brain Science Institute, RIKEN, Saitama 351-0198, Japan

Received 22 August 2001; accepted in final form 5 December 2001

Reichelt, W. and T. Knöpfel. Glutamate uptake controls expression of a slow postsynaptic current mediated by mGluRs in cerebellar Purkinje cells. J Neurophysiol 87: 1974–1980, 2002; 10.1152/jn.00704.2001. At the cerebellar parallel fiber-Purkinje cell synapse, isolated presynaptic activity induces fast excitatory postsynaptic currents via ionotropic glutamate receptors while repetitive, high-frequency, presynaptic activity can also induce a slow excitatory postsynaptic current that is mediated by metabotropic glutamate receptors (mGluR1-EPSC). Here we investigated the involvement of glutamate uptake in the expression of the mGluR1-EPSC. Inhibitors of glutamate uptake led to a large increase of the mGluR1-EPSC. d-aspartate (0.4 mM) and L-(−)-threo-3-hydroxyaspartate (0.4 mM) increased the mGluR1-EPSC ~4.5 and ~9-fold, respectively, while dihydrokainic acid (1 mM), had no significant effect on the mGluR1-EPSC. d-aspartate (0.4 mM) shifted the concentration-response curve of the depression of the mGluR1-EPSC by the low-affinity mGluR1 antagonist (S)-a-Methyl-4-carboxyphenylglycine [(S)-MCPG] to higher concentrations and decreased the stimulus intensity and the number of necessary stimuli to evoke an mGluR1-EPSC. Depression of the mGluR1-EPSC by rapid pressure application of (S)-MCPG at varying time intervals after tetanic stimulation of the parallel fibers indicated that the glutamate concentration in the peri- and extrasynaptic space decayed with time constants of 36 and 316 ms under control conditions and with inhibition of glutamate uptake, respectively. These results show that expression of the slow mGluR-mediated excitatory postsynaptic current is controlled by glutamate transporter activity. Thus in contrast to fast glutamatergic synaptic transmission, metabotropic glutamate receptor-mediated transmission is critically dependent on the activity and capacity of glutamate uptake.

INTRODUCTION

Chemical synaptic transmission involves diffusion of a transmitter from the presynaptic release site to the postsynaptic receptors. In the mammalian CNS, excitatory synaptic transmission is mainly mediated by the neurotransmitter glutamate via ionotropic and metabotropic glutamate receptors. During fast glutamatergic transmission, the concentration of glutamate in the synaptic cleft reaches millimolar levels and rapidly decays within milliseconds to concentrations below the binding constant of glutamate receptors (1–10 μM) (Bergles et al. 1999; Clements 1996). Ambient extracellular glutamate levels are maintained at low concentrations due to glutamate sequestered by glia cells and neurons, thus providing a steep gradient required for rapid clearance of diffusing glutamate from the synaptic cleft. Indeed, dilution by diffusion is sufficient to account for a rapid clearance of glutamate from the synaptic cleft, and only at some synapses are glutamate transporters thought to support fast clearance of glutamate (Barbour et al. 1994; Bergles et al. 1999; Clements 1996; Diamond and Jahr 1997; Eccles and Jeager 1958).

This scenario accounts well for the situation of single isolated synaptic events mediated by glutamate receptors localized in the synaptic cleft. Recent evidence suggests that the precise time course and spatial distribution of glutamate transients are of significant functional relevance during repetitive synaptic transmission and activation of extrasynaptic glutamate receptors. Trains of presynaptic activation can result in the diffusion of glutamate to neighboring synaptic specializations, a phenomenon that has been referred to as “spillover” (Barbour and Hauser 1997; Carter and Regehr 2000; Isaacson 2000; Mitchell and Silver 2000). At the parallel fiber-Purkinje cell synapse, synaptic trains of presynaptic activity are also required for inducing slow excitatory postsynaptic currents (EPSCs) mediated by metabotropic glutamate receptors (mGluRs) (Batchelor et al. 1994; Tempia et al. 1998). These mGluR-mediated EPSCs are mediated by the mGluR1 subtype (Batchelor et al. 1994, 1997; Tempia et al. 1998). It is not known why expression of the mGluR1-mediated EPSCs requires repetitive synaptic activity. One possibility is that the underlying intracellular signal transduction mechanism involves a threshold level of a yet unknown factor. Alternatively, in agreement with the peri- and extrasynaptic localization of mGluR1 in Purkinje cells (Martin et al. 1992; Mateos et al. 2000), one might speculate that the spillover or accumulation of glutamate is required for induction of the mGluR1-mediated EPSCs and, hence, glutamate uptake would regulate the expression of this potential.

The present experiments were designed to investigate the latter hypothesis. A preliminary report on this study appeared in abstract form (Reichelt and Knöpfel 1999).

METHODS

Sagittal slices, 150–200 μm thick, were cut from the cerebella of 18- to 36-day-old mice as described (Knöpfel et al. 2000). The slices were then transferred into artificial cerebrospinal fluid (ACSF) at 32°C for 20 min and finally stored at 20–24°C for 4–5 h. The ACSF was equilibrated with 95% O₂-5% CO₂ and contained (in mM) 118

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
NaCl, 3 KCl, 1 MgCl₂, 1 NaH₂PO₄, 2 CaCl₂, 25 NaHCO₃, and 10 glucose. For electrophysiological recordings, slices were placed in an immersion-type perfusion chamber (2–3 ml/min, 22–25°C) mounted on the stage of an upright microscope (Axioskope, Carl Zeiss, Göttingen, Germany) and visualized using a ×40 water-immersion lens. Whole cell currents were recorded from Purkinje cell somata. Pipettes (resistances 1–2 MΩ) were filled with a solution containing (in mM) 137 K-glucuronate, 10 HEPES, 0.5 EGTA, 4 ATP, and 0.5 GTP. The pH was adjusted to 7.3 by KOH. Holding potential was −60 mV throughout. The signals were filtered at 1 kHz and sampled at 10 kHz, except for the fast EPSC (Fig. 1A) and the climbing-fiber responses (Fig. 5B), which were filtered at 10 kHz and sampled at 100 kHz. Electrical stimulation was performed by passing current pulses (100 μs, 10–150 μA) through a sodalime glass capillary filled with ACSF (1 MΩ). For parallel fiber stimulation, the electrode tip was placed on the surface of the molecular layer above the distal dendrite of the patched Purkinje cell. Climbing-fiber stimulation was achieved by single current pulses applied to the granular cell layer; the location of the stimulation pipette was changed until an all-or-nothing climbing-fiber response was recorded.

In most experiments, substances were applied onto the slices by switching to extracellular bath solution between different reservoirs. In a series of experiments, (S)-a-Methyl-4-carboxyphenylglycine [(S)-MCPG] was pressure applied via a glass capillary pipette. The capillary (tip diameter of ~10 μm) was filled with ACSF containing 10 mM (S)-MCPG. The stream of fluid from the pressure capillary was directed toward an area close to the tip of the electrical stimulation electrode. Pressure was applied to the back of the capillary at a rate of 30–40 psi for a duration of 50–300 ms. These parameters were individually determined at the beginning of the current recording from each Purkinje cell by searching for a distinct effect and were held constant during recording from a Purkinje cell.

Synaptically induced currents were quantified in terms of their peak amplitude. Analysis of data and creation of graphics was performed using Origin (Microcal Software, Northampton, MA).

**FIG. 1.** The effect of the glutamate uptake blocker d-aspartate on ionotropic and mGluR1-excitatory postsynaptic currents (EPSCs) in a cerebellar Purkinje cell. A: ionotropic EPSC elicited by single stimuli delivered at different intensities. B: metabotropic EPSC elicited by 10 stimuli at different intensities in the presence of L-threo-3-hydroxysaspartate (NBQX). Note the different time scale in A and B. C: adding d-aspartate (0.4 mM) to the ACSF increases duration and amplitude of the metabotropic EPSC. Stimulus intensity was 20 μA. The effect of d-aspartate is completely reversible ~3 min of washout. Inset: responses scaled to the same amplitude. The average of 5 trials is shown in A; traces in B and C are not averaged. The numbers in A and B give the stimulus intensity in microampères. Arrowhead and square depict times of single and tetanic stimulation; stimulus artifacts have been blanked in this and in all following figures.

**RESULTS**

Cerebellar parallel fiber-to-Purkinje cell synapses mediate fast excitatory postsynaptic currents via AMPA receptors (AMPA-EPSC) as well as slow EPSCs via the metabotropic glutamate receptor mGluR1 (mGluR1-EPSC). Figure 1 illustrates the different dynamics of these responses and how they can be isolated from each other. A single electrical stimulus delivered to parallel fibers evoked a fast EPSC (Fig. 1A, refs) while a high-frequency train of stimuli (e.g., 5–10 stimuli at 100 Hz) evoked a slow mGluR1-EPSC (Fig. 1B) (Tempia et al. 1998). Because a single stimulus never evoked a significant mGluR1 response, isolated recording of the fast AMPA-EPSC was achieved by suppressing GABA A currents with 20 μM picrotoxin. To suppress the fast AMPA-EPSC during recordings of the slow mGluR1-EPSC, additional suppression of ionotropic glutamate receptors was necessary and achieved by using 20 μM NBQX and 50 μM d-APV.

In those experiments where no AMPA-EPSC was recorded, picrotoxin, NBQX, and d-APV were routinely added to the artificial cerebrospinal fluid (ACSF).

To investigate the dependence of the mGluR1-EPSC on glutamate uptake, we applied d-aspartate, an inhibitor of glutamate uptake by Bergmann glial cells (Clark and Barbour 1997). d-Aspartate (0.4 mM) led to a large increase of the current amplitude and duration of the mGluR1-EPSC, an effect that occurred in 100% of the cells tested (n = 88; Fig. 1C).

**Effect of different glutamate uptake blockers on the mGluR1-EPSC**

The d-aspartate-induced enhancement of the mGluR1-EPSC depended on the concentration of the uptake blocker. A dose-response relationship was measured and is shown in Fig. 2A. d-Aspartate at 0.4 mM increased the current to 445 ± 55% (mean ± SE, n = 6). This four- to fivefold increase is in
The preceding results suggest that inhibition of glutamate uptake caused an increased glutamate concentration at the level of the mGluR1 and, hence, an increased amplitude of the mGluR1-EPSC. To test this hypothesis, we used the competitive low-affinity glutamate antagonist (S)-MCPG (Hayashi et al. 1994) to probe glutamate concentration at the level of the receptor using the known potencies of glutamate and (S)-MCPG at mGluR1a (Hayashi et al. 1994). Assuming that receptor activity required binding of glutamate at two mGluR1a molecules (see METHODS), we estimated the effective glutamate concentration during the mGluR1-EPSC to be 30 and 141 μM under control conditions and in the presence of 0.4 mM d-aspartate, respectively.

Inhibition of glutamate uptake decreases the stimulus intensity to evoke a mGluR1-EPSC

The preceding data are consistent with the hypothesis that inhibition of the glutamate transporters increased the accumulated extracellular glutamate concentration on repetitive parallel fiber stimulation. Therefore we would expect that glutamate uptake blockers may lower the stimulation intensity necessary to evoke a mGluR1-EPSC. To test this hypothesis, the stimulation intensity necessary to evoke a discernable mGluR1-EPSC was increased in 5- to 10-μA steps until a response was discernible and then between 15 and 30 μA was added to achieve a stable and clear response. Consistent with our hypothesis, the (S)-MCPG concentration necessary to suppress the peak amplitude of the mGluR1-EPSC by 50% was higher when d-aspartate was present (Fig. 3). The concentration-response curve of the (S)-MCPG depression of the mGluR1-EPSC is shifted to higher concentrations by d-aspartate and the half-maximal inhibition increased from 138 ± 3 (SE) μM (n = 10) to 658 ± 81 μM (n = 5; Fig. 3). This shift of the concentration-response curve suggests that the glutamate concentration reaches higher values when glutamate uptake is blocked by d-aspartate. These data also permitted estimation of the local glutamate concentration at the site of the receptor using the known potencies of glutamate and (S)-MCPG at mGluR1a (Hayashi et al. 1994). Assuming that receptor activity required binding of glutamate at two mGluR1a molecules (see METHODS), we estimated the effective glutamate concentration during the mGluR1-EPSC to be 30 and 141 μM under control conditions and in the presence of 0.4 mM d-aspartate, respectively.

**FIG. 2.** A: dose-response curve of the d-aspartate induced enhancement of the mGluR1-EPSC. Data points are mean ± SE values. B: l(-)-threo-3-hydroxyaspartate (THA, 0.4 mM) strongly increased the mGluR1-EPSC; this effect was only partially reversed after wash with control solution. C: dihydrokainic acid (DHK, 1 mM) had no significant effect on the mGluR1-EPSC, whereas d-aspartate was effective in the same cell. Traces in B and C are averages of 3–6 trials.

**FIG. 3.** d-aspartate increases the K_i value of the competitive mGluR antagonist (S)-α-Methyl-4-carboxyphenylglycine [(S)-MCPG]. A: concentration-dependent inhibition of the mGluR1-EPSC in control artificial cerebrospinal fluid (ACSF) and in the presence of 0.4 mM d-aspartate. The numbers indicate the (S)-MCPG concentrations (nM). B: dose-response curves; ●, control ACSF; ○, ACSF containing 0.4 mM d-aspartate. Data points are means ± SE values. Lines represent fits to a function of the form y = (|A| * 41)/(|A|*41 + (4.1*41) + (4.1*(S)-MCPG)).

Agreement with the ~75% inhibition of transporter currents in cerebellar astroglia by 0.5 mM d-aspartate (Clark and Barbour 1997). The action of d-aspartate was rapid on wash in of the drug and completely reversed after return to control solution (Fig. 1C).

Another substrate of high-affinity glutamate transporters of cerebellar Bergmann glia cells is l(-)-threo-3-hydroxyaspartate (THA) (Gonzalez and Ortega 2000). THA increased the amplitude of the mGluR1-EPSC to 894 ± 122% (n = 5) at a concentration of 0.4 mM (Fig. 2B). Notably, the onset of THA action was slower compared with d-aspartate, and the THA effect was not completely reversed during a comparable period of time (n = 5; Fig. 2B).

A third blocker of glutamate transport, dihydrokainic acid (DHK; 1 mM), did not have a consistent effect on the mGluR1-EPSC (Fig. 2C). The mean mGluR1-EPSC amplitude were 289 ± 48 and 315 ± 52 (SE) pA (n = 6) in control ACSF and DHK-containing ACSF, respectively. These values are not significantly different (P > 0.1). The DHK unresponsive cells did, however, show the normal reaction to d-aspartate (Fig. 2C).

**Effect of glutamate uptake block on competitive mGluR antagonism**

The preceding results suggest that inhibition of glutamate uptake caused an increased glutamate concentration at the level of the mGluR1 and, hence, an increased amplitude of the mGluR1-EPSC. To test this hypothesis, we used the competitive low-affinity glutamate antagonist (S)-MCPG (Hayashi et al. 1994) to probe glutamate concentration at the level of mGluR1. The rationale of this approach was that at higher glutamate concentrations, a higher concentration of the competitive antagonist is necessary to displace glutamate and achieve suppression of the mGluR1-EPSC. For these experiments, the mGluR1-EPSC was evoked in the presence of varying concentrations of (S)-MCPG in control ACSF (n = 10 cells) and in the presence of 0.4 mM d-aspartate in the ACSF (n = 5 cells). The following stimulation protocol was adopted to ensure the comparable stimulation in all the experiments: The stimulation intensity was increased in 5- to 10-μA steps until a response was discernible and then between 15 and 30 μA was added to achieve a stable and clear response. Consistent with our hypothesis, the (S)-MCPG concentration necessary to suppress the peak amplitude of the mGluR1-EPSC by 50% was higher when d-aspartate was present (Fig. 3). The concentration-response curve of the (S)-MCPG depression of the mGluR1-EPSC is shifted to higher concentrations by d-aspartate and the half-maximal inhibition increased from 138 ± 3 (SE) μM (n = 10) to 658 ± 81 μM (n = 5; Fig. 3). This shift of the concentration-response curve suggests that the glutamate concentration reaches higher values when glutamate uptake is blocked by d-aspartate. These data also permitted estimation of the local glutamate concentration at the site of the receptor using the known potencies of glutamate and (S)-MCPG at mGluR1a (Hayashi et al. 1994). Assuming that receptor activity required binding of glutamate at two mGluR1a molecules (see METHODS), we estimated the effective glutamate concentration during the mGluR1-EPSC to be 30 and 141 μM under control conditions and in the presence of 0.4 mM d-aspartate, respectively.
EPSC was first determined in control ACSF ($n = 12$; Fig. 4A). Then the stimulation intensity was adjusted to 50% of this threshold intensity i.e. a condition under which no response could be resolved. When d-aspartate (0.4 mM) was added to the ACSF, this stimulation intensity was sufficient to produce clear mGluR1-EPSCs in all but one of the cells tested ($n = 11$). Thus, the inhibition of glutamate uptake caused by d-aspartate decreases the stimulation intensity necessary to evoke a mGluR1-EPSC. In six experiments, d-aspartate was washed out at the end of the experiment. This led to the subsequent disappearance of the slow EPSC on stimulation with 50% threshold intensity in five of the six cells.

For a more detailed comparison of the relationship between mGluR1-EPSC amplitude and stimulation intensity under control conditions and in the presence of d-aspartate, stimulation intensity was varied over a wide range (Fig. 4B, $n = 5$ cells). Following wash in of d-aspartate, significant mGluR1-EPSCs were obtained at stimulation intensities amounting to half of the threshold intensity under control conditions ($P < 0.05$). d-aspartate (0.4 mM) lowered the threshold intensity by $\geq 50\%$ (Fig. 4B).

**Inhibition of glutamate uptake decreases the necessary number of stimuli to evoke a mGluR1-EPSC**

To test whether the number of parallel fiber stimuli necessary to evoke a mGluR1-EPSC is also lowered by d-aspartate, parallel fibers were stimulated with a varying number of stimuli (1, 2, 3, 4, 7, and 10 stimuli at constant stimulation intensity) under control conditions and in the presence of d-aspartate (0.4 mM; Fig. 5, $n = 7$). Under control conditions, 1–3 stimuli did not induce any significant mGluR1-EPSC ($P > 0.05$). Significant currents were only obtained with 4 ($P < 0.05$) or more ($P < 0.01$) stimuli. In contrast, with d-aspartate, mGluR1-EPSCs could be readily induced with 2 ($P < 0.05$) or more ($P < 0.01$) stimuli (Fig. 5A). Thus d-aspartate decreases from four to two stimuli the minimal number of stimuli necessary to evoke a response. In the presence of d-aspartate, maximal responses were achieved between 7 and 10 stimuli whereas >10 stimuli are necessary for saturation under control conditions (Tempia et al. 1998).

**Repetitive climbing-fiber stimulation does not produce a slow EPSC when glutamate uptake is blocked**

The preceding results indicate that during high-frequency stimulation of parallel fibers perisynaptic glutamate concentration builds up, a process that is counteracted by glutamate uptake. Although mGluR1 receptors have been found in the vicinity of climbing fiber synapses (Martin et al. 1992), repetitive stimulation of climbing fibers does not produce a slow EPSC under normal conditions (Batchelor et al. 1994; Tempia et al. 1998). We asked whether this would still hold when glutamate uptake is inhibited. All-or-nothing climbing-fiber responses were evoked in Purkinje cells ($n = 5$) by stimulation in the granular layer in different locations (Fig. 5B, a–c). After a climbing-fiber response was unequivocally identified by its all-or-nothing nature, AMPA receptors were blocked by...

**FIG. 4.** A: d-aspartate lowers the stimulation intensity necessary to evoke an mGluR1-EPSC. A small mGluR1-EPSC was evoked in this Purkinje cell at a stimulation intensity of 30 $\mu$A in control ACSF (black traces). At 15 $\mu$A, there is no discernable mGluR1-EPSC, whereas at 22.5 $\mu$A, a mGluR1-EPSC could be just resolved. After addition of d-aspartate (0.4 mM) to the ACSF a mGluR1-EPSC was evoked at all 3 stimulation intensities (gray traces). B: plot of the amplitudes of mGluR1-EPSCs recorded at different stimulus intensities in control solution (filled circles) and in the presence of 0.4 mM d-aspartate (open circles). Data points are means ± SE of currents normalized to the current obtained at fourfold threshold stimulation intensity in d-aspartate-containing ACSF ($n = 6$ cells). d-aspartate significantly (*$P < 0.05$; **$P < 0.01$) increases the amplitude of the currents over the full range of stimulus intensities.

**FIG. 5.** A: d-aspartate lowers the number of stimuli required to induce a significant mGluR1-EPSC. Parallel fibers were stimulated with 1, 2, 3, 4, 7, and 10 stimuli before (b) and after (d) application of 0.4 mM d-aspartate. d-aspartate significantly increased the amplitude of the slow EPSC at $\geq 2$ stimuli. Note that in the presence of d-aspartate but not under control conditions, 2 stimuli were sufficient to induce an mGluR1-EPSC. Current amplitudes were normalized to the current obtained at 10 stimuli in the presence of d-aspartate. B: all-or-nothing climbing-fiber response induced in a Purkinje at stimulus intensities of $\geq 25$ $\mu$A (traces a–c). After blocking AMPA receptors, a train of 10 stimuli at an intensity of 40 $\mu$A did not evoke a slow EPSC in neither control (d) nor d-aspartate-containing solution (e). The traces a–c are averages of 5 trials and the traces d and e are averages of 3 trials.
NBQX and the climbing fiber was repetitively stimulated with 10 suprathreshold stimuli at 100 Hz. As illustrated in Fig. 5B, d and e, application of d-aspartate (0.4 mM) did not generate a change in the Purkinje cell’s response in contrast to the effect during parallel fiber stimulation. Slow EPSCs could not be evoked in climbing fibers using repetitive stimulation in the absence or presence of d-aspartate (n = 5).

**Expression of the mGluR1-EPSC is associated with slow clearance of extracellular glutamate**

The experiments described in the preceding text indicated that inhibition of glutamate uptake increases the glutamate concentration in the vicinity of the mGluR1 receptors and facilitates the expression of mGluR1-EPSCs. An increased accumulation of free extracellular glutamate can be explained by a reduced binding capacity of transporter molecules (Diamond and Jahr 1997). It is therefore not immediately clear if inhibition of glutamate uptake by d-aspartate also affects the time course of glutamate clearance in the vicinity of the mGluR1 receptors.

To investigate this question, experiments were performed in which (S)-MCPG was applied by rapid pressure application into the area around the electrical stimulation pipette at varying time intervals after tetanic stimulation of the parallel fibers. The timing scheme is illustrated in Fig. 6A. The fast pressure application began with a delay d1 (10–500 ms) relative to the electrical stimulation. In control solution (Fig. 6, B–D) and in d-aspartate-containing solution (Fig. 6, E–G), application of (S)-MCPG at a range of d1 periods reduced the mGluR1-EPSC. This reduction became significant (as determined by visual inspection, see 3rd vertical line in Fig. 6A) after a delay time d2 relative to the start of the (S)-MCPG application (Fig. 6, A–G). Interestingly, d2 was independent of d1 and of the presence of the glutamate uptake inhibitor with mean values of 200 ± 15 (SE) ms (n = 17) and 213 ± 14 ms (n = 33) for control and d-aspartate-containing solution, respectively (Fig. 6H). The mechanism determining d2 is not clear, but d2 involves the time required for displacement of glutamate from the receptor and the signal transduction time between receptor and effector activities (Canepari et al. 2001).

When (S)-MCPG was pressure applied to Purkinje cells without d-aspartate in the bath solution (n = 5 cells, 42 trials), a reduction of mGluR1-EPSC amplitude was observed with d1 values of 10 ≤ 100 ms (Fig. 6, D and I). However, in the presence of 0.4 mM d-aspartate, this situation changed dramatically (n = 5 cells, 38 trials). Consistent with the lower potency of (S)-MCPG for depression of the mGluR1-EPSC in the presence of d-aspartate (see preceding text), mGluR1-EPSCs were less affected by (S)-MCPG. However, antagonist applications were effective with d1 values as long as 350 ms. To quantify the effect of the fast (S)-MCPG application, we measured the mGluR1-EPSC amplitude at times d1 + 300 ms [i.e., at times where the effect of (S)-MCPG, if present, was clearly developed]. Fig. 6f shows a plot of the (S)-MCPG induced inhibition of the mGluR1-EPSC as a function of d1. The values obtained in the absence of d-aspartate could be fitted well to single decay time constants of 36 ± 5 ms (Fig. 6f). In the presence of d-aspartate, the quantitative effect of (S)-MCPG was clearly less robust. Fitting resulted in an apparent decay time constant of 430 ± 350 ms. In contrast, in the presence of D-aspartate the mGluR1-EPSC was reduced by (S)-MCPG at d1 values ≥ 350 ms. In A–G the black traces represent control recordings and the gray traces are recordings with fast application of (S)-MCPG. H: d2 was not dependent on d1 or the presence of d-aspartate. Solid circles, control conditions; open circles, 0.4 mM d-aspartate. I: plot of the amplitude reduction at d1 + 300 ms vs. d1. Solid circles, control conditions; open circles, 0.4 mM aspartate; solid and broken line represent exponential decays with time constants of 36 ± 5 and 430 ± 290 ms, respectively.

**FIG. 6.** Fast application of (S)-MCPG during induction of the mGluR1-EPSC reveals the time course of glutamate clearance. A: the relationship among times of stimulation, (10 mM) MCPG application delay (d1), and delay between MCPG application and the occurrence of an effect (d2). B–D: under control conditions (S)-MCPG application affected the mGluR1-EPSC up to a d1 value of 100 ms. E–G: In contrast, in the presence of d-aspartate the mGluR1-EPSC was reduced by (S)-MCPG at d1 values ≥ 350 ms. In A–G the black traces represent control recordings and the gray traces are recordings with fast application of (S)-MCPG. H: d2 was not dependent on d1 or the presence of d-aspartate. Solid circles, control conditions; open circles, 0.4 mM d-aspartate. I: plot of the amplitude reduction at d1 + 300 ms vs. d1. Solid circles, control conditions; open circles, 0.4 mM aspartate; solid and broken line represent exponential decays with time constants of 36 ± 5 and 430 ± 290 ms, respectively.

**DISCUSSION**

The present work suggests that glutamate uptake is a critical determinant of the mGluR1-EPSC in cerebellar Purkinje cells and that during short trains of tetanic stimulation, extracellular glutamate accumulates in the peri- and extrasynaptic space decays within tens of milliseconds under control conditions.
where it reaches levels in the order of tens of micromolar and clears relatively slowly to basal levels. Accumulation and “spillover” of glutamate from synaptic clefts into the perisynaptic extracellular space has been described at several synapses (Carter and Reghr 2000; Mitchell and Silver 2000). The underlying mechanism may simply be based on diffusion dynamics (Destexhe and Sejnowski 1995); however, the saturation of transporter binding sites has also been proposed as a possible mechanism (Diamond and Jahr 1997). Indeed, glutamate transporters have a relatively long turnover time (10–70 ms) (Clements 1996), the time required to displace glutamate from the extracellular to the intracellular space after it binds to the transporter. Thus on the time scale of milliseconds, uptake capacity is essentially limited by the number of available transporter molecules.

Does glutamate in the vicinity of mGlur1 accumulate simply because residual amounts of glutamate sum up during clearing by diffusion (Destexhe and Sejnowski 1995)? Or, do glutamate-binding sites at transporter molecules become saturated during repetitive stimulation (Diamond and Jahr 1997)?

The present data suggest that the latter is the case. This can be seen when considering the enhancement of the mGlur1-EPSPs by d-aspartate as a function of stimulus number and stimulus intensity (Figs. 4 and 5). At very low stimulus intensities, a mGlur1-EPSP only develops with transporters occupied by d-aspartate. The absence of mGlur1-EPSC at low stimulus intensities, and the number of stimuli in the absence of d-aspartate, suggests that under these conditions, glutamate uptake capacity is large enough to handle all released glutamate. When stimulus intensity and/or stimulus number increase, the relative contribution of d-aspartate to the mGlur1-EPSC decreases, suggesting that the binding capacity of the glutamate transporters approaches saturation. This situation differs from that in hippocampal CA1 stratum radiatum where measurements of transporter-mediated currents indicated that trains of ≤10 stimuli delivered at 100 Hz did not overwhelm transporter capacity (Diamond and Jahr 2000).

There is a large body of data on the measured or calculated time constant of glutamate clearance in the synaptic cleft at the site of ionotropic glutamate receptors. Glutamate released into the center of a synaptic cleft clears with a time constant of ~1 ms or faster (Clements 1996; Diamond and Jahr 1997; Eccles and Jeager 1958). Information about the dynamics of extrasynaptic glutamate concentration following synaptic activity is sparse. The dynamics of glutamate transporter currents indicated a time constant of 17.3 ms for the extracellular decay of glutamate transients induced by single climbing fiber stimulation in the cerebellar molecular layer (Bergles et al. 1997), and of 25 ms when induced by 10 stimuli at 100 Hz in the hippocampal CA1 str. radiatum (Diamond and Jahr 2000). These values are close to our measurements of ~30 ms. Using a different approach, our present data demonstrate that the clearance of glutamate from the peri- and extrasynaptic extracellular space is in the order of tens of milliseconds, even with uninhibited glutamate uptake.

Our experiments with fast pressure application of (S)-MCPG revealed a relatively long delay between the start of antagonist application and the effect of the antagonist on the mGlur1-EPSC. It is unlikely that this delay was due to a delayed arrival of the pressure applied antagonist at the site of the receptor because a similar delay was recently observed between the time point of fast photolytic release of L-glutamate and the onset of the slow mGlur1-mediated current in rat cerebellar Purkinje cells (Canepari et al. 2001). The delayed response to rapid photolytic release of L-glutamate suggests that there is a significant delay between ligand binding at the receptor and expression of the mGlur1-EPSC. It has previously been discussed that induction of the mGlur1-EPSC requires intracellular activation or accumulation of a factor that primes a subsequent induction of this current (Batchelor and Garthwaite 1997). Interestingly, d-aspartate did not reduce the delay between the start of stimulation and the onset of mGlur1-EPSCs (Fig. 1), even though d-aspartate reduced the number of stimuli required to induce the mGlur1-EPSC (Fig. 5).

Our observation that application of d-aspartate and THA, but not of DHK, led to a large increase of the mGlur1-EPSC is consistent with the hypothesis that the glutamate uptake relevant for controlling the mGlur1-EPSC is largely mediated by the glutamate transporter EAAT-1, which is expressed at high levels in Bergman glial cells (Bergles et al. 1997; Lehre and Danbolt 1998; Rothstein et al. 1994). THA and d-aspartate are also substrates for glutamate transporters expressed by Purkinje cells (Auger and Attwell 2000; Canepari et al. 2001; Otis et al. 1997). The absence of any detectable postsynaptic glutamate uptake current in the presence of (S)-MCPG (Fig. 3) suggests, however, a minor contribution of postsynaptic glutamate uptake under our experimental conditions (Auger and Attwell 2000; Canepari et al. 2001).

The slow mGlur1-EPSC is observed only with high-frequency stimulation of a bundle of parallel fibers. Two nonexclusive mechanisms have been described for extracellular accumulation of transmitter taking into account diffusion dynamics. The first mechanism is spatial summation (pooling) of transmitter released from several neighboring sites (Destexhe and Sejnowski 1995). In the case of parallel fibers making single en-passant contacts with Purkinje cells, this mechanism relates to co-activation of neighboring axons. The second mechanism is based on temporal summation of residual levels during repetitive stimulation (Destexhe and Sejnowski 1995) with or without saturation of local uptake capacity (Diamond and Jahr 1997). We observed that uptake inhibition has effects on both intensity and frequency necessary to elicit an mGlur1-EPSC. Therefore, we conclude that both effects are involved. However, with intact uptake, only repetitive stimulation but not single high-intensity stimulations induce an mGlur1-EPSC.

This is in contrast to GABAergic-mediated responses in the hippocampus where sufficient amounts of GABA are achieved by cooperation between neighboring synapses as indicated by a strong dependence on stimulation intensity. Synapses formed by parallel fibers on Purkinje cells differ from hippocampal GABAergic synapses in several aspects. Parallel fiber-Purkinje cell synapses are localized on spines completely ensheathed by astroglia while hippocampal GABAergic synapses are formed side-by-side on dendritic shafts with fewer intervening glial processes (Lehr et al. 1998). This may explain the differences in local accumulation of transmitter and intersynaptic crosstalk.

What are the physiological conditions under which a mGlur1-EPSC is induced? Expression of an mGlur1-EPSC indicates synchronous activation of neighboring synapses as well as bursts of presynaptic activity. There is no evidence that
parallel fiber inputs carrying a common signal converge on the same dendritic site of a single Purkinje cell. Although this lack of evidence does not exclude such a possibility, high-frequency bursts of granule cell activity are known. Indeed, the mossy fiber-granule cell-Golgi cell system might be specialized to pass high-frequency burst (Gabbiani et al. 1994; Mitchell and Silver 2000). Furthermore, from the dependence on glutamate transporter activity of mGluR1 activation and expression of mGluR1-EPSCs, we would predict that glutamate transporters also control parallel fiber long-term depression, a phenomenon requiring mGluR1 activation in Purkinje cells (Ichise et al. 2000). Such a control is conceivable in view of the fact that glutamate transporters are themselves regulated by glutamate (Gonzalez and Ortega 2000).

While this paper was under review, Brasnjo and Ottis (2001) reported that inhibition of glutamate uptake could indeed facilitate the induction of long-term depression at rat parallel fiber-Purkinje cell synapses. These authors also provided evidence suggesting that neuronal glutamate transporters significantly contribute to the limitation of glutamate concentration transient responsible for the mGluR1-EPSC. Their data also suggested that inhibition of both neuronal and glial glutamate uptake has a more pronounced effect on the expression of the mGluR1-EPSC than inhibition of neuronal glutamate uptake alone. We argued in the preceding text that the absence of any detectable postsynaptic glutamate uptake current suggested a smaller contribution of postsynaptic glutamate uptake as compared with glial glutamate uptake under our experimental conditions. Considering our data together with those of Brasnjo and Ottis (2001), we would conclude that both postsynaptic and glial glutamate uptake cooperatively influence the expression of mGluR1-EPSCs in Purkinje cells.

We thank A. Takada for expert administrative and secretarial assistance. This work was supported by an intramural grant from the RIKEN Brain Science Institute.

REFERENCES


